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PLASTICITY OF PRIMARY AFFERENT NEURONS AND SENSORY PROCESSING AFTER SPINAL CORD INJURY

Topic Editors
Alexander G. Rabchevsky, Jeffrey C. Petruska and Charles H. Hubscher

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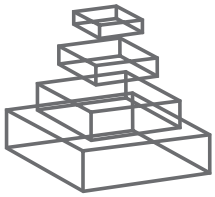

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PLASTICITY OF PRIMARY AFFERENT NEURONS AND SENSORY PROCESSING AFTER SPINAL CORD INJURY

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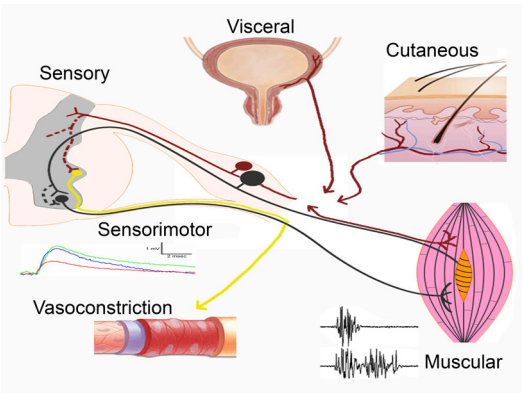

Following spinal cord injury, the primary afferent (dorsal root ganglion) and intraspinal neuronal circuitries adapt and continuously convey and process sensory information resulting in altered outputs to the peripheral motor, visceral, cutaneous, and vascular targets.

Traumatic injury of the spinal cord affects the entire organism directly and indirectly. Primary injury destroys neurons and severs axons which participate in neural circuits. Secondary injuries and pathologies arise from numerous sources including systemic inflammation, consequential damage of cutaneous, muscular, and visceral tissues, and dysregulation of autonomic, endocrine and sensory-motor functions. Evidence is mounting that spinal cord injury (SCI) affects regions of the nervous system spatially remote from the injury site, as well as peripheral tissues, and alters some basic characteristics of primary afferent cell biology and physiology (cell number, size/frequency, electrophysiology, other). The degree of afferent input and processing above the lesion is generally intact, while that in the peri-lesion area is highly variable, though pathologies emerge in both regions, including a variety of pain syndromes. Primary afferent input to spinal regions below the injury and the processing of this information becomes even more important in the face of complete or partial loss of descending input because such spared sensory processing can lead to both adaptive and pathological outcomes.

This issue hosts review and research articles considering mechanisms of plasticity of primary afferent neurons and sensory processing after SCI, and how such plasticity contributes to sparing and/or recovery of functions, as well as exacerbation of existing and/or emergent pathologies. A critical issue for the majority of the SCI community is chronic above-, peri-,

and below-level neuropathic pain, much of which may arise, at least in part, from plasticity of afferent fibers and nociceptive circuitry. For example, autonomic dysreflexia is common hypertensive syndrome that often develops after SCI that is highly reliant on maladaptive nociceptive sensory input and processing below the lesion. Moreover, the loss of descending input leaves the reflexive components of bladder/bowel/sexual function uncoordinated and susceptible to a variety of effects through afferent fiber plasticity. Finally, proper afferent feedback is vital for the effectiveness of activity-dependent rehabilitative therapies, but aberrant nociceptive input may interfere with these approaches since they are often unchecked due to loss of descending modulation.

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Challenges and opportunities of sensory plasticity after SCI

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Even in cases of spinal cord injury (SCI) where sensory perceptions do not arise from stimuli applied to below-level regions, sensory input to the spinal cord, carried by spinal sensory afferents, still occurs and influences the central and autonomic nervous systems (CNS, ANS). This is true also of the vagal system which provides non-spinal innervation of viscera below many spinal cord injuries. It is therefore important to consider (1) how the neurochemical, anatomical, and electrophysiological properties of these sensory neurons, and the processing of the inputs by the CNS and ANS, is altered by SCI, (2) whether and how they may play a role in pathologies, and (3) how they may interact with treatment strategies. This Research Topic addresses plasticity of sensory systems after SCI, with a non-exclusive focus on systems below the level of the injury.

POST-SCI AUTONOMIC DYSFUNCTIONS

The ANS controls systems below the level of consciousness and this is often taken for granted until something goes awry. Those living with SCI are acutely aware of the functions regulated, or more often dysregulated, by the ANS. One of the most pressing of these issues is autonomic dysreflexia (AD), a chronic and common hypertensive syndrome essentially unique to the high-level SCI community. It rarely arises acutely after injury (Krassioukov et al., 2003; Krassioukov, 2004), suggesting mechanisms beyond just loss of spinal sympathetic outflow regulation by the brain, and experimental evidence suggests that various forms of plasticity in numerous cell-types may contribute (e.g., Taylor and Schramm, 1987; Chau et al., 2000; Teasell et al., 2000; Rabchevsky, 2006; Schramm, 2006; Brown and Weaver, 2012). AD is generally considered an episodic pathology with bouts initiated and maintained by a physiological trigger, and is treated symptomatically and by finding and removing the stimulus. Continuing refinements in our understanding and measurements suggest that the severe clinical bouts that garner the most attention may be the tip of the iceberg of a much more insipid and persistent condition (e.g., Claydon et al., 2006; Krassioukov and Claydon, 2006). The most frequent triggers of AD appear to be noxious stimuli below the injury level [anything from a full bladder, an impacted bowel or a pressure ulcer to an ingrown toenail or simply having new shoes tied too tightly (e.g., Krassioukov et al., 2009)], placing focus onto plasticity in nociceptive sensory neurons (Ramer et al., 2012) for identifying potential mechanisms and treatments (Rabchevsky et al., 2012), though fundamental questions remain regarding the actual trigger in humans and experimental model systems (Macefield et al., 2012).

Additional autonomic functions are served and mediated by the vagal system, which is not directly impacted by experimental SCI and most clinical SCI. This vital and widespread system is nonetheless affected by SCI in terms of changes to electrical and chemical properties of neurons and changes in their connectivity (Kaddumi and Hubscher, 2007a,b; Holmes, 2012).

PAIN MECHANISMS AND TREATMENT

Chronic pain is not a consequence of SCI that is obvious to the casual observer, yet it is one of the most common post-SCI conditions and most impactful on the quality of life of SCI individuals (e.g., Finnerup and Baastrup, 2012). There are numerous mechanisms by which SCI-related pain can arise, some of which we are only now identifying, yet these are still poorly understood and there are few reliable treatments (e.g., Felix et al., 2007; Cruz-Almeida et al., 2009). The effect of SCI on primary sensory neurons is an emerging topic of investigation (Huang et al., 2006; Shortland et al., 2006; Ramer et al., 2012; Walters, 2012) as a possible mechanism of SCI-related pain and other pathologies such as AD (e.g., Widerstrom-Noga et al., 2004). New approaches to applying knowledge of nociceptive mechanisms are also being tested as potential treatments for SCI-related sensory pathologies (Gupta and Hubscher, 2012; Lee et al., 2012; Rabchevsky et al., 2012; Ramer et al., 2012).

EFFECTS OF POST-SCI TRAINING

In addition to a variety of forms of maladaptive plasticity, the spinal cord caudal to an injury which largely or completely separates it from the brain is capable of considerable and lasting adaptive plasticity, particularly activity-dependent plasticity (e.g., Edgerton et al., 1992; Hodgson et al., 1994; De Leon et al., 1999; Edgerton et al., 2001; Frigon and Rossignol, 2006), with some of this plasticity involving the sensory neurons (e.g., De Leon et al., 2001; Petruska et al., 2007). The spinal cord is capable of interpreting afferent input to learn a task and to counter perturbing forces or avoid obstacles placed in the path of hindlimbs stepping on a treadmill, and even retaining this information for a short time without reinforcement (Zhong et al., 2012). This collective work suggests that the spinal cord is capable of learning (see also Ferguson et al., 2012a,b; Grau et al., 2012), and may be capable of processes akin to formation of short- and long-term memory.

Generally the effects of training appear to be task-specific. For example, when an SCI animal is trained to step on a treadmill, this behavior improves, but the performance of other

tasks, such as standing, does not improve (Edgerton et al., 1997; De Leon et al., 1998, 1999). However, training does appear to have effects on some processes outside of the trained task. In animal models there are demonstrations of reduced spasticity (Bose et al., 2012), and reduced nociception (Wolpaw and Tennissen, 2001; Hutchinson et al., 2004; Martin Ginis and Latimer, 2007; Herrity et al., 2012).

More recently, principles identified from animal experiments have been applied to human experiments and clinical treatment with some success (Behrman et al., 2005; Barbeau et al., 2006; Dobkin et al., 2006; Harkema, 2008; Edgerton and Roy, 2009; Harkema et al., 2011). However, the field still has much to discover in terms of the characteristics of spinal plasticity, the necessary and sufficient influencing factors, as well as certain measures of systems, molecular, and cellular mechanisms that enable, facilitate, and inhibit such adaptive plasticity.

MECHANISMS REGULATING SPINAL LEARNING

Research on post-SCI training focuses on optimizing functional recovery and identifying relevant principles from the sensorimotor integration perspective. Another approach has examined the principles of learning that may be at play in the spinal cord (Ferguson et al., 2012a,b; Grau et al., 2012), with important concepts emerging about extrinsic factors *interfering* with successful spinal learning (i.e., training). Given the relative success of activity-based therapies in both animal and human experiments and the significant effort and resources dedicated to optimizing these approaches for clinical gain, we must also identify factors that inhibit recovery (e.g., Caudle et al., 2011; Ferguson et al., 2012a,b).

In this context it is intriguing that many clinical trials have exclusion criteria related to conditions that would be painful for spinal-intact individuals (bladder infection, pressure ulcer, tissue damage, etc.). Common among front-line therapists are anecdotes of discovering skin abrasions, treadmill harnesses pinching skin, bladder infections, and other covert noxious conditions in patients whose training sessions were unexpectedly going poorly. These anecdotes suggest that the powerful influence of the spinal nociceptive system on the spinal motor system known from animal work is also at play in SCI patients/subjects. Unfortunately, these accounts are not regularly included in data collection, limiting assessments of the role of nociception in activity-dependent therapies.

These concepts may be involved in other spinal processes. For example, systems that are accustomed to a certain level and pattern of activity can "fall out of tune" (e.g., Lundbye-Jensen and Nielsen, 2008). Also, growth of nociceptive afferent terminals within the cord contributes to AD (e.g., Cameron et al., 2006; Brown and Weaver, 2012). However, repetitive natural stimulation, determined to be accompanied by intraspinal sprouting of afferents, reduces nociceptive reflexes (Conde and Komisaruk, 2012). Collectively this suggests that the functional outcome of intraspinal afferent growth may be dependent on the pattern of information carried by those afferents and the context of the intraspinal growth. Perhaps intraspinal growth that is uncoupled from specific patterned input becomes maladaptive, while growth associated with patterned input is associated with adaptive outcomes [Conde and Komisaruk, 2012; Ferguson et al., 2012a,b; Grau et al., 2012; and discussed in Petruska et al. (2007) and Maier et al. (2009)].

EFFECTS OF SCI ON NEURAL TISSUE REMOTE FROM THE INJURY

Considering points of similarity and difference among experimental observations makes it clear that many characteristics of the injury model can have significant impact on the outcomes being measured (e.g., Cote et al., 2012; Hougland et al., 2012). Injury to one part of the spinal cord can have significant impact on systems that were not directly affected (Cote et al., 2012). The nervous system is particularly susceptible to such bystander effects because of the close physical proximity of neurons involved in diverse functions and the array of circuit interconnections, some of which may not be obvious until there is an injury. It is therefore beneficial to consider multiple elements of a system (such as examining sensory neurons and the spinal cord together when considering sensorimotor responses to SCI), as they can act differently in response to the same injury (Hougland et al., 2012).

The SCI condition involves pathologies beyond the spinal cord itself and the spinal cord disconnected from the brain can still generate output which relies heavily on the input it receives from the periphery. Understanding the status of the afferents providing input to the spinal cord and brainstem is of paramount importance. If the "below-level" spinal cord and the post-SCI vagal system are to be maintained in a healthy condition, then we must understand the vital roles that gateway primary afferent neurons play in both acute and chronic post-SCI pathologies in order to prevent sensory-based pathologies and direct these neurons to enhance recovery of function.

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Plasticity of TRPV1-expressing sensory neurons mediating autonomic dysreflexia following spinal cord injury

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Spinal cord injury (SCI) triggers profound changes in visceral and somatic targets of sensory neurons below the level of injury. Despite this, little is known about the influence of injury to the spinal cord on sensory ganglia. One of the defining characteristics of sensory neurons is the size of their cell body: for example, nociceptors are smaller in size than mechanoreceptors or proprioceptors. In these experiments, we first used a comprehensive immunohistochemical approach to characterize the size distribution of sensory neurons after high- and low-thoracic SCI. Male Wistar rats (300 g) received a spinal cord transection (T3 or T10) or sham-injury. At 30 days post-injury, dorsal root ganglia (DRGs) and spinal cords were harvested and analyzed immunohistochemically. In a wide survey of primary afferents, only those expressing the capsaicin receptor (TRPV1) exhibited somal hypertrophy after T3 SCI. Hypertrophy only occurred caudal to SCI and was pronounced in ganglia far distal to SCI (i.e., in L4-S1 DRGs). Injury-induced hypertrophy was accompanied by a small expansion of central territory in the lumbar spinal dorsal horn and by evidence of TRPV1 upregulation. Importantly, hypertrophy of TRPV1-positive neurons was modest after T10 SCI. Given the specific effects of T3 SCI on TRPV1-positive afferents, we hypothesized that these afferents contribute to autonomic dysreflexia (AD). Rats with T3 SCI received vehicle or capsaicin *via* intrathecal injection at 2 or 28 days post-SCI; at 30 days, AD was assessed by recording intra-arterial blood pressure during colo-rectal distension (CRD). In both groups of capsaicin-treated animals, the severity of AD was dramatically reduced. While AD is multi-factorial in origin, TRPV1-positive afferents are clearly involved in AD elicited by CRD. These findings implicate TRPV1-positive afferents in the initiation of AD and suggest that TRPV1 may be a therapeutic target for amelioration or prevention of AD after high SCI.

Keywords: capsaicin, colo-rectal distension, dorsal horn, dorsal root, dorsal root ganglion, high blood pressure, hypertension, hypertrophy

INTRODUCTION

The original formulation of the neurotrophic hypothesis by Hamburger and Levi-Montalcini (1949) asserted that the survival of neurons depends on cues produced in limiting amounts in target tissues. The subsequent discovery of nerve growth factor (NGF) as a survival factor for sympathetic and sensory neurons (Cohen et al., 1954) not only validated this hypothesis, but also extended the role of neurotrophic factors beyond developmental neuronal survival: target-derived NGF and numerous other neurotrophic molecules maintain the phenotype of neurons during adulthood. This has been arguably best demonstrated in experiments on primary afferent neurons of the dorsal root ganglion (DRG), a heterogeneous population of neurons responsible for transmission of information from the periphery (somatic) and internal milieu (visceral) to the spinal cord and brainstem.

Early classification of DRG neuronal subsets was based on cytoplasmic staining with standard histological techniques and electrophysiological recording followed by dye-filling of neurons (Willis and Coggeshall, 2004). These methods revealed "small dark" neurons which have slowly conducting axons (corresponding to thermoceptors and nociceptors, approximately 70% of all neurons in the DRG), and "large light" cells with rapidly conducting fibers (30% of all DRG neurons, representing skin and muscle mechanoreceptors). In terms of their neurochemistry, DRG neurons are remarkably diverse, and much effort has gone into establishing particular neurochemical phenotypes as functional proxies (Willis and Coggeshall, 2004). Among the small dark cells, for example, there are peptidergic and non-peptidergic subtypes, which also happen to be sensitive to different neurotrophic factors (NGF, and glial cell line-derived neurotrophic factor, GDNF,

respectively; Braz et al., 2005). Ascribing functional relevance to this neurochemical distinction has proven difficult, in part because both populations express transducers of thermal and noxious stimuli: one example is the capsaicin-sensitive cation channel transient receptor potential vanilloid receptor (TRPV1), a remarkably versatile receptor that is also activated by protons and noxious heat (Caterina et al., 1997; Tominaga et al., 1998).

Dorsal root ganglia neurons undergo marked changes in neuronal phenotype following their disconnection from their target tissues by axotomy. Importantly, and in-line with the neurotrophic hypothesis, these changes are mimicked by depletion of the relevant factors in the absence of axotomy, and reversed by supplying axotomized neurons with trophic support from exogenous sources (Rich et al., 1984; Yip et al., 1984; Johnson and Yip, 1985; Wong and Oblinger, 1991; Matheson et al., 1997; Bennett et al., 1998). Uninjured DRG neurons undergo hypertrophy following *in vivo* delivery of NGF or GDNF (Ramer et al., 2001, 2003). Likewise, transgenic over-expression of NGF (Goodness et al., 1997) and the GDNF family member artemin (Elitt et al., 2006) cause sensory neuronal hypertrophy.

Spinal cord injury (SCI), while not disconnecting DRG neurons from their peripheral targets, nevertheless has profound effects on multiple tissues which might be expected to influence neuronal phenotype. Among the most dramatic of these are skeletal muscle atrophy, and in the lower urinary tract (LUT), detrusor hypertrophy. In the latter case, increased NGF production by the bladder has been correlated with hypertrophy of innervating DRG neurons (Yoshimura, 1999). For complete lesions above T5/6, SCI is almost always accompanied by cardiovascular disturbances including orthostatic hypotension (OH; a sudden fall in blood pressure upon assuming an upright position) and autonomic dysreflexia (AD; potentially life-threatening elevations in blood pressure triggered by sensory stimulation below the injury; Krassioukov and Claydon, 2006). There is increasing evidence that blood vessels, a peripheral target of sensory as well as sympathetic axons, also undergo SCI-induced alterations which may evoke phenotypic changes in their innervating neurons (McLachlan and Brock, 2006; Alan et al., 2010; Rummery et al., 2010; Al Dera et al., 2011; Tripovic et al., 2011).

Here we took a systematic immunohistochemical approach to studying one of the defining phenotypic features of sensory neurons – soma size – in different populations of sensory neurons after T3 SCI. We characterized hypertrophy in a specific subset of nociceptors, those that are sensitive to capsaicin and artemin. We examined the effect of rostro-caudal level and injury level; we also examined the density of central projections of hypertrophied neurons and the role of those neurons in AD. We hypothesized that SCI would induce nociceptor hypertrophy and/or terminal sprouting and that these effects would be more pronounced after high-thoracic injury. After identifying a specific subset of sensory neurons that responded to SCI, we hypothesized that these would be involved in induction and/or development of AD.

MATERIALS AND METHODS

SPINAL CORD INJURY SURGERY

Complete transection of the spinal cord at the third (T3) or tenth (T10) thoracic segments was performed in adult male Wistar rats (250–350 g, Charles River Laboratories, Inc., Wilmington, Canada). T3 SCI promotes the development of cardiovascular dysfunction, including AD, while T10 SCI does not. Sham surgeries were performed at T3 and were identical up to and including durotomy, without transection of the cord.

Rats were housed in a secure conventional rodent facility, on a 12-h reversed light-dark cycle. The surgical procedures and post-operative animal care have been described in detail (Ramsey et al., 2010), but the essential steps are included here.

Animals received prophylactic enrofloxacin (Baytril; 10 mg/kg, s.c., Associated Veterinary Purchasing; AVP, Langley, Canada) for 3 days prior to SCI surgery. On the day of surgery, anesthesia was induced with ketamine hydrochloride (Vetalar®; 70 mg/kg, i.p., University of McGill Animal Resources Centre, Montreal, Canada) and medetomidine hydrochloride (Domitor®; 0.5 mg/kg, i.p., AVP). Enrofloxacin (10 mg/kg, s.c.), buprenorphine (Temgesic®; 0.02 mg/kg, s.c., McGill University), and ketoprofen (Anafen®, 5 mg/kg, s.c., AVP) were administered pre-operatively.

After the skin at the surgical site was shaved, scrubbed, and treated with iodine, the animal was placed in the prone position. The spinal cord was exposed *via* a midline incision in the skin and superficial muscles, and blunt dissection of the muscles overlying the C8–T3 (T3 transection) or T8–T11 (T10 transection) vertebrae. At the T2–T3 intervertebral space or following a small laminectomy at T9, connective tissues were removed, the dura was opened and the spinal cord was completely transected with surgical scissors. Complete transection was verified by the clear separation and retraction of the cut ends of the cord, visualized under the surgical microscope. After hemostasis was achieved, the muscle and skin were closed with continuous, 4-0 Vicryl sutures, and interrupted 4-0 Prolene sutures, respectively.

POST-OPERATIVE ANIMAL CARE

Animals received warmed Lactated Ringer's solution (5 ml, s.c.) and recovered in a temperature-controlled environment (Animal Intensive Care Unit, HotSpot for Birds, Los Angeles, CA, USA). Anesthesia was reversed with atipamezole hydrochloride (Antisedan; 1 mg/kg, s.c., Novartis, Mississauga, Canada). Enrofloxacin (10 mg/kg, s.c.), buprenorphine (0.02 mg/kg, s.c.), and ketoprofen (5 mg/kg, s.c.) were administered once per day for 3 days following SCI.

Home cages for animals with complete T3 SCI were fitted with rubber matting (under woodchips, to facilitate movement), low-reaching water bottles, and food scattered on the cage bottom to encourage foraging (Ramsey et al., 2010). Animals were supported with an enriched diet, including meal replacement shake (Ensure; Abbott, Saint-Laurent, Canada), nutritive transport gel (Charles River), fruit, cereals, commercially available rat treats and kibble (LabDiet, Rodent Diet 5001). The bladder was manually expressed three to four times daily until spontaneous bladder function returned (about 10 days post-injury). Animal health was formally monitored daily for the first 2 weeks after SCI and every 2 days thereafter, using objective criteria to assess body weight, activity level, social behavior, healing at the surgery site, and clinical signs of morbidity.

SURVIVAL TIMES

Animals survived for 1 or 3 months after SCI. In our initial experiments examining SCI-induced hypertrophy in the DRG (data in **Figures 1–3** and **5**), T3 sham-, and spinal cord-injured animals survived for 3 months. In all subsequent experiments (data in **Figures 4** and **6–8**), T3 and T10 sham and spinal cord-injured animals survived for 1 month following surgery.

INTRATHECAL CAPSAICIN INJECTION

At 48 h (early cap.) or 28 days (late cap.) after SCI, animals were anesthetized with inhalant isoflurane (AErrane®, AVP; 5% induction, 2–3% maintenance). The skin overlying the lumbar enlargement of the spinal cord was shaved, scrubbed, and treated with iodine. The lumbar enlargement was exposed *via* midline incision in the skin and superficial muscles, blunt dissection of deeper muscles, and a midline laminectomy. Polyurethane tubing (PU, 3 French, Instech, Plymouth Meeting, USA) was introduced subdurally and 50 μg of capsaicin (Sigma-Aldrich Inc., St. Louis, USA) in 10 μl 50% dimethyl sulfoxide (DMSO; Sigma) was injected intrathecally. Control animals received 50% DMSO only, at 28 days after SCI. The muscle and skin were closed with sutures (4-0 Vicryl and 4-0 Prolene, respectively).

CARDIOVASCULAR ASSESSMENT

Thirty days after SCI, a cannula (PU, 3 French, Instech) was implanted into the left carotid artery in all animals for continuous beat-to-beat blood pressure recording. Arterial cannulation was performed under isoflurane anesthesia and the cannula was tunneled subcutaneously to exit the skin dorsally, through small incision at the base of the skull. The cannula was filled with a lock solution of 1:10 heparin (Hepalean®, AVP) and 5% dextrose in Lactated Ringer's.

Two hours after carotid cannulation, the cannula was connected to a fluid-filled pressure transducer (SP844, MEMScAP, Norway). Animals were conscious and the cannula was long enough to permit them to move freely in a cage during cardiovascular assessment. Beat-to-beat arterial pressure was monitored using PowerLab and Chart™5 for Windows (ADInstruments, Colorado Springs, USA). When blood pressure was stable (typically 5–10 min after connecting the cannula to the transducer), baseline blood pressure was recorded over 5 min.

The severity of colo-rectal distension (CRD)-induced AD was examined using a protocol that is well-established in our laboratory (Krassioukov and Weaver, 1995). A pediatric silicone balloon-tipped catheter (10 French; Coloplast, Denmark) was inserted into the rectum and secured to the tail. After stabilization of arterial pressure, the colon was distended *via* inflation of 2 ml of air, over 10 s. Distension was maintained for 1 min; upon deflation of the balloon, arterial pressure was allowed to recover over 10 min. Blood pressure was recorded during two episodes of AD in each animal, with a minimum of 10 min of recovery intervening.

TISSUE PROCESSING AND IMMUNOHISTOCHEMISTRY

Animals were euthanized with an overdose of chloral hydrate (1 g/kg, i.p.) and perfused through the heart with room temperature phosphate-buffered saline (PBS) followed by cold 4% paraformaldehyde (PF). Spinal segments and DRGs were removed, post-fixed in 4% PF for 12 h and cryoprotected in 20% sucrose in 0.1 M phosphate buffer for ≥24 h. Tissue was embedded in Tissue Tek (Fisher Scientific, Ottawa, Canada), frozen over liquid nitrogen, sectioned on a cryostat at 16 μm (DRG) or 20 μm (cord), thaw-mounted on to glass slides and stored at −80°C.

For immunohistochemistry, slides were incubated in 10% normal donkey serum in PBS plus Triton X-100 (0.1%) for 20 min. We used five antibodies to delineate different subsets of nociceptors: these included antibodies raised against TRPV1 (Neuromics, Edina, MN, USA; 1:2,000), the ionotropic ATP purinoceptor $P2X_3$ (Millipore, Billerica, MA, USA; 1:1,000), Substance P (Neuromics, 1:1,000), the glycoprotein isolectin B4 (IB4; Neuromics; 1:2,000), the glial cell line-derived neurotrophic factor family receptor α 3 (GFRα3; R&D Systems; 1:500). Subsets of non-nociceptive sensory neurons were identified by expression of the vanilloid transient receptor potential vanilloid-2 (TRPV2; Abcam, Cambridge, UK; 1:200), parvalbumin (Millipore, Etobicoke, Canada; 1:1,000), stage-specific embryonic antigen-4 (SSEA-4; Stem Cell Technologies, Vancouver, Canada; 1:100), and heavy neurofilament (NF200; Millipore, 1:500). Pan-neuronal markers – microtubule-associated protein-2 (MAP-2; Abcam, 1:5:000) or β-III-tubulin (Neuromics; 1:500) – were used to label all neuronal profiles in the DRG.

All primary antibodies were applied in PBS plus Triton X overnight. After three 15-min washes in PBS, secondary antibodies raised in donkey and conjugated to Cy3 (Jackson ImmunoResearch, West Grove, USA), Alexa 488 (Invitrogen, Eugene, USA), or AMCA (7-amino-4-methylcoumarin-3-acetic acid; Jackson ImmunoResearch) were applied at 1:100–1:400, in PBS-Triton X for 2 h. Epifluorescent images of DRGs were captured with an Axioplan 2 microscope (Zeiss, Jena, Germany) with a digital camera (Q Imaging, Burnaby, Canada) and Northern Eclipse software (Empix Imaging, Inc., Mississauga, Canada). Confocal images of TRPV1-positive axons in the spinal cord were captured on a spinning disk confocal microscope (an inverted Zeiss AxioObserver Z.1 equipped with an AxioCam CCD camera). All images for each antigen used for quantitative analysis were captured at identical imaging settings.

CARDIOVASCULAR DATA ANALYSIS

Systolic and diastolic blood pressures (SAP and DAP, mmHg) were obtained from maxima (max) and minima (min) respectively of beat-to-beat blood pressure recordings. Mean arterial pressure (MAP) was calculated as 1/3max + 2/3min and heart rate (HR, beats per minute, bpm) was calculated from inter-beat interval. Prior to data analysis, raw beat-to-beat blood pressure data were examined in the Chart view; false max/min readings created by muscle spasm were manually eliminated for each animal. Data were analyzed using SigmaPlot (SPSS Inc., Ashburn, USA). In SigmaPlot, raw pressure and HR values were averaged over 1 s, such that measurements represent a 1-s average, not a single beat. For each animal, baseline cardiovascular parameters represent the average of at least 3 min of recording time. The CRD-evoked changes in SAP and HR represent the average of two consecutive distensions for each animal.

IMAGE ANALYSIS

All images were analyzed using SigmaScan Pro 5.0 (SPSS Inc.). For analysis, L4 and L5 DRGs were pooled for each animal (denoted as L4/L5 throughout) and L6 and S1 DRGs were pooled for each animal (L6/S1 throughout). The size-frequency distributions of sensory neurons in the DRG were determined using recursive translation (Rose and Rohrlich, 1988), which converts neuronal profiles in section to the cellular population from which they were drawn (as described previously; Ramer et al., 2001). In five randomly selected sections from each DRG, all neuronal profiles were traced manually to create an artificial overlay. The average intensity across the cell body and soma diameter of each neuronal profile identified by the overlay was measured automatically; soma diameter was defined as Feret diameter, the theoretical diameter of the object if it were circular in shape. For proportional frequency measurements, the threshold intensity for expression was set manually for each image. Size-frequency and intensity-frequency distributions were generated to examine SCI-induced shifts in afferent size and TRPV1 signal intensity, respectively.

In the spinal cord, measurements of the distribution and intensity of TRPV1-positive axons in the dorsal horn were taken from tiled confocal projected z-stacks of the L4/L5 and L6/S1 dorsal horn in cross section. Terminal density was measured as a function of depth at two sites in the dorsal horn (mid and medial) as described previously (Ramer et al., 2001, 2004; MacDermid et al., 2004; Scott et al., 2005). Images were passed through a Laplacian omnidirectional edge-detection filter, which optimizes the signal-to-noise ratio and corrects for variations in background staining. Terminal profiles in filtered images were selected with a threshold overlay. In order to give all immunopositive pixels equal weight regardless of brightness, the threshold overlay was treated as a new image. Density measurements for each animal represent average density at each depth across five sections.

Density of TRPV1-positive axons in the spinal parasympathetic nucleus was measured from confocal images of the L6/S1 dorsal gray commissure (DGC). These images were filtered through horizontal and vertical Sobel edge-detection filters, with the results added through image math prior to thresholding. Density was measured in the spinal parasympathetic nucleus by selecting an area of gray matter that was centered on the midline, immediately ventral to the dorsal corticospinal tract and rostral to the central canal. Density measurements for each animal represent average density across three to five sections.

STATISTICS

For cardiovascular data, baseline parameters and stimulus-evoked changes were compared among groups using a one-way analysis of variance (ANOVA). The Holm–Sidak Test was used for pair-wise comparisons when significant differences were detected. For size distribution data from the DRG, Kolmogorov–Smirnov (K–S) goodness-of-fit tests were used to determine whether neuronal cumulative size-frequency distributions differed between groups (sham- versus spinal cord-injured). Proportions of neurons labeled with each antigen were compared using Student's *t*-test. The K–S goodness-of-fit test was also used to detect inter-group differences in cumulative intensity-frequency distributions of TRPV1-expressing cells in the DRG. Density of TRPV1-positive terminals in the dorsal horn of sham- and spinal cord-injured animals was compared using a one-way ANOVA on ranks, followed by Dunn's test to detect pair-wise differences. Density of TRPV1-positive terminals in the DGC of sham-injured and SCI rats was compared between groups using Student's *t*-test. In assessing the effects of early and late intrathecal capsaicin on TRPV1 axons in the DGC, a one-way ANOVA was used. For all physiological and anatomical analyses, group averages represent five to seven animals per group, results are expressed as mean ± standard error of the mean (SEM), image, and data analyses were performed in a blinded fashion (using coded image and data files) and P values less than 0.05 were considered significant.

RESULTS

COMPLETE HIGH-THORACIC SPINAL CORD INJURY PROVOKED HYPERTROPHY IN SENSORY NEURONS THAT EXPRESS THE CAPSAICIN RECEPTOR

Neuronal phenotype, of which size is a defining characteristic, is governed in large part by trophic influences of target tissues. These undergo profound changes following SCI, including atrophy of skeletal muscle and bladder hypertrophy. Therefore, we examined changes in the size distribution of all sensory neurons in lumbosacral DRGs 3 months after T3 complete SCI. Neuronal profiles in the L4/L5 DRG were labeled with neuron-specific β-III-tubulin (**Figure 1A**) and size-frequency analysis was performed following recursive translation (**Figure 1B**). There was a subtle but statistically significant right-shift (i.e., hypertrophy) in the size distribution of all sensory neurons in DRGs of animals with T3 SCI.

We next sought to determine which population(s) of sensory neurons responded to T3 SCI. We began by examining subsets of small-diameter sensory neurons in the L4/L5 DRG (**Figure 2**). Peptidergic (NGF-sensitive) nociceptors were identified by expression of Substance P (SP; **Figure 2A**), while non-peptidergic nociceptors were identified by binding of the glycoprotein isolectin B4 (IB4, from *Bandeiraea simplicifolia*) and the ionotropic ATP purinoceptor $P2X_3$ (**Figures 2B,C**). At 3 months after T3 SCI, neither of these minimally overlapping populations of nociceptors exhibited hypertrophy after T3 SCI, suggesting that sensory hypertrophy caudal to T3 SCI was selective to another subset of sensory neurons.

In rats, TRPV1 expression occurs in subsets of both peptidergic and non-peptidergic nociceptors (Tominaga et al., 1998). TRPV1 expression also defines a subpopulation of neurons which are neither $P2X_3$-expressing/IB4-binding nor neuropeptide-expressing (Michael and Priestley, 1999). When we examined the size distribution of TRPV1-positive neurons in the L4/L5 DRG, we found a pronounced hypertrophy in DRGs from animals with T3 SCI (**Figure 2D**). TRPV1-positive cells increased in size after SCI, but their proportional frequency did not change (**Figure 2D**, inset). Approximately 50% of TRPV1-positive sensory neurons also express the artemin-specific glial cell line-derived neurotrophic factor family member GFRα3 (Baloh et al., 1998; Bennett et al., 2006). GFRα3-positive sensory neurons (of which >80% express TRPV1; Bennett et al., 2006) also exhibited hypertrophy, in the

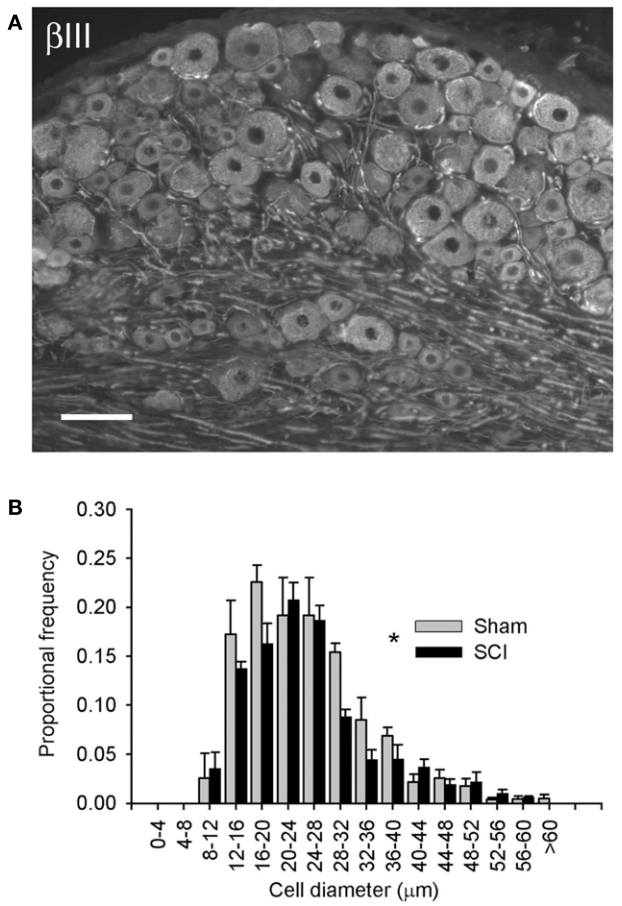

FIGURE 1 | High-thoracic (T3) spinal cord injury provoked hypertrophy of neurons in the L4/L5 dorsal root ganglion. (A) βIII-tubulin immunohistochemistry illustrating neuronal profiles in the L5 DRG. **(B)** Size-frequency distribution of pooled L4/L5 DRG neurons, reconstructed from profile distributions using recursive translation. Ganglia were harvested 3 months after sham-injury (gray) or complete T3 SCI (black). There was a small but significant rightward shift in size-frequency distribution in animals with T3 SCI ($P < 0.05$, K–S goodness-of-fit test). Scale bar = 70 μm.

absence of change in proportional frequency, in DRGs distal to T3 SCI (**Figure 2E**).

We performed size-frequency analysis on medium-to-large sensory neurons in the same (L4/L5) DRGs by examining neurons expressing heavy neurofilament (NF200; **Figure 3**). At 3 months after T3 SCI, there was no evidence of injury-induced hypertrophy in NF200-positive cells, nor did the proportion of NF200-expressing cells change (**Figure 3A**). In agreement with previous findings (e.g., Yamamoto et al., 2008), we found that the large majority of TRPV1-positive neurons were NF200-negative; only occasional neurons co-expressed TRVP1 and NF200 (**Figure 3B**, arrow). The extent of TRPV1 and NF200 co-expression did not increase after T3 SCI (**Figure 3B**).

To confirm that SCI-induced hypertrophy was specific to small-diameter DRGs, we also performed size-frequency analysis on three subpopulations of medium-to-large sensory neurons in the L4/L5 DRG (data not shown). Expression of TRPV2 was used to identify larger sensory neurons that are heat-sensitive but TRPV1-negative (Caterina et al., 1999; Tamura et al., 2005). Proprioceptors and cutaneous mechanoreceptors were labeled with parvalbumin (Celio, 1990) and SSEA-4 (Dodd et al., 1984), respectively. In contrast to SCI-induced hypertrophy in TRPV1-expressing nociceptors, there were no detectable changes in the size distributions of these subpopulations of medium- to-large DRG neurons following T3 SCI.

SPINAL CORD INJURY-INDUCED HYPERTROPHY WAS MOST PRONOUNCED IN LUMBOSACRAL SENSORY GANGLIA

Since our initial observations were made in DRGs far distal to T3 SCI (**Figures 1–3**, L4/L5 DRG), we examined the extent of SCI-induced hypertrophy in TRPV1-positive neurons at different rostro-caudal levels (**Figure 4**). We examined tissue harvested 1 month after T3 durotomy (sham-injury) or SCI (**Figures 4–6**). Of the levels examined, SCI-induced hypertrophy was restricted to ganglia below the injury: TRPV1-positive afferents did not exhibit hypertrophy in the T1 DRG, despite their proximity to the injury site (**Figure 4A**). Equidistant but caudal to SCI (in the T5 DRG), the size distribution of TRPV1-positive sensory neurons was right-shifted relative to sham-injured controls (**Figure 4B**). However, the effect of SCI was most dramatic in lumbosacral DRGs, remote from the site of injury. TRPV1-positive cells exhibited pronounced hypertrophy in both L4/L5 and L6/S1 DRGs (**Figures 4C,D**). The rightward shift in size distribution was most dramatic in L6/S1 DRGs, containing afferents innervating the urinary bladder and the distal colon (Nadelhaft and Booth, 1984).

CAPSAICIN-SENSITIVE AFFERENTS HYPERTROPHIED AND UPREGULATED THE CAPSAICIN RECEPTOR AFTER SPINAL CORD INJURY

While there was no change in the proportion of sensory neurons expressing TRPV1 after T3 SCI, this does not negate an intracellular upregulation of TRPV1. To investigate this possibility, we examined the intensity of TRPV1 expression in lumbosacral DRGs. Sections of L4/L5 and L6/S1 DRG from sham-injured controls and animals with T3 SCI were processed for TRPV1 immunohistochemistry and examined at the microscope by a blinded investigator, with all imaging parameters (exposure time, gain, and offset) set at constant levels across groups. With identical immunohistochemical processing and imaging, there was an obvious increase in intensity of TRPV1 expression in DRGs from animals with SCI (**Figure 5**). This was confirmed through blind quantification: when images were processed to generate size-intensity distributions of TRPV1-positive neuronal profiles, the intensity distribution in DRGs from animals with T3 SCI was right-shifted relative to sham-injured controls (**Figures 5A,B**). Akin to SCI-induced hypertrophy, the shift in TRPV1 signal intensity was most pronounced in the L6/S1 DRGs.

INJURY-INDUCED HYPERTROPHY WAS MODEST AFTER LOW-THORACIC SPINAL CORD INJURY

The large majority of work in animal models of SCI examines injury-induced changes after low-thoracic SCI (Ramsey et al., 2010). For example, data describing SCI-induced changes in

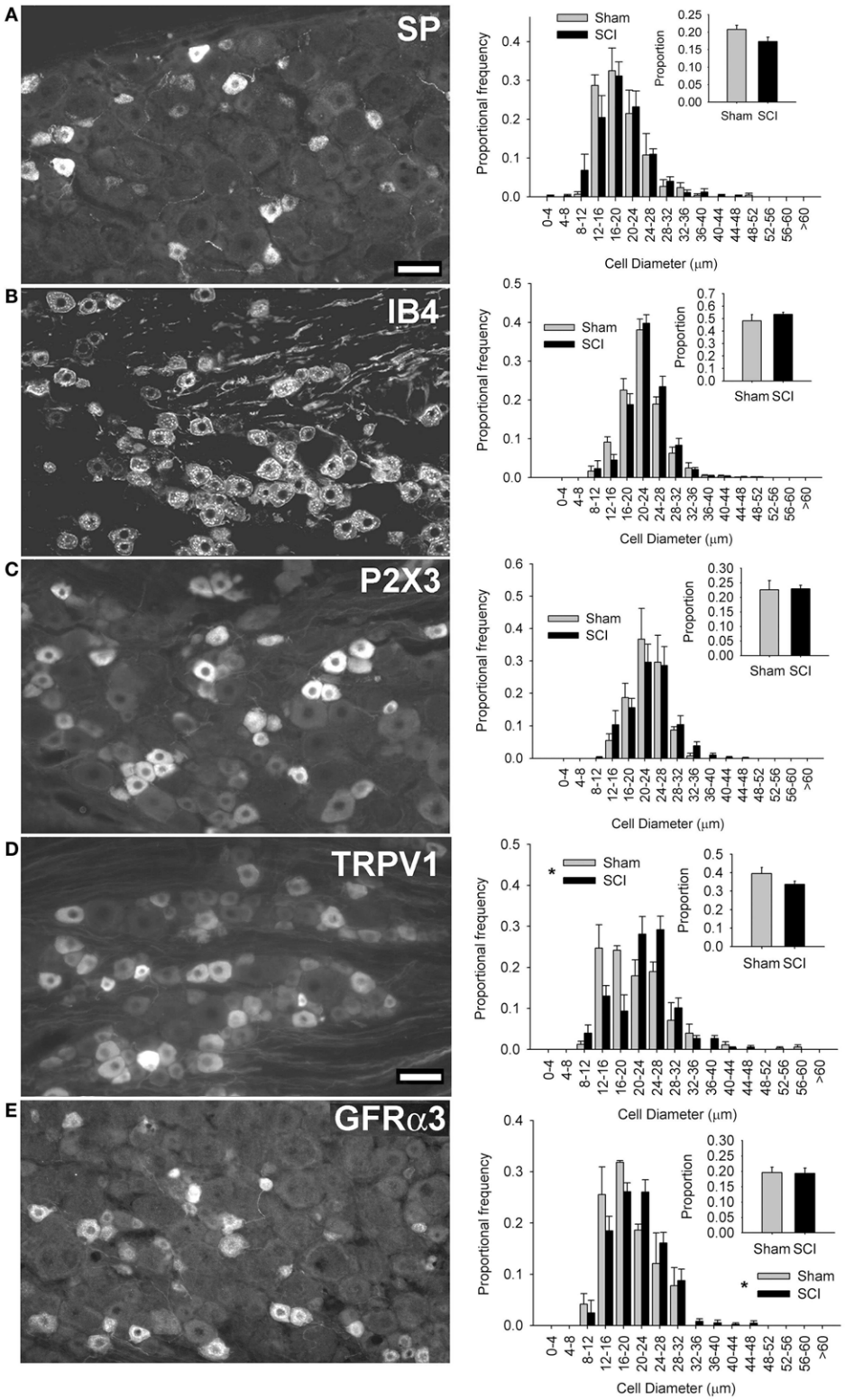

FIGURE 2 | High-thoracic (T3) spinal cord injury-induced selective hypertrophy of sensory neurons expressing the capsaicin receptor (TRPV1) and the artemin receptor (GFRα3) in the L4/L5 DRG. (A) Substance P (SP) – positive DRG neurons and their size-frequency distributions. **(B)** IB4-binding DRG neurons. **(C)** P2X3-positive DRG neurons. **(D)** TRPV1-positive DRG neurons. **(E)** GFRα3-expressing DRG neurons, known to express TRPV1. The overall proportions of immunopositive neurons [insets **(A–E)**] did not change for any subpopulation. Ganglia were harvested 3 months after sham-injury (gray) or complete T3 SCI (black). Scale bar = 50 μm. Asterisks indicate $P < 0.05$, K–S goodness-of-fit test.

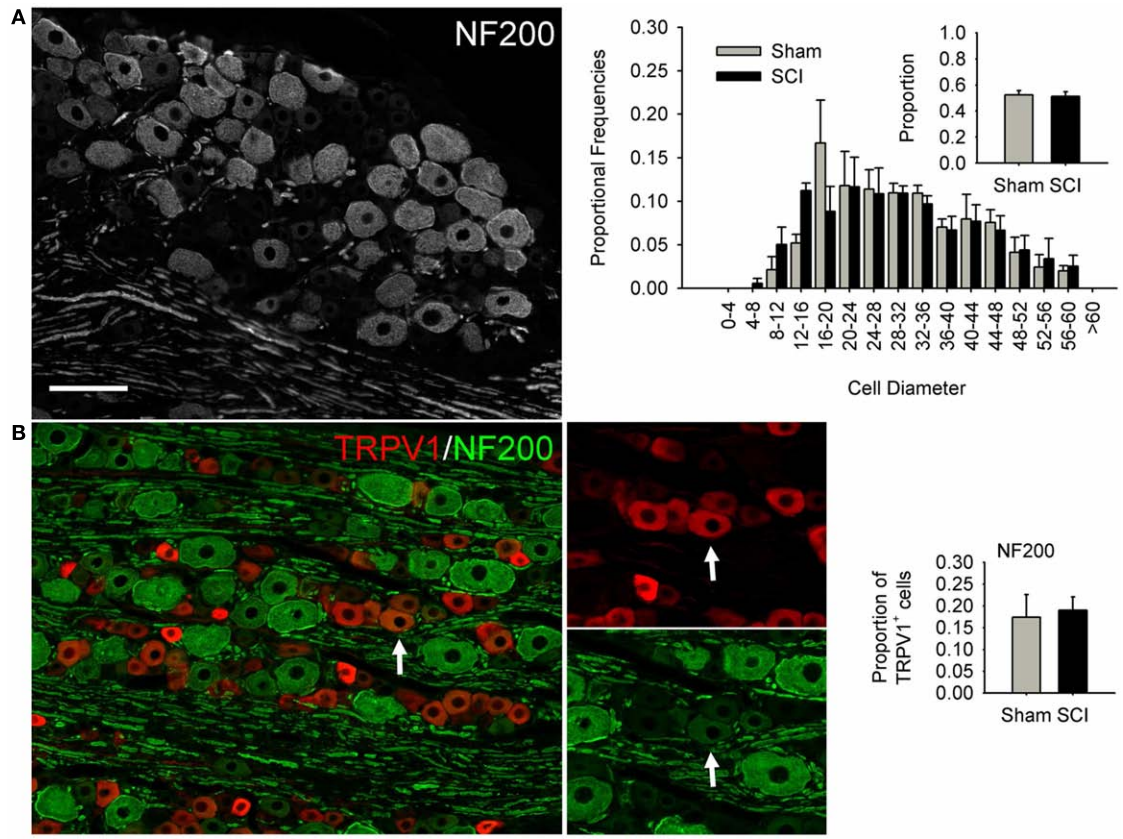

FIGURE 3 | High-thoracic (T3) spinal cord injury had no effect on medium-to-large sized neurons in the L4/L5 DRG expressing heavy neurofilament (NF200). (A) NF200-positive neurons did not undergo SCI-induced hypertrophy, nor did the proportion of neurons expressing NF200 change. **(B)** Hypertrophy of TRPV1-expressing DRG neurons was not accompanied by increased co-localization of TRPV1 and NF200. Ganglia were harvested 3 months after sham-injury (gray) or complete T3 SCI (black). Arrow: DRG neuron immunopositive for both TRPV1 and NF200. Scale bar = 70 μm.

DRGs with bladder-projecting afferents (L6/S1), in the absence of changes in DRGs with somatic afferents (L4/L5), were derived from rats with low-thoracic SCI (Zvarova et al., 2005). We therefore examined the size distribution of TRPV1-positive afferents in lumbosacral DRGs from animals with T10 complete SCI (**Figure 6**). One month after T10 complete SCI, TRPV1-positive neurons did not exhibit hypertrophy in L4/L5 DRGs; size distribution of TRPV1-expressing neurons was similar between animals with T10 SCI and sham-injured controls (sham; **Figure 6A**). In the L6/S1 DRGs, there was a small but significant rightward shift in the size distribution of TRPV1-positive neurons after T10 SCI (**Figure 6B**). Interestingly, hypertrophic changes induced by low-thoracic SCI were much less dramatic than those triggered by T3 SCI (compare **Figures 6B** and **4D**). This was surprising, given that both injuries induce hind limb paralysis and LUT dysfunction.

DRAMATIC SOMATIC HYPERTROPHY IN CAPSAICIN-SENSITIVE AFFERENTS WAS NOT REFLECTED IN PLASTICITY OF THEIR CENTRAL PROJECTIONS

Multiple studies have demonstrated that severe SCI triggers intraspinal sprouting of nociceptors (Krenz and Weaver, 1998; Weaver et al., 2001) and that sprouting of CGRP-expressing afferents in the dorsal horn is correlated with severity of AD (Krenz et al., 1999; Cameron et al., 2006). SCI also prompts a subset of DRG neurons, those expressing the pituitary adenylate cyclase activation peptide (PACAP), to expand their territory in the lumbosacral dorsal horn in segments containing visceral circuitry (L1, L2, L6, and S1; Zvarova et al., 2005). Since PACAP and CGRP partially co-localize with TRPV1 in DRG neurons (Moller et al., 1993), we measured the density of TRPV1-expressing terminals in the L4/L5 and L6/S1 dorsal horn, the central projections of afferents exhibiting the most pronounced hypertrophy after T3 SCI. Working from tiled mosaics of confocal z-stack projections (**Figure 7A**), we detected a slight but significant increase in density of TRPV1-positive terminals in the superficial laminae at two locations in the L4/L5 dorsal horn. Density of TRPV1-positive projections was increased superficially, in lamina I of the lateral dorsal horn and lamina II–III of the medial dorsal horn. There was no evidence of TRPV1-positive afferents sprouting into deeper laminae after SCI. There was also no difference in density of TRPV1-expressing afferents in the L6/S1 dorsal horn between sham-injured animals and animals with T3 SCI (**Figure 7B**). These results indicate that the CGRP- and PACAP-positive axons which were previously shown to sprout after SCI are not those which contain TRPV1.

We also examined density of TRPV1-positive projections to the DGC in the L6/S1 spinal cord, a region that receives input from visceral afferents, including those in the distal colon (Morgan et al., 1981; Hou et al., 2009). Densitometric analysis demonstrated that there was no effect of SCI on projections to the DGC (**Figure 7B**). Since we were working from cross-sections of spinal cord, we did not attempt quantitative measurements of TRPV1-positive projections to the lateral parasympathetic preganglionic nucleus (which is rostro-caudally periodic in the lumbosacral spinal cord; Morgan et al., 1981). There were no qualitatively apparent changes in the density of TRPV1-positive projections to parasympathetic preganglionic neurons after T3 SCI.

INTRATHECAL CAPSAICIN ATTENUATED COLO-RECTAL DISTENSION-INDUCED AUTONOMIC DYSREFLEXIA

The dramatic effects of T3 SCI on TRPV1-expressing afferents in L6/S1 DRGs prompted us to examine their contribution to the development of AD (**Figure 8**). We administered 50 μg of capsaicin in a single intrathecal injection at the L4 spinal cord, 28 days after T3 SCI ("late Cap.") or 48 h after T3 SCI ("early Cap."). Since TRPV1-positive sensory neurons exhibit spontaneous activity *de novo* as early as 24 h following SCI (Bedi et al., 2010), we hypothesized that early capsaicin treatment might have particularly pronounced effects on development of AD. Vehicle injections ("Veh.") were performed 28 days after SCI.

Consistent with previous findings, capsaicin injection produced a permanent degeneration of spinally projecting TRPV1-positive axons (**Figure 8A**; Yaksh et al., 1979). Efficacy of capsaicin was confirmed *via* densitometric measurements within the DGC (**Figure 8A**), which demonstrated a rapid and sustained depletion of TRPV1-positive projections. At 30 days after T3 SCI, carotid cannulae were implanted for beat-to-beat blood pressure measurements (**Figure 8B**). Capsaicin injection had no effects on blood pressure or HR at rest (**Figure 8C**). Animals that received capsaicin after T3 SCI exhibited much less severe AD in response to CRD (**Figure 8C**). There was no effect of differential timing of capsaicin injection following SCI: early and late capsaicin treatment produced equivalent reductions in CRD-induced hypertension and bradycardia. In both groups, the CRD-evoked change in SAP was reduced by approximately 50% relative to vehicle-treated animals, and CRD-induced bradycardia was dramatically attenuated.

DISCUSSION

These experiments demonstrate injury-induced hypertrophy in a specific subset of TRPV1-positive sensory neurons caudal to SCI. The response was most pronounced in lumbosacral DRGs and after high-thoracic SCI. Finally and notably, eliminating the central projections of TRPV1-expressing axons after T3 SCI *via* intrathecal capsaicin injection had pronounced mitigating effects on the severity of CRD-induced AD. Here we discuss the potential mechanisms of this SCI-provoked hypertrophy, its relationship to level of injury and the relevance for sensory-autonomic dysfunction following SCI.

SPINAL CORD INJURY-INDUCED HYPERTROPHY WAS RESTRICTED TO A SUBSET OF CAPSAICIN-SENSITIVE NEURONS

The selectivity of sensory neuron hypertrophy following SCI provides important clues about the underlying trophic mechanism.

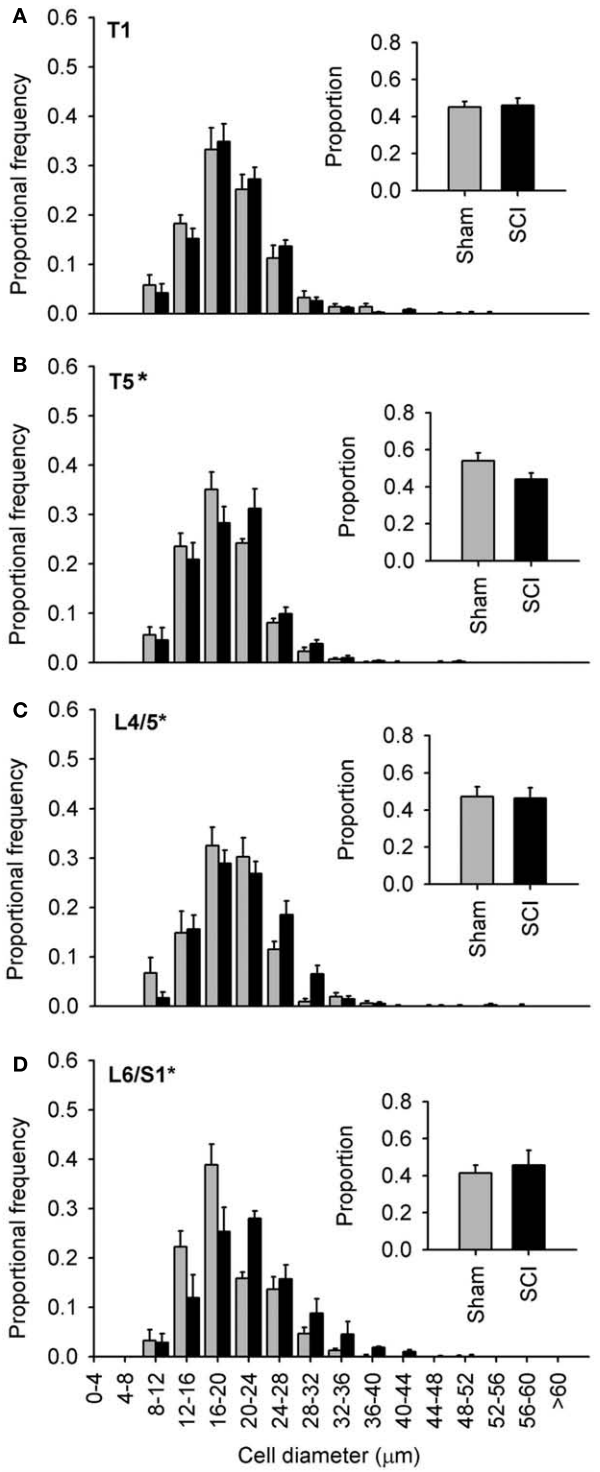

FIGURE 4 | Capsaicin-sensitive dorsal root ganglion neurons increased in diameter caudal to, but not rostral to, high-thoracic spinal cord injury. (A–D) Size-frequency distributions of TRPV1-positive neurons from a rostral DRG (T1) and caudal (T5, L4/L5, L6/S1) DRGs, harvested 1 month after sham- (gray) or complete T3 SCI (black). The increase in size is the most pronounced in the most caudal ganglia. Proportions of TRPV1-positive neurons at each level are shown in the insets **(A–D)**. Asterisks indicate $P < 0.05$, K–S goodness-of-fit test.

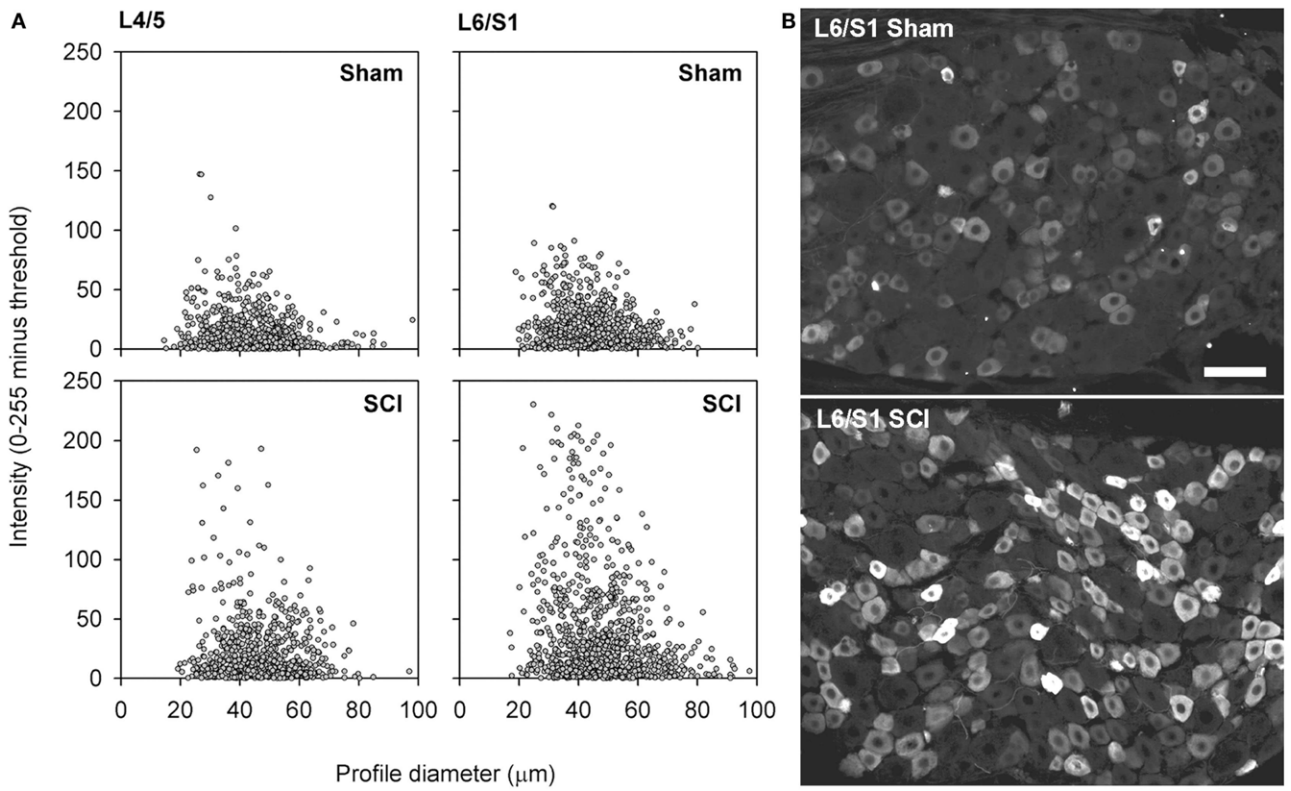

FIGURE 5 | Capsaicin-sensitive neurons in the dorsal root ganglion exhibited increased TRPV1 signal intensity following high-thoracic spinal cord injury. (A) Size-intensity scatter plots of TRPV1-positive DRG neurons showing a marked upward scatter with T3 SCI. The increase in TRPV1 intensity was particularly pronounced in L6/S1 DRGs. There was a significant increase in signal intensity at 3 months after T3 SCI for both L4/L5 DRGs and L6/S1 DRGs (K–S goodness-of-fit tests on cumulative intensity-frequency distributions). **(B)** Representative images of TRPV1-expression in the L6/S1 DRG, from a sham-injured control (top) and an animal with T3 SCI (bottom). Scale bar = 50 μm.

While the results demonstrate T3 SCI-induced hypertrophy of TRPV1-positive DRG neurons, it is clear that this is not universally true: that is, there must be a specific subset of TRPV1 neurons which responds to SCI by increasing in size. This assertion is based on the fact that a rightward shift in size-frequency distribution is detectable in the analysis of all (βIII-tubulin-labeled) neurons (**Figure 1**), but not for IB4/$P2X_3$ or SP subpopulations (**Figure 2**), each of which partially co-localizes with TRPV1 (Tominaga et al., 1998). Thus, the TRPV1-expressing neurons of interest express neither standard peptidergic nor non-peptidergic nociceptor markers (Michael and Priestley, 1999); these would have been included in the analysis of βIII-tubulin-positive profiles (i.e., all DRG neurons), but omitted from those involving SP, $P2X_3$, and IB4-positive cells. Thus, hypertrophy is largely or entirely restricted to a unique population of TRPV1-expressing neurons.

Analysis of neurons expressing GFRα3 (∼85% of which co-express TRPV1; Bennett et al., 2006) provides a further clue to the identity of this specific subset of nociceptors. The hypertrophic neurons are most likely sensitive to artemin, a GDNF family neurotrophic factor. Previous experiments have shown that peripheral over-expression of artemin in mouse keratinocytes not only leads to hypertrophy of DRG neurons, but also to upregulation of TRPV1 mRNA and increased capsaicin sensitivity (Elitt et al., 2006). Artemin is therefore a likely candidate for inducing hypertrophy following SCI. While there are no data describing expression of endogenous artemin after SCI, the pattern of injury-induced hypertrophy is suggestive.

SPINAL CORD INJURY-INDUCED HYPERTROPHY WAS PARTICULARLY DRAMATIC IN CAUDAL GANGLIA, FAR DISTAL TO INJURY

The ganglia in which size changes occurred also give cause for speculation on the molecular underpinnings of this response to injury. The inflammatory response to SCI is prolonged and well-characterized (Alexander and Popovich, 2009). The character of the inflammatory milieu evolves over time and is accompanied by production of cytokines and trophic factors which could act on the ganglia attached to the cord to induce hypertrophy. However, injury-induced hypertrophy was absent or modest in T1 and T5 DRGs (respectively) which argues against a central role for a factor produced at the site of SCI.

Pronounced hypertrophy occurred far distal to the site of T3 SCI, in lumbosacral DRGs. Interestingly, TRPV1-positive somata were enlarged in DRGs supplying predominately somatic (L4/L5) and predominately visceral (L6/S1) peripheral targets. This scenario represents a departure from the phenotypic changes that are restricted to visceral afferents after low-thoracic injury (Kruse et al., 1995; Qiao and Vizzard, 2002). TRPV1-expressing sensory neurons innervate skin, epithelia (notably in the bladder), and

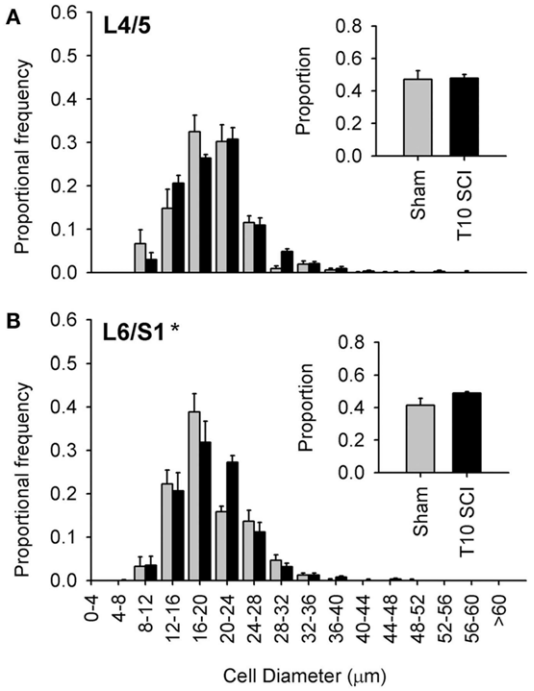

FIGURE 6 | Low-thoracic (T10) spinal cord injury elicited only modest changes in size of the most caudal capsaicin-sensitive dorsal root ganglion neurons. (A) No difference in size distributions of TRPV1-expressing DRG neurons in L4/L5 DRGs. **(B)** There was a small but significant rightward shift in the size-frequency distribution of L6/S1 DRG neurons ($P < 0.05$, K–S goodness-of-fit test), but this was much less dramatic than that which occurred after T3 SCI (see **Figure 4**). Ganglia were harvested 1 month after sham (gray) or complete T3 SCI (black).

both skeletal and smooth muscle in a variety of targets, including the colon and the bladder (Willis, 2007; Everaerts et al., 2008; Malin et al., 2011; Skryma et al., 2011; Yu et al., 2011). The structure and function of these target tissues are dramatically altered following SCI. Paralyzed skeletal muscle undergoes rapid and profound atrophy (Qin et al., 2010). In contrast, the smooth (detrusor) muscle of the bladder becomes hypertrophic over time following supraconal SCI, due to the combined effects of detrusor hyperactivity and detrusor-sphincter dyssynergia (Yoshimura, 1999). The bladder epithelium is also altered in chronic SCI (Smith et al., 2008). Less is known about remodeling of smooth muscle in the lower gastrointestinal (GI) tract after SCI. However, given the pronounced changes in lower GI function after SCI, including increased transit time that manifests clinically as constipation (Brading and Ramalingam, 2006), it seems reasonable to speculate that intrinsic smooth muscle of the distal colon also undergoes inactivity-induced remodeling following injury. The same logic applies to skin below the injury: while there are few data describing structural changes in skin, pressure ulcers are a common complication of SCI (Cardenas et al., 2004; Gelis et al., 2009). In view of the diverse changes in peripheral targets of TRPV1-positive lumbosacral DRG afferents after SCI, it is very likely that these afferents are exposed to different (or different amounts of) neurotrophic factors post-injury.

INJURY-INDUCED HYPERTROPHY WAS NOT ACCOMPANIED BY PRONOUNCED INTRASPINAL SPROUTING

The pronounced somatic hypertrophy in TRPV1-expressing afferents was not accompanied by an equally dramatic expansion of their central terminals in the spinal dorsal horn. Previous findings indicate that low-thoracic SCI prompts an increase in TRPV1 mRNA, but a reduction in TRPV1 expression demonstrated immunohistochemically, at and around the site of injury (DomBourian et al., 2006). Distal to SCI, the only available data report that there is no change in spinal distribution of TRPV1-positive projections after low-thoracic injury (Cruz et al., 2008). We observed a relatively minor increase in density that was restricted to the superficial laminae of the L4/L5 dorsal horn after T3 SCI. While somatic hypertrophy and axonal sprouting do not necessarily occur together, most trophic factors are capable of eliciting both. For example, in bladder afferents caudal to low-thoracic SCI, bladder-, and/or spinal cord-derived NGF is thought to mediate somatic hypertrophy (Yoshimura, 1999; Seki et al., 2002; Yoshimura et al., 2006), and intraspinal NGF also contributes to SCI-induced sprouting of peptidergic nociceptors in the dorsal horn (Christensen and Hulsebosch, 1997; Krenz et al., 1999; Cameron et al., 2006). In contrast to the distribution of CGRP-positive terminals in the dorsal horn caudal to high-thoracic SCI (Ondarza et al., 2003), TRPV1-positive afferents did not invade deeper laminae of the dorsal horn. In these experiments, we demonstrate something quite different after SCI: afferents that are constitutively present in the spinal dorsal horn undergo dramatic hypertrophy and receptor upregulation at the level of their soma (i.e., in the periphery). While the majority of data describe dramatic changes within the spinal cord caudal to high-thoracic SCI, creating the potential for altered connectivity within central sensory-sympathetic circuits, we demonstrate pronounced effects in the periphery. The potential for pathological sensory-sympathetic interactions in the periphery exists, and may also contribute to AD (Ramer et al., 2006). The absence of robust sprouting of TRPV1 axons in the spinal cord suggests that the stimulus is also peripheral, possibly in the DRG itself. An intraganglionic source of artemin, for example, might be satellite cells in the DRG: artemin is expressed in Schwann cells, which are phenotypically similar to satellite cells, and is upregulated in Schwann cells after peripheral nerve injury (Baloh et al., 1998).

INJURY-INDUCED HYPERTROPHY WAS MORE PRONOUNCED AFTER HIGH-THORACIC THAN LOW-THORACIC SPINAL CORD INJURY

The differential effects of complete SCI at T3 and T10 are interesting, given the similarities in many aspects of functional outcome. For example, T3 and T10 SCI induce hind limb paralysis and bladder dysfunction that is grossly similar: the bladder is initially areflexic and requires manual emptying until reflexive micturition is restored. The issue of LUT function is certainly relevant, since TRPV1-positive sensory neurons are critically involved in physiological bladder function (Araki, 2011) and appear to contribute to bladder dysfunction after SCI (Cruz et al., 2008). TRPV1-expressing afferents also mediate a number of pathological phenomena in the LUT, including bladder pain and overactive bladder (Eid, 2011; Kissin and Szallasi, 2011); recent findings suggest that these afferents also participate in pelvic organ cross-sensitization

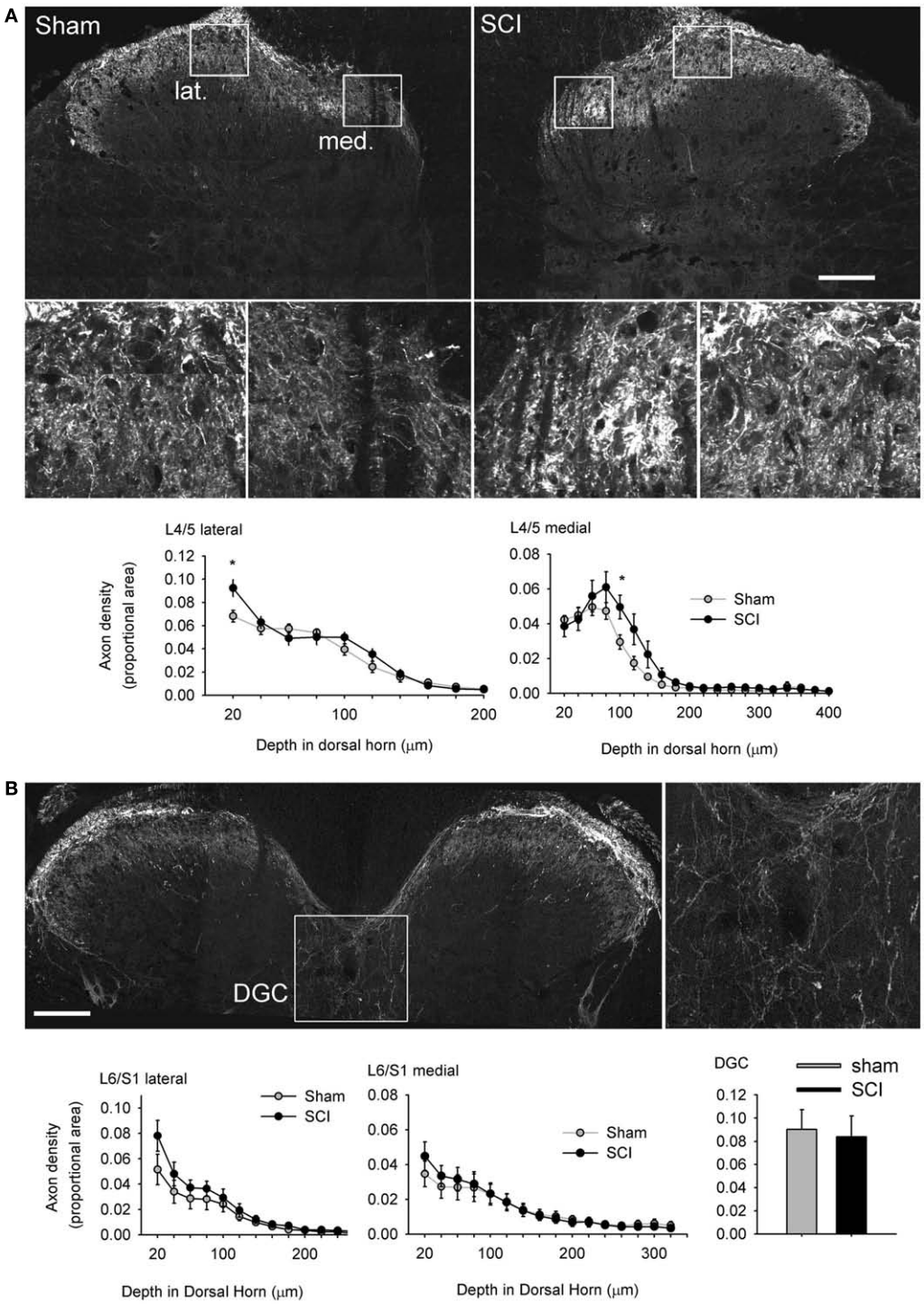

FIGURE 7 | Hypertrophy of capsaicin-sensitive afferents caudal to high-thoracic spinal cord injury was not accompanied by pronounced plasticity of their spinal projections. (A) One month after T3 complete SCI, there was a small but significant increase in TRPV1-positive axon density in the medial (med.) and lateral (lat.) parts of the most superficial laminae of the L4/L5 dorsal horn (boxed regions are shown enlarged). There was no evidence of TRPV1-positive axon extension into deeper laminae. **(B)** In the L6/S1 cord, there were no differences between sham-injured or T3 SCI animals in the dorsal horn, or in the dorsal gray commissure (DGC, boxed image enlarged to the right), the terminal field of the visceral medial collateral pathway. Asterisks: $P < 0.05$, Student's *t*-test. Scale bars = 200 μm.

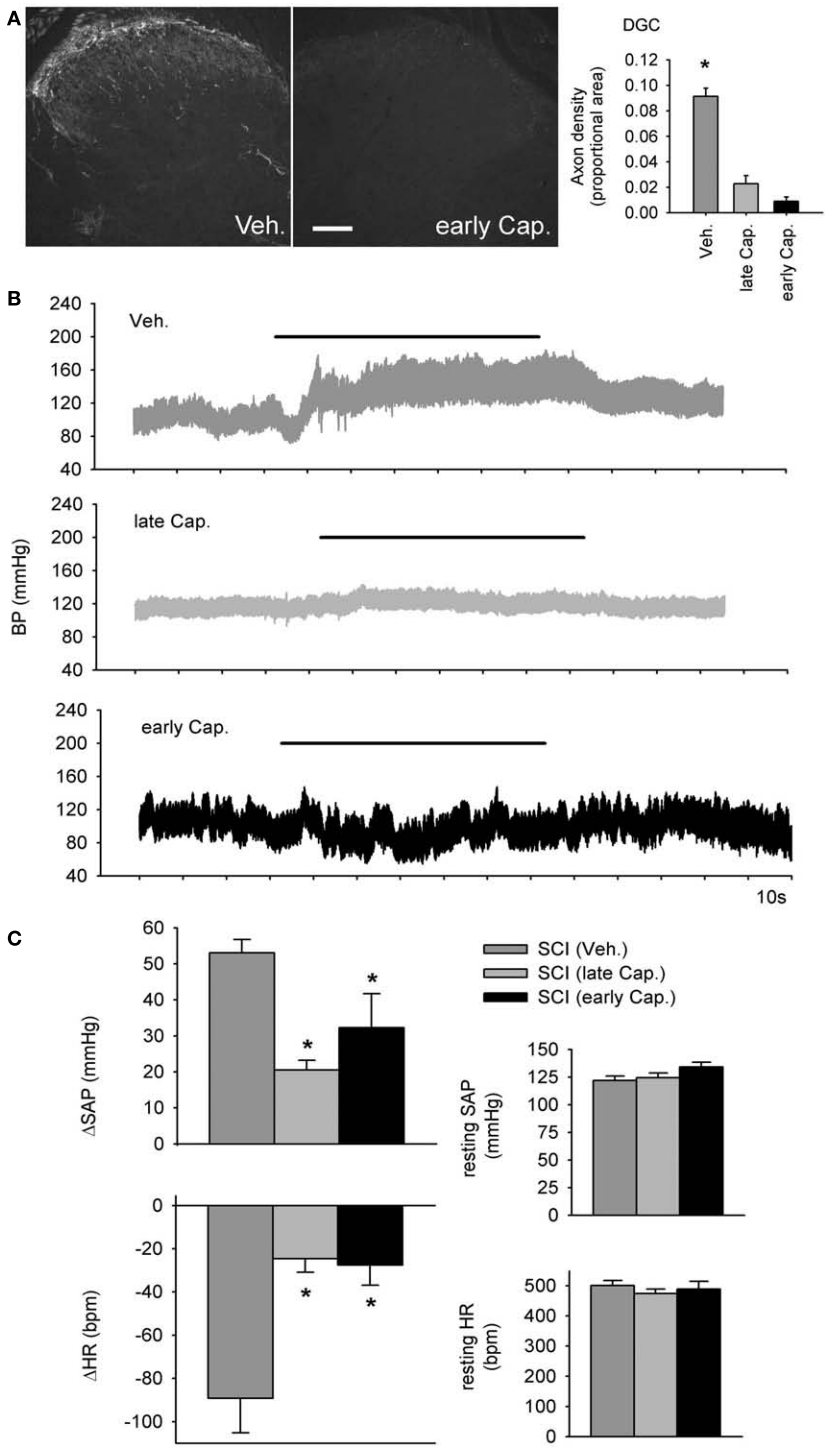

FIGURE 8 | Intrathecal capsaicin attenuated colo-rectal distention (CRD) -induced autonomic dysreflexia in animals that survived for 1 month after complete T3 spinal cord injury. (A) A single intrathecal bolus of capsaicin (10 μl of 5 mg/ml in 50% DMSO) resulted in permanent degeneration of spinally projecting TRPV1-positive axons. Quantification shows TRPV1 axon density in the dorsal gray commissure (DGC). Asterisk indicates significant difference between vehicle-treated T3 SCI animals and both capsaicin-treated groups ($P < 0.05$, one-way ANOVA). Scale bar = 200 μm. **(B)** Beat-to-beat changes in blood pressure in response to colo-rectal distension (horizontal bars) from animals treated with intrathecal vehicle (Veh.), early capsaicin (48 h following T3 SCI, early Cap.), and late capsaicin (48 h prior to physiological recording, 28 days post-T3 SCI, late Cap.). **(C)** Quantitative cardiovascular responses to CRD in vehicle and capsaicin-treated rats, 30 days post-SCI. Capsaicin treatment, whether administered 48 h or 28 days after SCI, mitigated CRD-induced increases in systemic arterial pressure (SAP) and decreases in heart rate (HR). Resting SAP and HR were unaffected by intrathecal capsaicin. Asterisks: $P < 0.05$, one-way ANOVA.

(Asfaw et al., 2011). However, SCI-induced changes were not restricted to bladder afferents after T3 SCI, since hypertrophy was also apparent in L4/L5 DRGs. One explanation might be that signals from the hypertrophic bladder contribute to injury-induced hypertrophy, but the predominant trigger is present in high-, but not low-thoracic SCI.

One notable difference in high- versus low-thoracic SCI that has been identified lies in the immune response to injury. In mice, immune suppression induced by SCI is level-dependent, such that mice with T3 SCI exhibit impaired antibody synthesis and elevated splenic norepinephrine, neither of which develop in mice with T9 injury (Lucin et al., 2007). If level-dependent immune suppression also occurs in rats with SCI, the inflammatory response in the DRG may also vary with level of injury, influenced by systemic activity of the immune system. The limited data that are available describe immune cell infiltration in DRGs caudal to T8 SCI (McKay and McLachlan, 2004): in this study, intra-ganglionic immune cell density was highest in DRGs closer to the lesion site (i.e., greater in T12 DRGs than in L6 DRGs). This pattern does not seem to fit with our results, but may be different after T3 SCI; alternatively, other factors modifying the environment of the DRG (such as satellite cells activation) may not vary in step with the local immune response.

The most dramatic difference in the functional outcomes of T3 and T10 SCI is the development of AD after the former, but not the latter injury. In AD, sensory stimulation evokes sympathetic contractions of vascular smooth muscle. It is not insignificant that artemin is developmentally expressed in vascular smooth muscle where it acts as a guidance cue for sympathetic (and probably also sensory) axons (Honma et al., 2002). In essential hypertension, chronic constriction of the blood vessels induces maladaptive remodeling of the vasculature (Rizzoni et al., 2007, 2009; Rehman and Schiffrin, 2010). Remodeling resulting in an increase in media-lumen ratio can occur *via* different mechanisms, including rearrangement of the same wall material around a narrowed lumen (eutrophic remodeling) or vascular smooth muscle cell growth (hypertrophic remodeling; Intengan and Schiffrin, 2001). While less is known about the effects of intermittent or episodic hypertension on vascular structure, one recent study indicates that carotid intima-media thickness is increased in individuals with SCI (Matos-Souza et al., 2009). The possibility of artemin upregulation in vascular smooth muscle has yet to be explored, but may contribute to the more pronounced hypertrophy of TRPV1/GFRα3 neurons following T3 SCI compared to injury at T10. TRPV1-positive afferents have collateral branches that supply blood vessels, particularly arterioles, in the submucosa of the gastrointestinal (GI) tract (Holzer, 2006). The vasculature of the GI tract is part of the splanchnic bed that is critically involved in blood pressure control, including the development of AD (Lujan et al., 2010): these arteries are known to be altered after high-thoracic SCI (Brock et al., 2006; Alan et al., 2010).

CAPSAICIN-SENSITIVE AFFERENTS CONTRIBUTED SUBSTANTIALLY TO COLO-RECTAL DISTENSION-INDUCED AUTONOMIC DYSREFLEXIA

Selective elimination of TRPV1-expressing afferents in the spinal dorsal horn dramatically reduced the severity of AD. Peripheral projections of TRPV1-positive afferents in the DRGs innervating the rectum and distal colon are found in smooth muscle and the mucosa and are mechanosensitive and/or chemosensitive (Lynn and Blackshaw, 1999; Berthoud et al., 2001; Ward et al., 2003). Neurons in the capsaicin-sensitive, mechanosensitive subset are known to respond to CRD (Brierley et al., 2005). Intrathecal capsaicin injected at L4 eliminated the central projections of TRPV1-positive spinal colonic afferents, which constitute approximately 50% of the lumbosacral colonic DRG neurons (Brierley et al., 2005). This is reflected in our data, demonstrating that CRD following intrathecal capsaicin injection still activates a subset of mechanosensitive afferents to elicit AD, although AD is dramatically reduced in severity (**Figure 8**).

We hypothesized that early elimination of TRPV1-expressing afferents would have even more pronounced effects on AD (than late capsaicin, administered at 28 days post-SCI). This premise was based in part on recent findings demonstrating spontaneous activity arising in the soma develops in DRGs after SCI (Bedi et al., 2010). There are several striking similarities between the patterns of *de novo* spontaneous activity and hypertrophy that emerge following SCI. Both phenomena develop caudal, but not rostral to SCI, and are most pronounced in distal DRGs, remote from the site of injury. Both occur in nociceptors, and most intriguingly, a high percentage of the afferents that exhibited spontaneous activity after T10 SCI were capsaicin-sensitive (Bedi et al., 2010). Cumulatively, these findings suggest that SCI has specific effects on TRPV1-expressing primary afferents. In bladder afferents, SCI-induced somatic hypertrophy is accompanied by increased excitability, including reduced thresholds for activation (Yoshimura, 1999). If hypertrophy is an anatomical surrogate for spontaneous activity after SCI, injury-induced ongoing activity in TRPV1-positive neurons might be more even more pronounced and/or prevalent after T3 SCI.

However, early and late capsaicin treatment had equivalent effects on AD (**Figure 8B**). From these findings, we cannot reliably determine whether TRPV1-positive afferents only instigate CRD-evoked AD, or both instigate AD and contribute to its development over time following SCI. A reversible TRPV1-block might distinguish between these two possibilities. Given the evidence for spontaneous activity in capsaicin-sensitive afferents caudal to SCI, this is a future direction with important clinical implications for AD, and potentially for pain.

CONCLUSION

Previous work has identified numerous mechanisms that might contribute to induction and progression of AD, and the list of putative mechanisms includes injury-induced changes in the vasculature and multiple components of the spinal sensory-sympathetic circuitry caudal to SCI (Krenz and Weaver, 1998; Krassioukov et al., 1999; Krenz et al., 1999; Brock et al., 2006; McLachlan and Brock, 2006). In terms of sensory plasticity, prior findings demonstrate that severity of AD is closely correlated to the extent of intraspinal nociceptor sprouting (Cameron et al., 2006). However, this is the first study to demonstrate AD mediated by a specific subset of afferents that exhibit pronounced somatic, but only slight central, injury-induced plasticity. Given the array of pronounced changes in peripheral targets of sensory neurons after SCI, it is not surprising that they respond to injury. Plasticity occurring outside the

CNS may represent a new and more accessible target for limiting sensory-autonomic dysfunction following SCI.

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Effects of gabapentin on muscle spasticity and both induced as well as spontaneous autonomic dysreflexia after complete spinal cord injury

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We recently reported that the neuropathic pain medication, gabapentin (GBP; Neurontin), significantly attenuated both noxious colorectal distension (CRD)-induced autonomic dysreflexia (AD) and tail pinch-induced spasticity compared to saline-treated cohorts 2–3 weeks after complete high thoracic (T4) spinal cord injury (SCI). Here we employed long-term blood pressure telemetry to test, firstly, the efficacy of daily versus acute GBP treatment in modulating AD and tail spasticity in response to noxious stimuli at 2 and 3 weeks post-injury. Secondly, we determined whether daily GBP alters baseline cardiovascular parameters, as well as spontaneous AD events detected using a novel algorithm based on blood pressure telemetry data. At both 14 and 21 days after SCI, irrespective of daily treatment, acute GBP given 1 h prior to stimulus significantly attenuated CRD-induced AD and pinch-evoked tail spasticity; conversely, acute saline had no such effects. Moreover, daily GBP did not alter 24 h mean arterial pressure (MAP) or heart rate (HR) values compared to saline treatment, nor did it reduce the incidence of spontaneous AD events compared to saline over the three week assessment period. Power spectral density (PSD) analysis of the MAP signals demonstrated relative power losses in mid frequency ranges (0.2–0.8 Hz) for all injured animals relative to low frequency MAP power (0.02–0.08 Hz). However, there was no significant difference between groups over time post-injury; hence, GBP had no effect on the persistent loss of MAP fluctuations in the mid frequency range after injury. In summary, the mechanism(s) by which acute GBP treatment mitigate aberrant somatosensory and cardiophysiological responses to noxious stimuli after SCI remain unclear. Nevertheless, with further refinements in defining the dynamics associated with AD events, such as eliminating requisite concomitant bradycardia, the objective repeatability of automatic detection of hypertensive crises provides a potentially useful tool for assessing autonomic function pre- and post-SCI, in conjunction with experimental pharmacotherapeutics for neuropathic pain, such as GBP.

Keywords: neuropathic pain, colorectal distension, power spectral density, telemetry, blood pressure, heart rate

INTRODUCTION

Spinal cord injury (SCI) is a serious health care problem in the United States striking, on average, 12,000 individuals each year. Approximately 270,000 Americans are living with the typically devastating neurological deficits and debilitating somatic and autonomic reflexes in chronic SCI (see https://www.nscisc.uab.edu). In particular, complete as well as incomplete SCI above high-thoracic levels can lead to a potentially life-threatening hypertensive condition termed autonomic dysreflexia (AD) that is often triggered by noxious somatic or visceral stimuli below the injury level (Karlsson, 1999). Due to the disruption of descending modulating pathways from the brainstem, this syndrome is characterized by episodic, sympathetically-driven reflexive hypertension which is usually accompanied by intact baroreflex-mediated bradycardia (Rabchevsky, 2006). Episodes of AD often cause debilitating symptoms including pounding headache, acute anxiety, shivering, flushing and sweating (Kewalramani, 1980). One of the most common triggers of AD is the distension of pelvic viscera (bladder and bowel) (Snow et al., 1978; Harati, 1997; Krassioukov et al., 2003).

The development of animal models of noxious colorectal distension (CRD)-induced AD (Krassioukov and Weaver, 1995; Rivas et al., 1995) to mimic clinical manifestations of fecal impaction have enabled the detailed analysis of temporal

dynamics of CRD-induced hypertension (Maiorov et al., 1997b, 1998). Accordingly, the development and severity of AD has been correlated with extent of aberrant sprouting of nociceptive C-fibers into the spinal cord below the injury (Krenz et al., 1999; Marsh et al., 2002; Cameron et al., 2006), and glutamatergic neurotransmission has been shown to contribute to spinal viscerosympathetic initiation of episodic hypertension (Maiorov et al., 1997a).

There is currently no single clinical intervention which effectively attenuates the manifestation of both muscle spasticity and AD stemming from SCI (Rabchevsky and Kitzman, 2011). Notably, however, one compound that has been shown to interfere with glutamatergic transmission and is safe for clinical use is gabapentin (GBP, Neurontin®; Pfizer, New York, NY, USA), which is approved for the treatment of epilepsy and is widely used off-label for the treatment of neuropathic pain (Kitzman et al., 2007). Accordingly, we have been testing the hypothesis that GBP can alleviate both spasticity and AD by impeding neurotransmission of noxious stimuli into the spinal cord, thus eliminating a critical physiological link between these aberrant reflexes. To this end, we recently reported that acute GBP administration significantly attenuates both AD and tail spasticity induced by noxious stimuli compared to saline-treated cohorts at 2–3 weeks post-injury (Rabchevsky et al., 2011).

In order to further characterize the effects of GBP and determine whether it can be administered as a prophylactic, here we report the results of long-term telemetry experiments designed to assess (1) whether daily GBP versus saline alters baseline cardiovascular parameters, (2) the efficacy of chronic, daily GBP treatment versus acute administration in modulating experimentally-induced AD and tail spasticity over 3 weeks post-injury, and (3) whether daily GBP modulates spontaneous AD events detected using a novel algorithm we have developed based on blood pressure telemetry data.

MATERIALS AND METHODS

SURGICAL METHODS; IMPLANTATION OF BLOOD PRESSURE TELEMETRY DEVICES IN DESCENDING AORTA

As described in detail (Rabchevsky et al., 2011), seven days prior to T4 transection SCI, naïve anesthetized (ketamine, 80 mg/kg, i.p.; xylazine 7 mg/kg, i.p.) rats ($n = 12$) were implanted with telemetric pressure transmitters (model TA11PA-C40, Data Sciences International, Inc., St. Paul, MN) into the descending aorta after its brief occlusion and securing the probe to the abdominal wall with silk sutures. The skin was closed with surgical staples after rinsing abdominal cavity with saline. The animals were then treated post-operatively, as described below, and blood pressure was monitored 24/7 to ensure patency of the probes and to obtain pre-injury baseline data.

SURGICAL METHODS; SPINAL CORD INJURY

All surgical procedures were performed under aseptic conditions using sterilized instruments, following the University of Kentucky IACUC and the NIH guidelines. One week following telemetry implantation, the T4 spinal segment of anesthetized (ketamine, 80 mg/kg, i.p.; xylazine 7 mg/kg, i.p.) female Wistar rats (~225 g) was exposed by T3 laminectomy ($n = 12$) and the spinal cord was completely transected with a scalpel blade before hemostasis was achieved with gelfoam placed into the resection site, as previously detailed (Cameron et al., 2006; Rabchevsky et al., 2011). Wounds were then irrigated with sterile saline, the muscles sutured using 3-0 vicryl and skin openings stapled with wound clips. Injured rats were housed one per cage with food and water *ad libitum*, placed on a heating pad during recovery, and injected with 10 ml Lactated Ringer's solution s.c. for fluid replacement. Upon regaining consciousness, post-surgery pain was alleviated by administering buprenorphine (0.02–0.05 mg/kg, s.c., Reckitt Benckiser, Hull, UK) twice a day for three days. The injured animals required manual bladder evacuation twice a day for 2–3 weeks post-injury until spontaneous bladder voiding returned with no signs of urinary tract infection. They also received twice daily injections (s.c.) of antibiotics (33.3 mg/kg Cefazolin, s.c., SoloPak Laboratories, New Gove, IL) and Ringer's for 5 days.

TELEMETRIC MONITORING OF BLOOD PRESSURE BEFORE AND AFTER SCI

Following transection SCI, the Dataquest A.R.T. system (Data Sciences International, Inc., St. Paul, MN) was used for 24/7 telemetric monitoring of pulsatile arterial blood pressure (PAP), as well as on-demand monitoring of blood pressure prior to, during, and after noxious CRD. CRD was performed on four separate days following SCI. Specifically, on days 14, 15, 21, and 22, injured animals in both chronic treatment groups were injected with either acute GBP or saline 1 h prior to cardiophysiology in response to CRD (see **Table 1**). For each gently restrained conscious rat, a period of 15 min was allowed to pass to allow them to become quiet after carefully inserting a latex balloon-tipped catheter (Swan-Ganz Paceport catheter; Baxter Healthcare Corporation, CA) 2 cm inside the rectum and securing it to the tail with tape. Prior to each initiation of spinal viscero-sympathetic reflexes, 30 s of baseline arterial pressure was recorded. Measurements continued during the gradual (15 s) inflation of the 10 mm long balloon inflation (CRD) with 2 ml of air for a period of 60 s, followed by another 30 s following balloon deflation. Such CRD expands the colon as would several large fecal boluses. During a recording period, animals within individual cages were placed upon receiver plates and PAP readings were transmitted to a receiver (PhysioTel Receiver, RPC-1 from Data Sciences International) as a radio frequency signal integrated by a data exchange matrix. The two traces for each animal trial were saved and stored in a selected file for subsequent analyses. Thus, two separate trials were conducted for each animal separated by ~30 min on each of the four testing days. The average MAP and HR values over the entire dynamic 60 s CRD period were calculated prior to averaging the values over the two trials for each animal; the mean MAP and HR values across each treatment group were then derived from summing individual animal averages.

BEHAVIORAL ASSESSMENT FOR SPASTICITY IN THE TAIL MUSCLES

Between the two trials for each of the four separate days that CRD was performed (~30 min), spasticity in the tail was assessed behaviorally using an established scale (Kitzman, 2006; Kitzman

et al., 2007). Specifically, the response of the tail muscles to a quick stretch, light stroking (non-noxious stimulus) and a light pinch (noxious stimulus) applied approximately 10 cm from the tip of the tail were assessed. Tail manipulations were performed with the animals lightly restrained and the tail was free to move over its full length. In this study, the animals displayed either a stage-2, stage-3, or stage-4 spasticity prior to initiating pharmacological intervention (see **Table 2**). For the purpose of statistical analysis, responses to quick stretch and pinch were graded using a five point scale in which 0 = minimal ($\leq$45° flexion) response to the stimulus, 1 = 45–90° flexion, 2 = >90–180° flexion, 3 = >180–225° flexion, 4 = >225–360° flexion, and 5 = significant coiling of the tail and/or activation of flexors, extensors, and abductors (writhing) lasting >2 s and the presence of clonus. The response to light touch was scored using a three point grading scale in which 0 = no response, 1 = minimal flexion of the tail away from the stimulus, and 2 = pronounced flexing of the tail away from the stimulus.

DRUG ADMINISTRATION

Every morning at 9:00 am, beginning the day after SCI, animals received a daily i.p. injection of either GBP (50 mg/kg; Neurontin) or saline vehicle. Notably, however, on days 15 and 22 the treatment groups were reversed to assess the effects of acute versus chronic GBP administration on the cardiophysiological responses to CRD and tail manipulations (see **Tables 1** and **2**). Importantly, the half-life of GBP is 5–9 h in humans, which is unaltered following multiple dosing (Goa and Sorkin, 1993; McLean, 1995). Accordingly, when a person receives GBP 3–4 times per day, the half-life is still reported as being 5–9 h; thus the required multiple dosages each day. If the half-life altered with multiple dosages, then once a patient reached the therapeutic dosage (typically 3–4 dosages per day), over time one might expect to be able to decrease the number of dosages per day since the therapeutic half-life would increase. However, this does not appear to be the case with GBP.

DETECTING SPONTANEOUS INCIDENCES OF AUTONOMIC DYSREFLEXIA

An algorithm was developed to automatically detect spontaneous AD events based on the 24 h blood pressure and heart rate (HR) telemetry data. Concurrent values for mean arterial blood pressure (MAP) and HR were recorded using DataQuest (Data Sciences International, Inc., St. Paul, MN) at 2 s intervals for 4 days pre-injury and 22 days following T4 spinal cord transection. Example waveforms of MAP and HR are shown in **Figures 1** and **4**. The algorithm processed these waveforms to effectively estimate the number of instances where an abnormally sharp MAP increase was accompanied by HR decrease, as illustrated in **Figure 3**. This was implemented with a program written in Matlab (The MathWorks, Inc., Natick, MA).

The MAP and HR signals were initially low-pass filtered at a 0.04 Hz cut-off frequency with a 6th order Butterworth filter to smooth out transient spikes (less that 12.5 s duration) and limit their impact on threshold crossings. A baseline comparison for MAP was created by a moving average window of 240 s. This was used for comparing the MAP values at 25 s after the end of the averaging window. Note that in **Figure 3**, the MAP values were delayed by 25 s relative to the baseline average so comparisons could be more easily visualized. A MAP peak was associated with a detected AD event when simultaneous numerical conditions were met, as illustrated in **Figure 3**. The following are the conditions with values used in the algorithm for results presented in this report:

(1) MAP peak exceeds the baseline by $T_p = 10$ mm Hg or greater.
(2) The difference between the MAP peak value and the MAP minimum value within the previous $T_r = 35$ seconds must be $T_s = 20$ mm Hg or greater than this peak (i.e., MAP swing must be sufficiently large and fast).
(3) The HR must drop by $T_h = 10$ bpm or greater within the MAP event interval, defined as staring with the MAP exceeding T_p and ending when it drops below this same value (see example in **Figure 3A**). The maximum HR associated with the drop must occur within the first 75% of this interval and the minimum HR value must occur after this maximum value and within 5 s beyond the end of the MAP interval (i.e., the HR drop must be sufficiently close to the elevated MAP event).

SPECTRAL ANALYSIS

To examine persistent MAP dynamics between groups, a power spectral density (PSD) analysis was performed. This analysis complements the result for the spontaneous AD events in that it detects differences in persistent dynamics, since it averages over the observation interval. Sparse transient events are averaged out in this case. The PSDs were computed using Welch's method (Proakis and Manolakis, 1996) from data recorded for 4 h following the daily injections. The raw pulsatile blood pressure signal was originally sampled at 512 Hz, and re-sampled to 50 Hz after an anti-aliasing filter was applied. Spectral magnitudes were computed from 128 s segments with linear trends removed, and a 50% overlap was used between consecutive segments.

SPINAL CORD TISSUE PROCESSING AND HISTOLOGY

After final analyses, all injured rats were overdosed with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde in PBS. Dissected spinal cords from T4-transected rats were stored for long term storage at 4°C in 20% sucrose/PBS containing 0.02% sodium azide.

STATISTICS

All data were both collected and analyzed in a blinded manner, as routinely performed in our published pharmacological studies. Notably, all analyses were performed by individuals blinded with respect to treatment. For comparisons of CRD-induced MAP and HR changes, unpaired Student's *t*-tests, with bonferroni correction factor when appropriate, were used between saline- and GBP-treated groups. Specifically, we compared the average MAP and HR values over the entire 60 s CRD period, and averaged the values over the two trials for each animal. To compare AD events over time between groups, a repeated measures analysis of

variance (ANOVA) was performed, followed by Fischer's PLSD when appropriate. Each behavioral test (tail responses to stretch, noxious pinch, and non-noxious light touch, as well as presence of clonus) was compared using the Mann-Whitney U test for ordinal data. PSD data were analyzed via power ratios between low and mid frequencies ranges and 95% confidence limits for each group were computed. Statistical significance was set *a priori* at $p < 0.05$ for all analyses.

VALIDATION OF ALGORITHM

The parameters of the AD algorithm were optimized using a data set consisting of 156 h of data labeled by an observer. The level of agreement between human observation and the automatic algorithm was assessed by estimating the probability of agreement on humanly detected AD events (47) from 169 h of telemetry data from 3 SCI rats. The results are described by two metrics, a probability of positive agreement (when the algorithm and observer identified the same time segment as containing an AD event), as well as a negative agreement rate (how often per second the algorithm identifies an AD event when the human observer did not). The test resulted in a positive agreement probability of 0.87 and a negative agreement rate of 6.0×10^{-5} per second (approximately five detections per day). In addition, the algorithm was applied to 72 h of pre-injury data (where no AD events are expected) and resulted in two detections per day, which is considered a false-detection rate of 2.3×10^{-5} per second. Given the expected level of human error, especially for large data sets, this is considered a good agreement.

RESULTS

EFFECTS OF DAILY GBP TREATMENT ON RESTING BLOOD PRESSURE AND HEART RATE

During the experiment, one GBP-treated animal died shortly after surgery for uncertain reasons. All animals were housed one per cage and **Figure 1** demonstrates blood pressure recordings gathered telemetrically from a rat prior to SCI over a 24 h period. Every morning, beginning the day after SCI, the injured animals received an injection (i.p.) of either GBP or saline vehicle. As depicted in **Figure 2**, assessments over 24 h periods across days post-injury revealed no significant treatment effect ($p > 0.1$) on average daily MAP and HR values, but there was a significant effect of days post-injury on both MAP [$F_{(1,\ 21)} = 7.282$, $p < 0.0001$] and HR [$F_{(1,\ 21)} = 13.23$, $p < 0.0001$] values. *Post-hoc* analyses revealed a significant ($p < 0.05$) decrease in MAP values at 1 day post-injury (DPI) compared to pre-injury values, as well as compared to 3 and 4 DPI. The ensuing MAP values between 5 and 13 DPI were again significantly ($p < 0.05$) lower compared to pre-injury. Notably, however, from 14 to 22 DPI there were significant ($p < 0.05$) elevations of daily MAP compared to 5–13 DPI that approximated pre-injury values. For accompanying HR values, there were significant ($p < 0.05$) decreases from 8 to 13 DPI compared to 1 DPI which was followed by significant ($p < 0.05$), diametrically opposite increases in HR values from 14 to 22 DPI compared to 1 DPI. However, there was no significant treatment by days effect on either daily MAP [$F_{(1,\ 21)} = 0.865$; $p = 0.636$] or HR [$F_{(1,\ 21)} = 0.383$; $p = 0.994$]. Overall, there appeared to be a

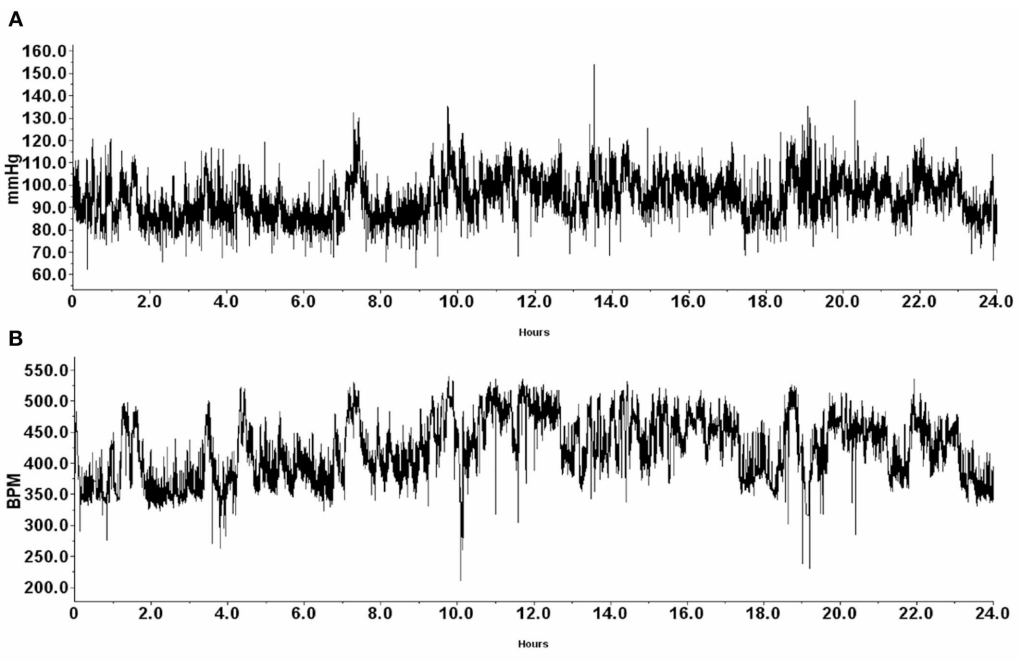

FIGURE 1 | Representative traces of mean arterial pressure (A; mmHg) and heart rate (B; BPM) recorded from a naïve animal 1 day prior to T4 spinal cord transection. Data was gathered telemetrically over a 24-h period using an implantable probe that was inserted into the descending aorta. Animals were housed one per cage to allow for individual telemetric monitoring. Receivers placed beneath each animal's cage telemetrically relayed signals into a data exchange matrix, where the signal was integrated into files to be opened with the Dataquest A.R.T. software package for subsequent analyses.

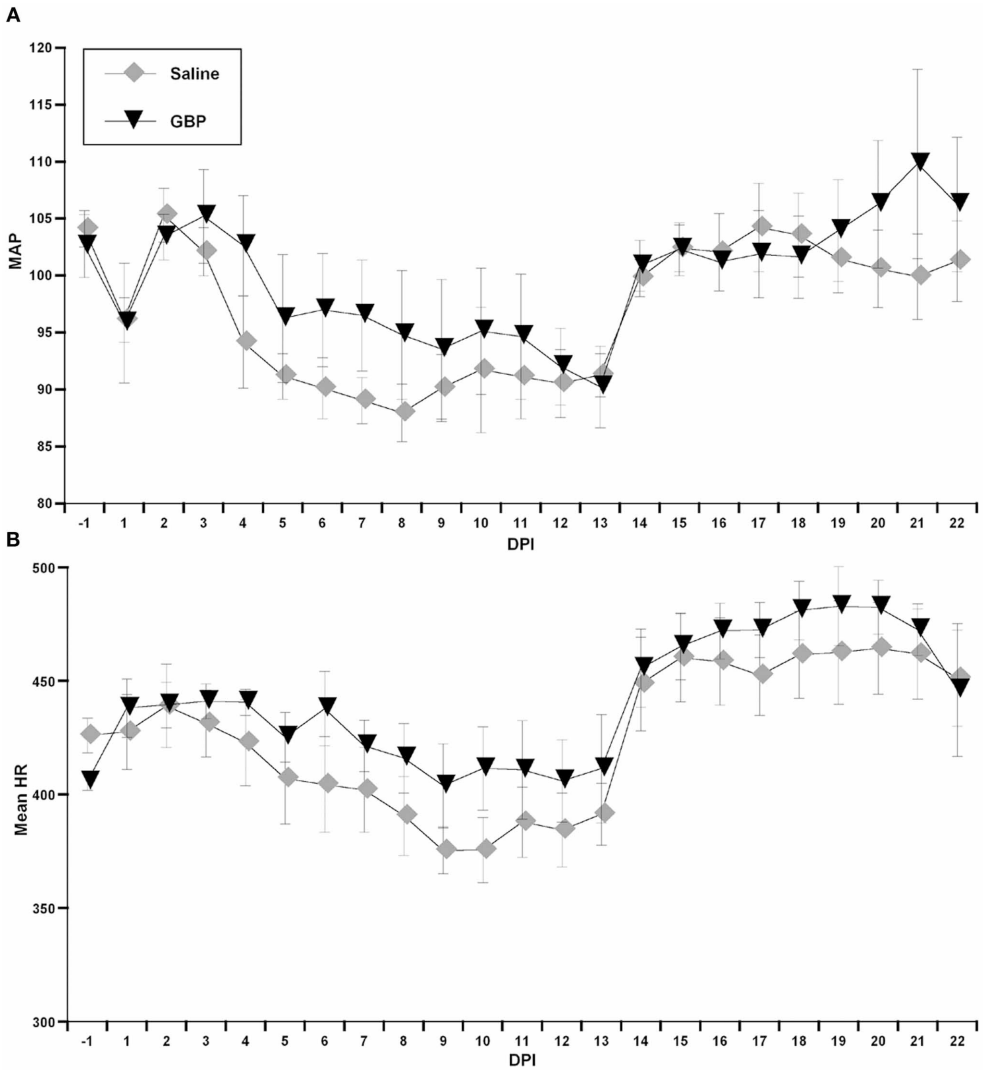

FIGURE 2 | Graphs representing both mean arterial pressure (A, MAP) and heart rate (B, HR in beats per minute) across days post-injury (DPI) gathered telemetrically over 24 h periods, beginning at 9:00 am following injections of either Saline or gabapentin (GBP). Due to the overall variability in both outcome measures, there were no significant differences between treatment groups for daily MAP and HR values. The conspicuous sustained elevation in both daily MAP and HR values beginning on days 14–15 post-injury that approached pre-injury values are interesting. $n = 6$ Saline; $n = 5$ GBP (50 mg/kg). Symbols represent group means ± SEM error bars.

conspicuous elevation in both MAP and HR values beginning two weeks post-injury that remained elevated, approaching pre-injury values; the significance of this alteration is uncertain, although it does correspond to the development of AD in this model.

ACUTE, NOT CHRONIC GBP TREATMENT REDUCES INDUCED AUTONOMIC DYSREFLEXIA AND TAIL MUSCLE SPASTICITY

As detailed in "Materials and Methods" and depicted in **Table 1**, on days 15 and 22, the animals receiving GBP every morning were injected with a single dose of saline (acute saline) 1 h prior to assessments, whereas the animals receiving saline every morning were given a single dose of GBP (acute GBP) 1 h prior to assessments. It was found that only acute GBP treatment significantly reduced CRD-induced MAP increases by approximately two-fold compared to acute saline treatment, irrespective of chronic morning treatments. Bradycardia also appeared reduced with GBP at all time points examined, but variability precluded significant differences. As detailed in "Materials and Methods" and depicted in **Table 2**, when tail spasticity was assessed in the same injured animals in response to light touch, stretch and noxious pinch, only acute GBP treatment had a significant and striking effect in virtually abolishing all three measures of tail spasticity.

EFFECT OF DAILY GBP TREATMENT ON THE INCIDENCE OF SPONTANEOUS AUTONOMIC DYSREFLEXIA

We developed a novel algorithm to detect spontaneous events of AD based on the 24 h MAP and HR telemetry data. The algorithm was developed and thresholds determined from a sample data set

Table 1 | Quantified changes in mean arterial pressure (MAP) and heart rate (HR) measurements from baseline during one minute of noxious colorectal distension (CRD) in injured rats treated chronically (daily morning) with either Saline or GBP.

Acute administration (Time points)		Chronic treatment groups			
		MAP (mmHg)		HR (beats per min)	
		Saline	GBP	Saline	GBP
Saline	14 DPI	19.7 ± 2.5	–	−43.8 ± 12.2	–
GBP		–	9.0 ± 3.6*	–	4.1 ± 10.9*
Saline	15 DPI	–	26.3 ± 3.8	–	−13.7 ± 10.1
GBP		5.9 ± 1.1***	–	−16.0 ± 4.6	–
Saline	21 DPI	38.0 ± 3.4	–	−36.1 ± 19.5	–
GBP		–	14.7 ± 4.7**	–	−16.3 ± 15.5
Saline	22 DPI	–	34.3 ± 3.5	–	−50.9 ± 22.0
GBP		19.4 ± 3.1*	–	−6.8 ± 7.2	–

*Both chronic treatment groups were subjected to CRD after either Acute GBP or Acute Saline injections prior to assessments on alternating days (days 14–15 and 21–22). Acute GBP treatment significantly reduced CRD-induced MAP increases by approximately two-fold compared to Acute Saline treatment, irrespective of Chronic (daily morning) Treatment Groups. While bradycardia appeared to be reduced at all time points examined, the variability precluded significant differences. Values represent group means ± SEM. *$p \leq 0.05$, **$p \leq 0.005$ and ***$p \leq 0.001$ between Acute GBP versus Acute Saline groups at all Administration Time Points.*

Table 2 | Behavioral responses of the tail musculature 2–3 weeks following T4 spinal cord transection (same rats as in Table 1) in response to Touch (Left), Stretch (Middle) and Pinch (Right) 1 h following the administration of Saline or GBP.

Acute administration (Time points)		Chronic treatment groups					
		Saline	GBP	Saline	GBP	Saline	GBP
		Tail touch		Tail stretch		Tail pinch	
Saline	14 DPI	2.0 ± 0.2	–	5.0 ± 0.2	–	5.0 ± 0.0	–
GBP		–	0.0 ± 0.2*	–	0.0 ± 0.4*	–	1.0 ± 0.4*
Saline	15 DPI	–	2.0 ± 0.0	–	5.0 ± 0.4	–	5.0 ± 0.6
GBP		0.0 ± 0.2*	–	0.0 ± 0.3*	–	0.0 ± 0.2*	–
Saline	21 DPI	2.0 ± 0.2	–	5.0 ± 0.0	–	5.0 ± 0.0	–
GBP		–	0.0 ± 0.2*	–	0.0 ± 0.4*	–	0.0 ± 0.4*
Saline	22 DPI	–	2.0 ± 0.0	–	5.0 ± 0.0	–	5.0 ± 0.0
GBP		0.5 ± 0.3**	–	0.0 ± 0.8**	–	0.0 ± 0.8**	–

*Response to Tail Touch was scored on a 2 point scale in which 0 = no spasticity and 2 = severe spasticity. Responses to Tail Stretch and Tail Pinch were scored on a 5 point scale in which 0 = no spasticity and 5 = severe spasticity. Quantitative analyses confirmed the striking effect of Acute GBP treatment in virtually abolishing all three measures of tail spasticity, irrespective of the Chronic (daily) treatment over days post-injury (**DPI**). *$p < 0.05$ Values represent group medians ± SEM for visualization purposes only.*

with AD events labeled by human observers of the MAP, baseline MAP, and HR from several animals (about 60 h of data). An illustration of a detected AD event is shown in **Figure 3**. To establish the algorithm relative to human identified events, the algorithm was run on an independent set of test data where AD events were identified by human observers (see "Materials and Methods," Statistical analyses). Results showed an agreement of greater than 80% with humanly detected events. For the times corresponding to no humanly detected AD events, the algorithm detected events at a rate of 0.0004 events per second, which corresponds to about 1 event every 42 min.

Through validation of humanly observed events that the program detected (indicated by green and red lines in **Figure 4**), the optimal parameters which corroborated humanly observed events were subsequently used to calculate spontaneous events of AD in both treatment groups over days post-injury. Notably, when the pre-injury data was analyzed with these algorithm parameters, event detections were infrequent (1–3 events/day in the 4 days prior to injury) relative to post-injury detections (5–30/day). This infers that the algorithm detects aberrant physiology rather than typical MAP and HR dynamics.

When the algorithm was applied to the data sets from both treatment groups, it calculated the number of detected spontaneous AD events over days post-injury (**Figure 5**). In particular, it was used to generate both the number of detected AD events over 24 h periods between the chronic saline and GBP treatment groups (**Figure 5A**), as well as the number of detected AD events in the first 4 h following daily saline and GBP injections (**Figure 5B**). In both cases, negligible events were detected prior to injury, as expected based on the algorithm parameters.

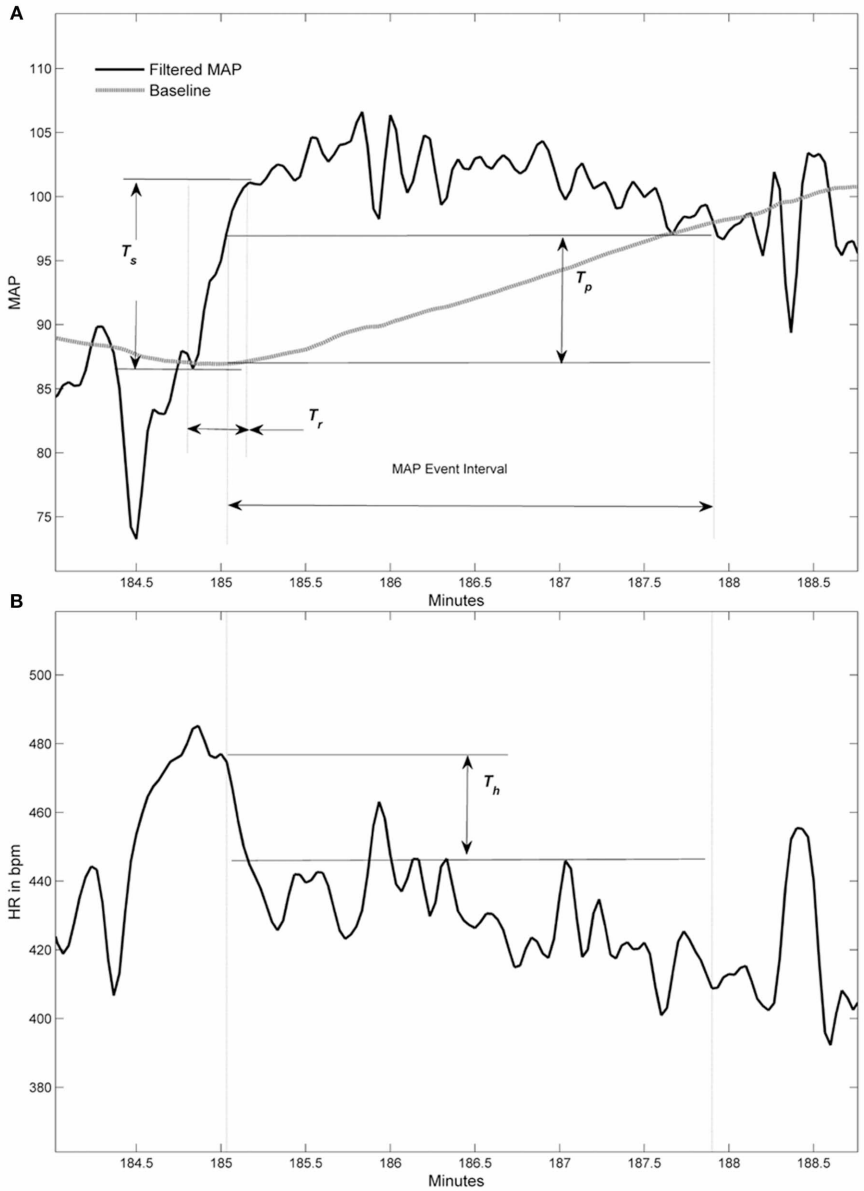

FIGURE 3 | Graphic examples of conditions used to detect an autonomic dysreflexia (AD) event. (A) Filtered mean arterial pressure (MAP) signal with baseline (gray line) and three thresholds used in conditions for event. **(B)** Corresponding filtered heart rate (HR) signal within MAP event interval shows threshold for HR drop condition. Heart rate signal does not have a baseline.

In the first several days following SCI, there appeared to be inexplicable detected AD events only in the GBP treatment group. Subsequently, however, until two weeks post-injury, the detected AD events remained only marginally elevated from pre-injury values for both groups. After 14 days post-injury, there appeared to be increases in the number of AD events detected in both treatment groups compared to pre-injury values; over 24 h or 4 h after daily injections. When a repeated measures ANOVA was run between treatment groups and the numbers of detected AD events across 22 days post-injury, there was no significant treatment effect ($p > 0.1$). When the data between treatment groups was collapsed and assessed similarly from 14 days post-injury onwards, a time when severe AD is known to be present, there was still no significant treatment effect ($p > 0.1$) over the 24 h or first 4 h periods (**Figures 5A,B**). While there was a significant effect of days post-injury for 24 h period values [$F_{(1,\ 8)} = 2.921$; $p < 0.01$], this was not the case for the first 4 h post daily injection [$F_{(1,\ 8)} = 1.170$; $p = 0.329$]. Accordingly, there was no significant treatment by days interaction for AD events over 24 h periods [$F_{(1,\ 8)} = 0.974$; $p = 0.463$] (**Figure 5A**) or during the first 4 h after daily injection [$F_{(1,\ 8)} = 1.67$; $p = 0.121$] (**Figure 5B**).

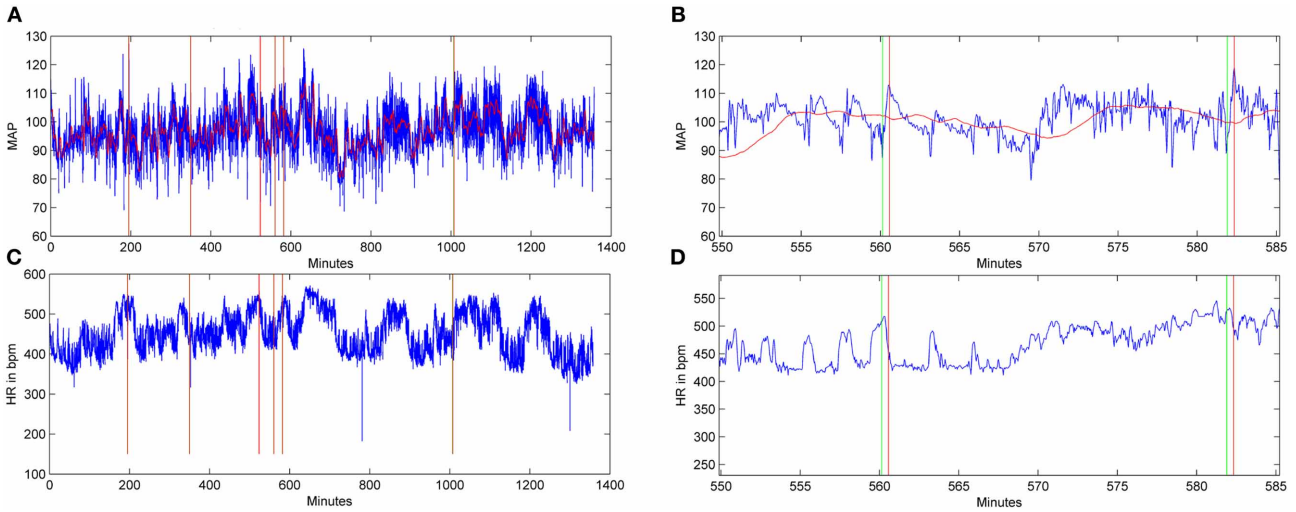

FIGURE 4 | Graphic examples of autonomic dysreflexia (AD) events detected over a 24-h period in an injured animal, employing the algorithm. Signals for mean arterial pressure (MAP) **(A,B)** and heart rate (HR) in beats per minute **(C,D)** from a saline-treated, injured rat 15 days following T4 spinal cord transection. In the MAP and HR traces, the horizontal red lines represent the baseline trace, while the blue lines correspond to the raw signals. The HR signal does not include a baseline, only the raw blue traces. In **(B)** and **(D)**, shown at higher magnification, the green lines indicate the beginning of an AD event, while red lines coincide with the end of the event.

SPECTRAL ANALYSIS OF BLOOD PRESSURE

We then sought correlations between the visible reductions of AD events in GBP-treated animals and trends in the spectral analysis of blood pressure. Critically, traditional spectral analyses do not pick up this clinically relevant event, highlighting the importance of the parameters we used to define an AD event in the algorithm. As depicted in **Figure 6**, spectral analysis of blood pressure only demonstrated the significant loss of a regulatory mechanism after SCI with dynamics in the 0.2–0.8 Hz (Mid) range (**Figure 6A**), with no consistent differences between treatment groups. Importantly, this does not negate the AD detections found using the algorithm and suggests that GBP did not impact the persistent changes resulting from the injury. The error bars in **Figure 6B** represent the 95% confidence limits of the mean power (PSD) ratio estimates from each group on each day. Note that for each day post-injury, the 95% confidence limits overlap; however for the pre- and post-injury days their 95% confidence limits do not overlap, indicating a significance difference ($p < 0.05$).

DISCUSSION

Here we report the results of long-term radio-telemetry experiments designed to determine, firstly, whether daily GBP administration for 3 weeks post-SCI versus saline altered baseline cardiovascular parameters, as well as AD elicited by noxious CRD. Notably, the same injured animals were also assessed for induced tail muscle spasticity, both distinct debilitating secondary reflexes following SCI that are often triggered by noxious stimuli below the SCI level (Rabchevsky and Kitzman, 2011). Daily GBP administration did not have significant effects on cardiovascular parameters, but there were trends for elevated MAP and HR compared to saline, notably in the first two weeks post-injury. On the contrary, and in support of our recent findings (Rabchevsky et al., 2011), acute GBP treatment significantly attenuated experimentally induced AD and tail spasticity, irrespective of chronic daily morning treatments.

GBP is currently provided as a prophylactic for neuropathic pain, indicating chronic administration. Therefore, we sought to determine whether GBP can be effectively taken orally near the onset of AD (acutely) to alleviate the debilitating reflexes. GBP possesses multiple cellular mechanisms and demonstrates the potential to help decrease the manifestation of spasticity in the chronic SCI population (Gruenthal et al., 1997; Priebe et al., 1997). Inhibition of glutamatergic transmission may be pre-eminent in mediating its therapeutic effects in epilepsy, neuropathic pain, and spasticity (Wheeler, 2002). Specifically, gabapentin has been shown to inhibit presynaptic glutamate release (Shimoyama et al., 2000; Maneuf and McKnight, 2001; Maneuf et al., 2004; Coderre et al., 2005, 2007). Moreover, glutamatergic neurotransmission has been shown to contribute to spinal viscerosympathetic initiation of episodic hypertension during experimental AD (Maiorov et al., 1997a) as well as induced tail muscle spasticity (Kitzman et al., 2007). Alternatively, GBP has been reported not to work by modulating presynaptic Ca^{2+} channel release; instead, it serves as a thrombospondin receptor which, when bound, inhibits new synapse formation in a murine model of whisker barrel de-afferentation (Eroglu et al., 2009). However, the latter study reported that GBP did not eliminate established synapses, inferring that inhibition of synapse formation with chronic daily GBP treatment cannot explain the mechanisms by which acute GBP treatments were effective in ameliorating nociceptive spinal reflexes after SCI.

Based on this and our previous findings (Rabchevsky et al., 2011), our initial expectation was that daily GBP may lower basal MAP, in part, by reducing the incidence of spontaneous AD. In order to address this intriguing possibility, we developed an

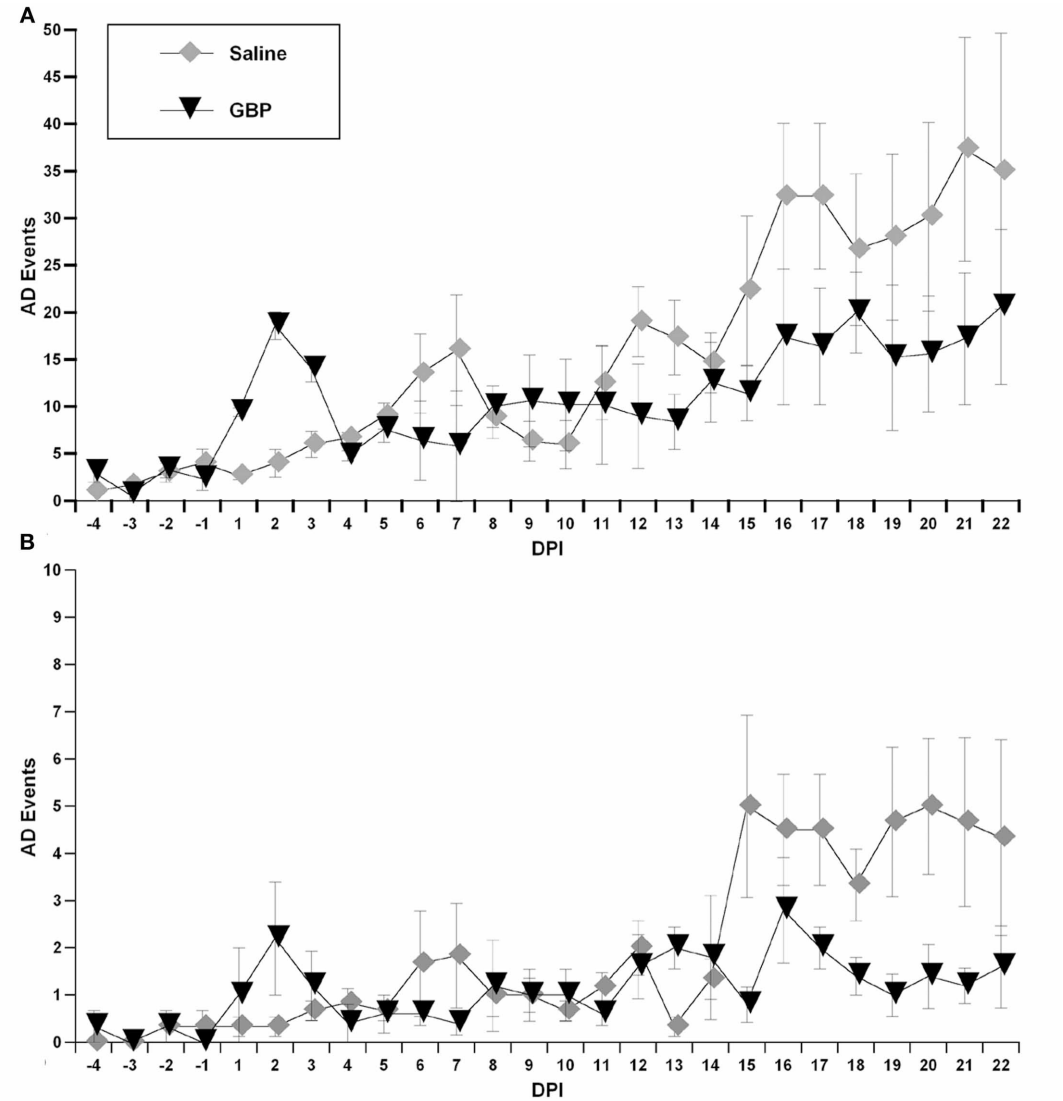

FIGURE 5 | Using the algorithm described in "Materials and Methods," these graphs illustrate detected autonomic dysreflexia (AD) events for each chronic (daily) treatment group over 24 h (A) versus the first 4 h (B) following daily injections of gabapentin (GBP) or Saline. Although there appeared to be fewer overall spontaneous AD events following GBP treatment at later days post-injury (**DPI**), variability precluded significant differences. Symbols represent group means ± SEM error bars.

algorithm to detect spontaneous events of AD based on 24 h MAP and HR data gathered over 3 weeks. Once validated, we found that chronic GBP treatment reduced daily spontaneous AD events and, more prominently, within the first 4 h after administration. The appearance of AD events and, accordingly, the suppressive effects of GBP became apparent two weeks after SCI, which corresponds to the time course of AD development (Krassioukov and Weaver, 1995; Mayorov et al., 2001; Rabchevsky, 2006). Interestingly, we observed remarkable cardiophysiological alterations in mean daily MAP and HR values in both treatment groups that began to appear following initial CRD trials on days 14–15. While, we do not have data for control injured rats, this suggests that noxious CRD may lead to profound adaptations in the spinal cord of animals with complete SCI, irrespective of treatment.

Several weeks following experimental SCI, CRD induces a rapid increase in MAP that are usually accompanied by varying degrees of bradycardia; the clinical definition of AD (Karlsson, 1999). Critically, such AD episodes can last as long as the noxious CRD is applied and the magnitude of MAP increases is typically in the range of 20–50 mm Hg above baseline, as shown in this and previous studies (Krassioukov and Weaver, 1995; Cameron et al., 2006; Hou et al., 2008). This is a key distinction between detecting spontaneous AD events versus those elicited by experimental CRD, the latter of which establishes a stable baseline MAP prior to assessments. Indeed, in developing the AD algorithm we could

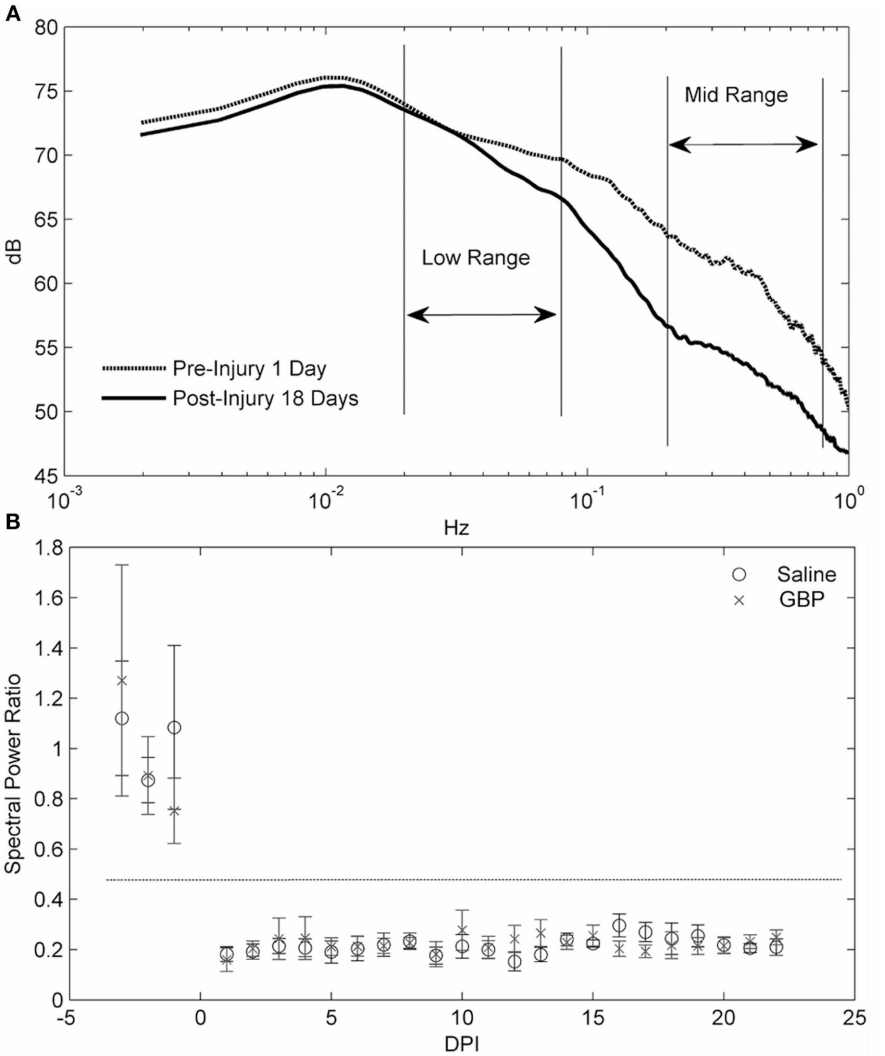

FIGURE 6 | (A) Power spectral density (PSD) analysis averaged over all animals for one day pre- and 18 days post-injury (DPI). The key difference resulting from the injury is the loss of mid frequency (Hz) dynamics. These differences were typical of the others days as well. **(B)** For day-by-day comparisons, an average ratio between the power in low frequency range (0.02–0.08) and mid frequency range (0.2–0.8) was computed for each group. A decrease in this ratio represents a shifting of power to the lower frequencies. The results are presented with 95% confidence limits for the error bars. Note that the horizontal dashed line indicates that the 95% intervals between pre-and post-injury are non-overlapping. The relative loss in mid frequency dynamics over DPI is clearly seen by the distance between means from the pre-injury cases. However, there is no consistent difference between treatment groups over DPI.

not find persistent prolonged MAP increases accompanied by bradycardia, most notably $\geq$15 mm Hg above baseline established by extended averaging windows and low-pass filtered MAP signals. It is uncertain whether the sparse transient AD events detected are a reflection of either fecal impaction or distended bladder, but the same injured rats responded to noxious CRD with significant AD, except for those receiving GBP acutely before assessments. Importantly, however, the spontaneous AD events detected over days post-injury were not significantly altered by daily, chronic GBP treatment.

Based on seminal rodent AD modeling studies (Krassioukov and Weaver, 1995; Rivas et al., 1995; Maiorov et al., 1997b; Krenz et al., 1999; Mayorov et al., 2001; Marsh et al., 2002), our own published reports have consistently reported CRD-induced changes in MAP versus changes in systolic arterial blood pressure (SAP). To our knowledge, only recently have SAP changes been reported as a primary indicator of experimentally-evoked AD (Inskip et al., 2012). Nevertheless, we reanalyzed our data sets for the current and other ongoing studies to establish the applicability of reporting changes in SAP values instead of MAP. While the magnitudes of CRD-induced SAP increases were found to be greater than MAP changes, the patterns and statistical differences were unaltered. Accordingly, we have reported the MAP changes evoked by CRD as well as spontaneous AD events detected with the algorithm.

Alternatively, it is documented in other reports, experimental and clinical, that AD episodes can also be accompanied by tachycardia, and we have observed many instances of such

occurrences in this and previous studies. However, regarding the algorithm, and in line with all our previous reports, we have operationally defined AD as a MAP increase concomitant with bradycardia (Krassioukov and Weaver, 1995; Rivas et al., 1995; Karlsson, 1999). It is also appreciated that based upon our required bradycardia inclusion, we may have missed otherwise detected AD episodes. While we did observe instances of tachycardia during CRD-induced MAP increases (see **Table 1**), the overall HR changes ranged from −60 to +4 bpm. Accordingly, we cannot predict how many spontaneous ≥10 mm Hg increases were accompanied by tachycardia since the algorithm parameters were not set to capture such events. Despite this caveat, such a limitation is based on the user-defined parameters applied to the algorithm and, therefore, speaks to its broad applicability. For example, to re-define an AD event the algorithm can be modified to detect any chosen supra-threshold MAP increase for any given duration, but when accompanied by either tachycardia, bradycardia or both.

We chose 50 mg/kg dosage (i.p.) for this study since we already documented that this dosage and route given acutely completely eliminated spasticity and significantly abrogated CRD-induced AD in spinal rat models (Kitzman et al., 2007; Rabchevsky et al., 2011). This dosage is on the low side of what has been employed in rat models of neuropathic pain, some up to 300 mg/kg (Yoon and Yaksh, 1999; Coderre et al., 2007), and in mice it has been shown that up to 1000 mg/kg does not affect motor performance (Czuczwar et al., 2003). Also, there is no reported change in Rotorod performance one hour after each of four consecutive daily injections of 100 mg/kg GBP (i.p.), and there are no reported differences in the half-life between GBP administered i.v. or i.p. (Xiao et al., 2007). The issue that remains unresolved, therefore, is whether a once daily injection of GBP is sufficient to have potential effects on spontaneous daily AD events detected by the algorithm.

The AD event detection algorithm has the advantage of consistently applying the same rules for every case, and while the human observers are aware of these rules, their ability to recognize the signal dynamics is limited by the need for sufficiently large swings in HR and MAP (relative to surrounding signals) to catch their attention. Therefore, the additional AD events captured by the algorithm were events that fit the definition, but were missed by the human observers either due to fatigue or the signal changes too close to the thresholds. Notably, there were insignificant detections in pre-injury data, validating injury-induced cardiovascular alterations. The missed AD events by the algorithm on humanly detected events were due, in part, to human error where a large jump in HR or MAP may have swayed a decision when both signals did not meet the criteria. On the other hand, there were cases where some transients were not entirely filtered out causing spurious threshold crossing and shortening event intervals. Humans would typically overlook these crossings, especially if other signal cues suggested the AD event was occurring. In summary, the algorithm showed good agreement with humanly identified events and provided a repeatable method for determining number of AD events, not dependent on human subjectivity or variability, and thus a useful tool when large amounts of data are available.

Although we found that daily GBP after SCI insignificantly elevated both MAP and HR values compared to daily saline treatment, notably between 1 and 2 weeks post-injury, in the current study design we did not include a sham injured GBP-treated group to determine its influences on MAP and HR compared to sham injured vehicle-treated rats. Importantly, however, we did analyze pre-injury data for each animal, which served as internal baseline controls for all cardiophysiological outcome measures in both injured treatment groups. Accordingly, such controls ensured reliability and validity of spectral analyses. The loss in the MAP fluctuations in the mid frequency range after SCI were shown to be significant, as demonstrated by the PSD comparisons of **Figure 6**. This strongly suggests that the regulatory mechanisms lost after SCI generate MAP fluctuations with periods on the order of 1.25–5 s (0.2–0.8 Hz). It is worth noting that comparison of spectra before and 18 days post-injury (**Figure 6A**) showed differences in the Mid frequency range, a region that has been associated in rat with sympathetically mediated effects upon blood pressure variability (Brown et al., 1994; Julien et al., 2003). Importantly, similar blood pressure fluctuations must occur within many intervals used in PSD estimation to detect such events. In other words, the blood pressure signals from the AD events were not sufficiently stationary to emerge in the spectral analysis and, accordingly, such analyses were blind to sparse transient events that we defined as AD. This stresses the importance and significance of detecting transient irregular AD events independently of spectral analysis, since frequency domain analyses are restricted to identify activity that is persistent over the observation epoch. Such analyses do indicate, therefore, that GBP does not affect the persistent dynamics of MAP related to SCI.

In summary, we designed a preclinical study to develop a treatment for chronic SCI individuals who continually suffer from secondary complications, notably abnormal muscle spasms and autonomic spinal reflexes. There is currently no pharmaceutical intervention which is known to effectively attenuate neuropathic pain as well as the manifestation of both muscular spasticity and AD after chronic SCI. While the clinical significance of the persistent MAP fluctuations associated with SCI is unclear, transient AD events directly impact the individual with SCI and, therefore, analyses directed at detecting and characterizing these event are critical for assessing treatments for SCI patients. Consequently, this quantitative experimental study was requisite to establish proof-of-principle prior to direct clinical application employing a two-pronged approach designed to alleviate dissimilar aberrant neurologic reflexes with a single drug, GBP. Such preclinical data appears to support the novel indication of GBP for the treatment and maintenance of both spasticity and AD following SCI.

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Somatosympathetic vasoconstrictor reflexes in human spinal cord injury: responses to innocuous and noxious sensory stimulation below lesion

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It is known that the sudden increases in blood pressure associated with autonomic dysreflexia in people with spinal cord injury (SCI) are due to a spinally mediated reflex activation of sympathetic vasoconstrictor neurons supplying skeletal muscle and the gut. Apart from visceral inputs, such as those originating from a distended bladder, there is a prevailing opinion that autonomic dysreflexia can be triggered by noxious stimulation below the lesion. However, do noxious inputs really cause an increase in blood pressure in SCI? Using microelectrodes inserted into a peripheral nerve to record sympathetic nerve activity we had previously shown that selective stimulation of small-diameter afferents in muscle or skin, induced by bolus injection of hypertonic saline into the tibialis anterior muscle or the overlying skin, evokes a sustained increase in muscle sympathetic nerve activity and blood pressure and a transient increase in skin sympathetic nerve activity and decrease in skin blood flow in able-bodied subjects. We postulated that these sympathetic responses would be exaggerated in SCI, with a purely noxious stimulus causing long-lasting increases in blood pressure and long-lasting decreases in skin blood flow. Surprisingly, though, we found that intramuscular or subcutaneous injection of hypertonic saline into the leg caused negligible changes in these parameters. Conversely, weak electrical stimulation over the abdominal wall, which in able-bodied subjects is *not* painful and activates large-diameter cutaneous afferents, caused a marked increase in blood pressure in SCI but not in able-bodied subjects. This suggests that it is activation of large-diameter somatic afferents, not small-diameter afferents, that triggers increases in sympathetic outflow in SCI. Whether the responses to activation of large-diameter afferents reflect plastic changes in the spinal cord in SCI is unknown.

Keywords: autonomic dysreflexia, innocuous stimulation, noxious stimulation, spinal cord injury, sympathetic nervous system

INTRODUCTION

While spinal cord injury (SCI) can cause devastating changes in the somatic nervous system below the lesion – paralysis and loss of sensation – compared to our knowledge of the sensory and motor disturbances following a spinal injury relatively little is known about changes to the sympathetic nervous system (McLachlan and Brock, 2006). Given that the sympathetic supply to the viscera and blood vessels is derived from the thoracic and lumbar segments of the spinal cord, an injury to the cord can – depending on the level and extent of the lesion – result in partial or complete loss of descending control of sympathetic function below the lesion. With high thoracic or cervical lesions the loss of sympathetic vasoconstrictor drive to blood vessels in muscles and the gut results in a low resting blood pressure and orthostatic hypotension; control of skin blood flow, and sweat release, and hence thermoregulatory control, is also compromised (Mathias and Frankel, 2002). However, because segmental spinal circuitry is intact, sensory stimuli originating below the lesion can cause a spinally mediated reflex activation of sympathetic vasoconstrictor neurons and a consequent increase in blood pressure. While it is known that visceral inputs, such as those from the bladder or bowel, can trigger these reflex vasoconstrictor responses, it is generally believed that non-visceral (somatic) inputs must be in the noxious range in order to generate similar vasoconstrictor responses. The purpose of this review is to contextualize recent experimental evidence that addresses the dogma surrounding the types of somatic inputs involved in the generation of spinal somatosympathetic reflexes.

AUTONOMIC DYSREFLEXIA

Following SCI reflex activation of sympathetic preganglionic vasoconstrictor neurons by sensory stimuli below lesion can cause arterial pressure to rise so suddenly and remain at dangerously high levels – leading to retinal and subarachnoid hemorrhage, stroke, and (occasionally) cardiac arrest – that this phenomenon, termed *autonomic dysreflexia*, is considered a medical emergency (Karlsson, 1999, 2006; Mathias and Frankel, 2002). It is common for midthoracic lesions and above (>T6, i.e., above the level of sympathetic outflow to the splanchnic circulation), where many vascular beds may have been decentralized and the only means available to offset the hypertension is to slow down the heart via

the vagus nerve (which, because it exits from the base of the skull, is spared in spinal cord injuries). Moreover, denervation supersensitivity of the vessels has been documented in human SCI, such that vascular responses to noradrenaline are greatly exaggerated (Mathias et al., 1976; Arnold et al., 1995), and work in spinalized rats has shown that neurovascular transmission is potentiated (Brock et al., 2006; Rummery et al., 2010).

Although autonomic dysreflexia has been reported in low thoracic (T8–T10) lesions (Gimovski et al., 1985), usually enough vascular beds are under supraspinal control that adequate vasodilatation can be brought about via the normal baroreceptor-mediated *withdrawal* of vasoconstrictor drive (Teasell et al., 2000). Autonomic dysreflexia is defined as an increase in systolic BP greater than 20 mmHg above baseline, combined with one or more subjective or objective clinical symptoms (Karlsson, 1999). The typical subjective signs of autonomic dysreflexia include a throbbing headache, tingling in the head or nasal congestion; sweating and flushing above the lesion, and (if observed) pupillary dilation, are clinical signs that prompt medical staff to measure blood pressure and to locate the source of sensory stimulation (usually a distended bladder or impacted colon, sometimes – according to clinical doctrine – a pressure sore or ingrown toenail). However, there is no correlation between the magnitude of the hypertensive response and the headache (Teasell et al., 2000). Moreover, by definition, subclinical episodes go undetected, and this syndrome of *silent dysreflexia* is of increasing concern, particularly for those living with SCI in the community (Linsenmeyer et al., 1996).

ASSESSING SYMPATHETIC OUTFLOW BELOW LESION FOLLOWING HUMAN SPINAL CORD INJURY

In intact individuals sympathetic control of blood vessels in muscle is specific to the maintenance of blood pressure, while sympathetic outflow to the skin primarily subserves thermoregulation through its actions on cutaneous blood vessels, sweat glands, and (phylogenetically) hairs. Direct recordings of muscle sympathetic nerve activity (MSNA), which are made routinely in our laboratory via tungsten microelectrodes inserted percutaneously into a peripheral nerve (microneurography), reveal ongoing bursts of MSNA that are phase-locked to the cardiac cycle via the baroreflex and which increase during maneuvres that unload the baroreceptors (Delius et al., 1972a). Conversely, bursts of skin sympathetic nerve activity (SSNA) are largely unrelated to the cardiac cycle but increase during whole-body cooling (which causes an increase in cutaneous vasoconstrictor drive) or whole-body heating (which brings about an increase in sudomotor drive); bursts of SSNA can also be evoked by arousal stimuli, such as unexpected loud sounds or electrical stimuli to the skin, and the overall level of SSNA is elevated during states of anxiety (Delius et al., 1972b). In patients with complete high thoracic or cervical spinal lesions there is essentially no spontaneous MSNA or SSNA below the lesion because the descending supraspinal excitatory drive to the preganglionic neurons – located in the thoracolumbar spinal cord – is interrupted, and there are no spontaneous fluctuations in skin blood flow or sweat release below the lesion (Wallin and Stjernberg, 1984; Stjernberg et al., 1986). Moreover, the differential control of MSNA and SSNA seen in intact individuals is lost: vasoconstrictor neurons supplying muscle and skin, as well as sudomotor and pilomotor neurons, are coactivated by visceral or somatic sensory inputs originating below the level of the lesion (Wallin and Stjernberg, 1984; Stjernberg et al., 1986). In intact individuals, distension of the bladder – caused by drinking water – causes an increase in both the amplitude and frequency of spontaneous bursts of MSNA and increases in systolic and diastolic pressure (Fagius and Karhuvaara, 1989). Although the same experiment has not been done following SCI, firm pushing over the lower abdomen to compress the bladder wall causes a very marked increase in blood pressure yet only brief bursts of MSNA that are modest in amplitude (Wallin and Stjernberg, 1984; Stjernberg et al., 1986). Despite this relatively weak sympathetic neural response, repeated bouts of bladder percussion cause a large increase in noradrenaline spillover below the lesion (Karlsson et al., 1998). This suggests that a given increase in sympathetic neural traffic causes a greatly increased release of noradrenaline (and co-transmitters) from the sympathetic terminals.

Given the importance of the sympathetic nervous system in the genesis of autonomic dysreflexia, it is surprising that assessment of injury in terms of autonomic involvement is still lacking in patients with SCI. Moreover, unlike studies of spinal injury in experimental animals – in which lesions are often complete transactions performed surgically – the diverse nature of accidents precipitating human SCI means that the lesions are usually incomplete. In addition, many individuals regain some functional recovery over time. Accordingly, just as it is important to know the status of the somatosensory and somatomotor nervous systems below a spinal lesion, it is equally important to know the status of the sympathetic nervous system. In 2009, an *Autonomic Standards Assessment Form* was released to document the impact of SCI on autonomic function (Alexander et al., 2009). However, this assessment tool does not give an autonomic level of injury, rather a framework for the assessment of specific autonomic functions following SCI. Level of injury is still based solely on motor and sensory function and is defined neurologically as the most caudal segment at which both motor and sensory functions are intact. The American Spinal Injury Association (ASIA) impairment scale (AIS; Marino et al., 2003) provides a score as to whether the injury is complete or incomplete, with a complete injury (ASIA A) being defined as complete loss of motor or sensory function in sacral segments S4–S5. An incomplete injury is classified as ASIA B, C, or D depending upon the degree of preservation of motor and sensory function. Various approaches have been used to assess the integrity of sympathetic pathways through and below a lesion in human SCI.

While microneurography can record sympathetic neural activity directly, it is a very time-consuming procedure to perform in the clinical setting and is not typically used for routine clinical diagnosis. Moreover, microneurography can be very difficult to undertake in individuals with SCI because of the decreased excitability of motor nerves, which makes finding muscle fascicles of the nerve difficult (Lin et al., 2007). Nevertheless, recording the effector-organ responses does provide a means of assessing sympathetic function indirectly: an increase in muscle and/or splanchnic vasoconstrictor drive can be inferred from an increase in blood pressure (assuming heart rate does not increase in parallel), while an increase in cutaneous vasoconstrictor or sudomotor drive can

be inferred by a decrease in skin blood flow (measured by laser Doppler or photoelectric plethysmography) or by an increase in sweat release (measured indirectly from changes in electrical resistance, conductance, or potential of the skin – termed the *galvanic skin response* or the *skin sympathetic response*). Indeed, there have been several attempts to assess the autonomic completeness of a spinal lesion by recording the electrodermal responses to electrical stimulation below and above lesion (Fuhrer, 1975; Cariga et al., 2002; Reitz et al., 2003; Ogura et al., 2004). While each of these studies found that a complete spinal lesion abolishes sudomotor responses in the lower limbs to electrical stimulation above lesion, Cariga et al. (2002) concluded that the isolated spinal cord cannot generate cutaneous sympathetic responses, as demonstrated by the absence of electrodermal responses in the lower limbs to stimuli delivered below lesion. While sudomotor responses to electrical stimulation below the lesion have *occasionally* been found (Fuhrer, 1975; Reitz et al., 2003), in our experience sudomotor responses in SCI are very unreliable markers of increases in cutaneous sympathetic outflow (Brown et al., 2007, 2009a,b; Brown and Macefield, 2008).

Recording of skin vasomotor responses below lesion to arousal stimuli has been suggested as a means of assessing the integrity of sympathetic pathways (Nicotra et al., 2005), and earlier studies had shown that skin vasoconstrictor responses can be elicited by sensory stimuli delivered below lesion (Wallin and Stjernberg, 1984; Stjernberg et al., 1986; Karlsson et al., 1998). Indeed, we recently showed that cutaneous vasomotor responses to stimuli delivered above and below lesion provide robust markers as to the integrity of cutaneous sympathetic pathways both through and below a spinal lesion (Brown et al., 2007, 2009a,b; Brown and Macefield, 2008). We have also used the blood pressure and heart rate responses to a maximal inspiratory breath-hold as a robust indicator of the integrity of muscle sympathetic outflow in SCI: in spinal injury this maneuvre causes a sustained fall in blood pressure and a compensatory increase in heart rate, the magnitudes of which depend on the level of the lesion, whereas in intact individuals the sustained increase in MSNA causes diastolic pressure to return to normal levels after an initial fall; heart rate remains stable (Brown and Macefield, 2008).

SOMATOSYMPATHETIC REFLEXES IN EXPERIMENTAL ANIMALS

The increase in blood pressure from sensory stimulation of the body wall is an example of a somatosympathetic reflex, while that to distension of the bladder or rectum is an example of a viscero sympathetic reflex. As noted above, while visceral inputs are known to be potent triggers of autonomic dysreflexia, it is generally assumed that any somatic stimuli must be noxious in order to induce significant increases in blood pressure. Indeed, the term "somatosympathetic reflexes" has been used specifically to refer to the autonomic responses to *noxious* stimuli originating below lesion (Saper and DeMarchena, 1986). However, it is known that light stroking of the skin is capable of inducing autonomic dysreflexia in spinalized rats, as is colonic distension at pressure levels lower than those that evoke signs of pain in intact rats (Marsh and Weaver, 2004), arguing against the idea that noxious stimulation is a requirement for evoking somatosympathetic reflexes in SCI. Somatosympathetic reflexes have been well characterized in the cat and rat, and have been shown to comprise both spinal and supraspinal components. Electrical stimulation of peripheral nerves or dorsal roots induces a biphasic response comprising a short-latency and a long-latency component: the latter depends on supraspinal circuitry, whereas the short-latency component is a spinal reflex that is arranged segmentally (Coote and Downman, 1966; Coote et al., 1969; Sato and Schmidt, 1971, 1973; Kerman and Yates, 1999). It has been shown that electrical stimulation of thoracic dorsal roots induces reflex activation of sympathetic preganglionic neurons with stimulation frequencies of up to 20 Hz, the amplitude of the responses decreasing at higher frequencies (Beacham and Perl, 1964). For some preganglionic neurons single afferent stimuli could induce reflex responses after an interval of only 12 ms, but tetanic stimulation of the afferents did not lead to augmented responses (Beacham and Perl, 1964). The afferent axons are believed to be myelinated, given that long-latency reflex responses can be evoked at stimulation intensities of 1.5× sensory (dorsal root) threshold (T), and short-latency components at 5T (Sato and Schmidt, 1971), yet most studies have used maximal stimuli (e.g., 50T) – intensities that also activate unmyelinated (C) fibers. Indeed, studies of somatosympathetic reflexes in the spinal cat have mostly used intensities of electrical stimulation that were within the noxious range, thereby activating both myelinated and unmyelinated afferents (Horeyseck and Jänig, 1974; Jänig and Spilok, 1978). Apart from these early studies on the relationships between the intensity of the somatic inputs and the sympathetic responses, little is known of the input-output relationships for somatosympathetic reflexes, although recent work has begun to redress this (McMullan et al., 2008). However, even less is known about these reflexes in humans.

SOMATOSYMPATHETIC REFLEXES TO NON-NOXIOUS INPUTS IN HUMAN SPINAL CORD INJURY

By definition, an increase in sympathetic outflow in response to sensory inputs originating below the level of a complete spinal lesion is spinally mediated, so human SCI provides a clinical model in which to examine spinal somatosympathetic reflexes. Interruption of the descending vasomotor pathways by a spinal lesion leaves the spinal sympathetic preganglionic neurons below the lesion deprived of supraspinal control, yet open to sensory inputs from below the lesion. We had previously shown (Brown et al., 2007) that by delivering brief (1 s) innocuous trains of electrical stimuli (3–10 mA, 0.2 ms pulses @ 20 Hz) to the forehead at unexpected times we could evoke sympathetically mediated cutaneous vasoconstrictor and sudomotor responses. These are simply arousal responses, but if we can record these from the toes in a patient with a high lesion this indicates that descending sympathetic pathways are intact.

Experimental records from a patient with a neurologically complete (ASIA A) cervical lesion (C6), sustained 4 months previously, are shown in **Figure 1**. Although not shown, electrical stimulation over the forehead failed to elicit cutaneous vasoconstriction (or sweat release) in the fingers and toes, indicating that descending sympathetic pathways were effectively interrupted. Conversely, electrical stimuli delivered to the abdominal wall (i.e., below the lesion) did evoke vasoconstriction in the fingers and toes. In this

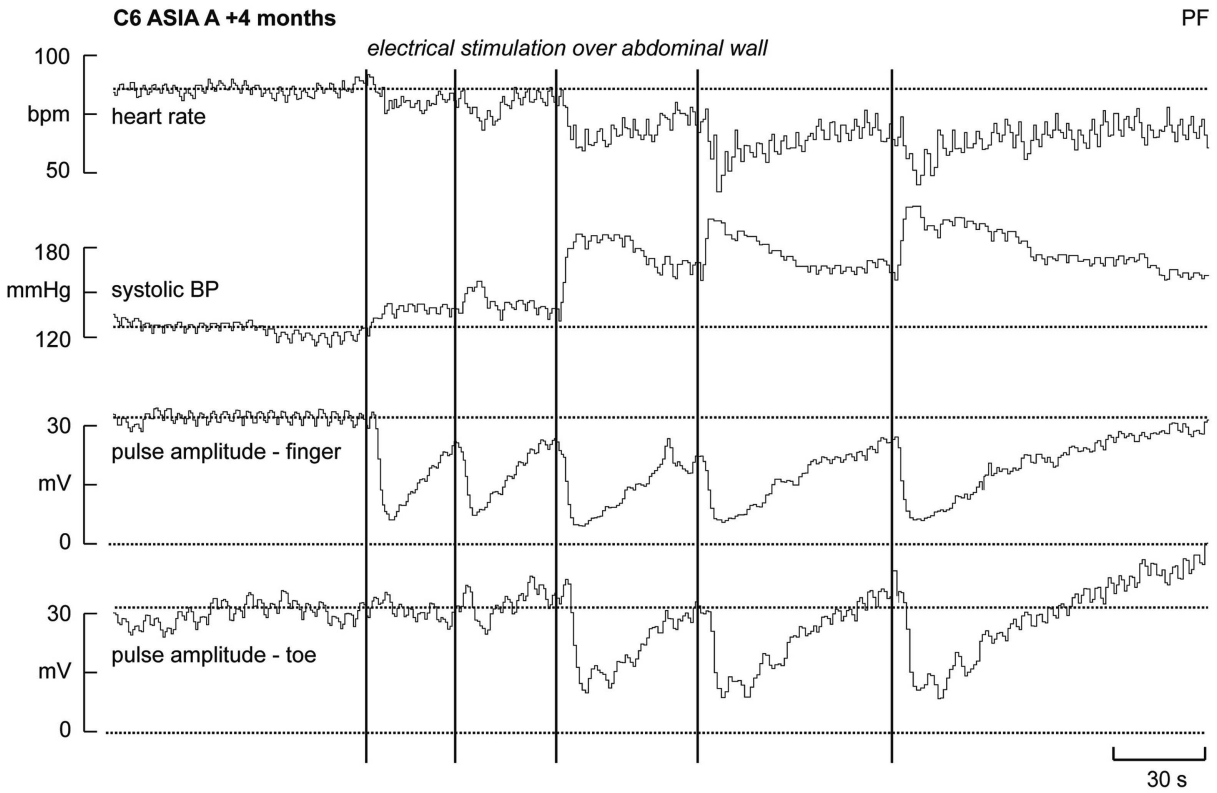

FIGURE 1 | Heart rate, systolic pressure, and skin blood volume recorded from a finger and toe in a patient who had sustained a C6 lesion. Electrical stimuli (vertical lines) applied to the abdominal wall (i.e., below the lesion) caused vasoconstriction in both the fingers and toes, an increase in systolic pressure and a baroreflex-mediated bradycardia (modified from Brown et al., 2007).

example we found evidence of "build-up," in which the effectiveness of the vasoconstrictor outflow increases with subsequent stimuli. This phenomenon could, perhaps, be seen as evidence of short-term plasticity in the spinal circuitry, though it might reflect changes at the level of neurovascular transfer. The first two stimuli applied to the abdominal wall – which in intact individuals were considered surprising but not noxious – caused no vasoconstriction in the toes and, while marked vasoconstriction was observed in the fingers, the stimuli caused negligible increases in arterial pressure. However, when vasomotor responses were observed in the toes (with the third stimulus) a significant increase in systolic pressure was generated. Presumably, this reflects constriction in a larger area of the vascular beds below the lesion. Moreover, it can be seen that the hypertensive response, and the resultant vagally mediated bradycardia, is cumulative. Clearly, because the subject could not feel the stimuli these vasoconstrictor responses represent spinal somatosympathetic reflexes rather than arousal responses. Unlike reductions in skin blood flow, sweat release – another marker of cutaneous sympathetic outflow – was rarely observed below the lesion. Indeed, sudomotor responses to abdominal stimulation could not be detected in the hands and feet in any of the patients with lesions above T7 (Brown et al., 2007).

In another study we examined the input-output relationships of the vasoconstrictor responses induced by electrical stimulation of the abdominal wall, testing the hypothesis that the magnitude and duration of the vasoconstriction was directly related to the duration of the stimulus. We predicted that a 20 s train of electrical stimuli would cause a longer period of vasoconstriction than that caused by a 1 s train of identical frequency, and that a train of 20 pulses delivered over 20 s (i.e., a stimulation frequency of 1 Hz) would cause a *smaller* vasoconstrictor response than that caused by delivering the 20 pulses over 1 s (stimulation frequency 20 Hz). Interestingly, we showed that the magnitude and duration of the somatosympathetic reflex response to electrical stimulation applied below lesion is not related to the duration and intensity of the sensory input; there was no difference in magnitude or duration of cutaneous vasoconstriction below the lesion for each of the stimulus parameters, suggesting that the recruitment of cutaneous (and muscle and splanchnic) vasoconstrictor neurons may be limited to the initial afferent barrage evoked by a stimulus train (Brown et al., 2009a). However, we do not know whether this is due to habituation of the spinal somatosympathetic reflex with longer trains or represents changes in neurotransmitter release and/or changes in vascular reactivity.

As noted above, Cariga et al. (2002) found that they could not evoke sudomotor responses in any patients during stimulation of peripheral nerves below the lesion. While we agree that sudomotor responses can only rarely be obtained, our demonstration of robust vasoconstrictor responses in the skin (and, by inference, other vascular beds) does not support the conclusion made

by these authors that the isolated spinal cord is unable to generate cutaneous sympathetic responses. Unlike the poor sudomotor responses, assessment of cutaneous vasomotor responses provides a very robust measure of the state of the sympathetic nervous system below lesion; moreover, one can plot the time course of the vasoconstrictor response by measuring the fall in cutaneous perfusion with every heart beat; this is a proxy marker of skin blood flow. We measured sudomotor and vasomotor responses, together with continuous blood pressure and heart rate, in another study from our laboratory that specifically targeted autonomic dysreflexia using non-noxious stimulation in a clinical setting. In six quadriplegic (C3–C7) and four paraplegic (T3–T6) men, vibroejaculation – brought about by vibration of the penis – was performed as a means of assessing fertility (Brown et al., 2009b). This is an inherently dangerous procedure, given the risks of triggering autonomic dysreflexia (Brackett et al., 1998); that the dysreflexia is abolished by anesthetic block of the dorsal penile nerve indicates that the increase in blood pressure is brought about by a somatosympathetic reflex (Wieder et al., 2000). In our study, vibration of the penis caused systolic pressure to increase by 18–90 mmHg in the quadriplegics and by 15–25 mmHg in the paraplegics (Brown et al., 2009b). Skin blood flow also fell during vibration, again representing an apparently generalized constriction of *all* blood vessels below lesion. Importantly, there was an inverse relationship between blood pressure and skin blood flow below the lesion, but a poor relationship between the clinical signs and the observed increase in blood pressure: some individuals reported severe headaches with reflex increases in systolic pressure of 35 mmHg, whereas others had no headache or other signs despite increases in systolic pressure exceeding 50 mmHg. For the sake of the current discussion, what is also important to recognize is that vibration of the penis is a non-noxious stimulus, one that generates marked somatosympathetic reflexes.

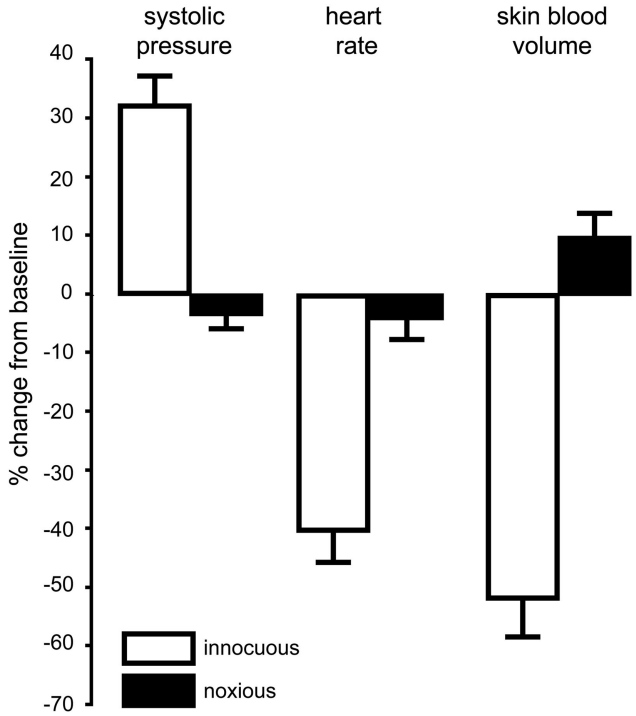

FIGURE 2 | Mean changes (±SE) in blood pressure, heart rate, and cutaneous vasoconstriction in the toes during innocuous stimulation (electrical stimulation of the abdominal wall with a 1 s 20 Hz train; $n = 12$) or noxious stimulation (intramuscular injection of 1 ml hypertonic saline into tibialis anterior; $n = 12$) below lesion in patients with spinal cord injury. Data combined from Brown et al. (2007) and Burton et al. (2008).

SOMATOSYMPATHETIC REFLEXES TO NOXIOUS INPUTS IN HUMAN SPINAL CORD INJURY

We recently showed that selective stimulation of muscle nociceptors, induced by bolus intramuscular injection of hypertonic saline into the leg of awake human subjects, caused a strong, dull ache that lasted ∼8 min and produced an increase in MSNA and modest increase in blood pressure and heart rate; these followed the time course of the subjective report of pain (Burton et al., 2009a). Conversely, SSNA showed only a *transient* increase, associated with a fall in skin blood flow, which we believe is simply a reflection of the *alerting* (arousal) response to the pain (Burton et al., 2009b). Interestingly, when similar injections were made into the legs of people with SCI (i.e., below the level of the lesion) the predicted increases in blood pressure and decreases in skin blood flow *did not occur*; in fact, there was a slight *fall* in blood pressure (Burton et al., 2008). **Figure 2** compares the changes in cutaneous vasoconstriction, blood pressure, and heart rate to noxious stimulation (produced by intramuscular injection of hypertonic saline below lesion) and innocuous stimulation (produced by low-intensity electrical stimulation over the abdominal wall) in patients with SCI. It is also worth pointing out that the absence of any sustained cutaneous vasoconstriction to noxious stimulation argues against the idea that an ongoing noxious stimulus would, via a spinal reflex, cause prolonged decreases in skin blood flow and thereby compromise wound healing.

Based on the above observations in able-bodied subjects, we now believe that the increase in sympathetic vasoconstrictor drive, and the resultant increase in blood pressure, evoked by noxious stimuli reflect part of the affective responses to pain, and are related to the emotional quality of the pain rather than to any spinal reflex. Indeed, brain imaging during intramuscular injection of hypertonic saline has revealed discrete activation of cortical areas involved in the affective responses to pain – the subgenual anterior cingulate and insular cortices – as well as the primary somatosensory cortex (Henderson et al., 2006). That selective stimulation of nociceptors in skin or muscle below lesion does *not* cause vasoconstriction or increases in blood pressure goes against existing dogma, in which autonomic dysreflexia is believed to be, in many cases, triggered by noxious inputs below the lesion (Karlsson, 1999). Indeed, here is a common definition found in the literature for the cause of autonomic dysreflexia: "*. . .any stimulus that might cause pain in a person without spinal cord injury is capable of triggering autonomic dysreflexia*" (Blackmer, 2003; Selcuk et al., 2004). Moreover, stimuli that would undoubtedly be noxious in intact individuals often fail to induce autonomic dysreflexia in those with SCI. For example, although pressure ulcers may be quite painful in intact individuals (Roth et al., 2004), and

there are reported cases of autonomic dysreflexia being associated with pressure sores (Teasell et al., 2000), clinical experience does not support the concept that most spinal patients who have pressure ulcers consistently and continually suffer from constant autonomic dysreflexia – the presence of an assumed continuous nociceptive stimulus does not necessarily translate into a continual hypertensive state. Another example from our clinical experience: a patient with T6 complete lesion and a known clinical history of autonomic dysreflexia experienced a spiral fracture of his right femur after a fall. Despite his predisposition to autonomic dysreflexia, regular observations of heart rate, and blood pressure were stable following the fall and he did not experience any symptoms of autonomic dysreflexia following the fracture.

PLASTIC CHANGES IN THE SPINAL CORD FOLLOWING SPINAL CORD INJURY

While SCI makes it easier to see spinal somatosympathetic reflexes in human subjects, are there changes in the spinal circuitry responsible for these reflexes following SCI? This is difficult to answer, because any reflexly generated increase in sympathetic vasoconstriction would be buffered by the baroreflex in intact individuals. We know that electrical stimulation of the abdominal wall (or anywhere on the body surface) in intact individuals causes cutaneous vasoconstriction and sweat release, but this does not mean it is spinally generated; conversely, the vasoconstrictor response to such stimulation in spinal patients can only be spinally mediated. Moreover, the fact that noxious stimulation below lesion – produced by intramuscular or subcutaneous injection of hypertonic saline – causes no cutaneous vasoconstriction, no sweat release, no increase in blood pressure, and no decrease in heart rate indicates that the increase in muscle and cutaneous sympathetic nerve activity produced by these stimuli in intact individuals is generated supraspinally. It is quite possible that the spinally mediated increase in sympathetic outflow to somatosensory stimulation following SCI is exaggerated: we know that the duration of the cutaneous vasoconstriction is increased in human SCI (Brown et al., 2007, 2009a), but this may well reflect an augmented peripheral – as opposed to central – response. Indeed, in the spinal rat blood vessels show an increased reactivity to direct stimulation of the vasomotor nerves (Yeoh et al., 2004a,b; Brock et al., 2006; McLachlan and Brock, 2006; Rummery et al., 2010). It is now believed that neurovascular transmission is potentiated by both an increase in neurotransmitter release and a reduction in noradrenaline reuptake, depending on the vessel (Brock et al., 2006; McLachlan, 2007; Rummery et al., 2010). So, while this can be interpreted as a reflection of "peripheral plasticity" following SCI, we are still left with the question as to whether there are plastic changes centrally.

Studies conducted in experimental animals have demonstrated anatomical changes in preganglionic neurons following a SCI – an initial loss but subsequent regrowth of synapses (Krassioukov and Weaver, 1995, 1996) – and plastic changes in the strength of the synaptic connections between preganglionic and postganglionic sympathetic neurons following section of the axons of preganglionic neurons: those neurons that remain exhibit extensive collateral sprouting (Murray and Thompson, 1957; Liestol et al., 1987). Moreover, by removing all but one set of segmental inputs to a paravertebral ganglion one can demonstrate plastic changes in synaptic strength over time: immediately following surgery strong synaptic inputs were present on only 10% of the postganglionic neurons, whereas after several weeks the number of these synapses had increased to some 70% (Ireland, 1999). This indicates that the residual preganglionic neurons had sprouted and formed strong synaptic connections, even on neurons that had been completely deprived of synaptic drive by the lesion. Indeed, it has been argued that formation of aberrant strong inputs may contribute to the exaggerated vasoconstrictor responses to sensory inputs below lesion in SCI (McLachlan, 2007), and evidence from rats has shown that sprouting of small-diameter primary afferent neurons occurs within the spinal cord following a severe but not a mild compression injury of the midthoracic cord (Weaver et al., 2001). Moreover, the magnitude of the autonomic dysreflexia induced by colonic distension is related to the degree of primary afferent sprouting (Weaver et al., 2001), and dysreflexia can be abolished by blocking this sprouting (Krenz et al., 1999). It is highly likely that such plastic changes, both afferent and efferent, also occur within the spinal cord in human SCI.

CONCLUSION

We conclude that robust somatosympathetic reflexes can be produced by innocuous stimuli below lesion in human SCI, whereas *specific* activation of nociceptors below lesion does not generate such reflexes. Not withstanding the importance of viscerosympathetic reflexes, this argues against the existing clinical dogma that autonomic dysreflexia is often triggered by noxious somatic inputs originating below a spinal lesion. Moreover, the absence of sustained cutaneous vasoconstriction to noxious inputs originating below a spinal lesion argues against the idea that an ongoing noxious stimulus, such as that produced by a pressure ulcer, would reduce skin blood flow and potentially compromise wound healing.

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Upper gastrointestinal dysmotility after spinal cord injury: is diminished vagal sensory processing one culprit?

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Despite the widely recognized prevalence of gastric, colonic, and anorectal dysfunction after spinal cord injury (SCI), significant knowledge gaps persist regarding the mechanisms leading to post-SCI gastrointestinal (GI) impairments. Briefly, the regulation of GI function is governed by a mix of parasympathetic, sympathetic, and enteric neurocircuitry. Unlike the intestines, the stomach is dominated by parasympathetic (vagal) control whereby gastric sensory information is transmitted via the afferent vagus nerve to neurons of the nucleus tractus solitarius (NTS). The NTS integrates this sensory information with signals from throughout the central nervous system. Glutamatergic and GABAergic NTS neurons project to other nuclei, including the preganglionic parasympathetic neurons of the dorsal motor nucleus of the vagus (DMV). Finally, axons from the DMV project to gastric myenteric neurons, again, through the efferent vagus nerve. SCI interrupts descending input to the lumbosacral spinal cord neurons that modulate colonic motility and evacuation reflexes. In contrast, vagal neurocircuitry remains anatomically intact after injury. This review presents evidence that unlike the post-SCI loss of supraspinal control which leads to colonic and anorectal dysfunction, gastric dysmotility occurs as an indirect or secondary pathology following SCI. Specifically, emerging data points toward diminished sensitivity of vagal afferents to GI neuroactive peptides, neurotransmitters and, possibly, macronutrients. The neurophysiological properties of rat vagal afferent neurons are highly plastic and can be altered by injury or energy balance. A reduction of vagal afferent signaling to NTS neurons may ultimately bias NTS output toward unregulated GABAergic transmission onto gastric-projecting DMV neurons. The resulting gastroinhibitory signal may be one mechanism leading to upper GI dysmotility following SCI.

Keywords: brain-gut axis, gastric emptying, gastric motility, thoracic spinal cord injury, vago-vagal reflexes

INTRODUCTION

The association of gastrointestinal (GI) pathology with neurological trauma dates back to the mid-nineteenth century observations of Rokitansky and subsequent descriptions by Schiff [both cited by Cushing (1932)]. Gastric stasis and ulceration, commonly known as "Cushing's ulcer," associated with severe trauma has been repeatedly described in the clinical literature in the 80 years since Harvey Cushing's original report. Regardless of the nature of the original traumatic insult, the absence of abnormal GI motility is a strong predictor of patient outcome and length of hospitalization.

Roughly 11,000 new spinal cord injury (SCI) cases occur each year and the current United States SCI population has been estimated as high as 1.2 million people (*The Christopher and Dana Reeve Paralysis Resource Center*). In addition to the immediate loss of sensation and motor function, SCI also profoundly affects the autonomic nervous system (Weaver et al., 2006; Inskip et al., 2009; Krassioukov, 2009). While attention to autonomic dysfunction has increased in recent years, studies targeting cardiovascular and bladder dysfunction outnumber those of GI dysfunction. While the former derangements present formidable challenges to the SCI individual, GI complications are typically responsible for 11% of hospitalizations in the SCI population (Middleton et al., 2004; Jaglal et al., 2009) and are consistently rated as serious quality of life issues (Anderson, 2004).

Functional GI motility disorders present as a broad range of symptoms which include delayed gastric emptying, early satiety and the sensation of nausea, bloating, abdominal pain and diminished propulsive transit along the entire length of the GI tract. Due to the segmental distribution of the spinal neurocircuitry regulating both visceral preganglionic and somatic motor neurons, the degree of disability, morbidity, and mortality following injury tends to be associated with the spinal level at which injury occurred.

Conventional division of the GI tract into designations of upper and lower compartments remains open to debate. Based upon embryological development, the GI tract can be classified along three divisions consisting of (1) the foregut, which gives rise to the esophagus, stomach, and duodenum as far as the major duodenal papilla; (2) the midgut, from where the bile duct enters at the major duodenal papilla to the mid-transverse colon; and (3) the hindgut, from the mid-transverse colon to the anus. For the present purposes of this review, the upper GI tract is defined as the esophagus, stomach and proximal duodenum. The scope of this review precludes discussion of post-SCI pathologies along the entire GI tract. This is especially true for

the intestines and rectoanal junction, since the loss of descending input to the sympathetic, parasympathetic, and pudendal nuclei located throughout the thoracic, lumbar, and sacral spinal cord presents a pattern of neural control that is fundamentally different from upper GI innervation.

NEURAL CONTROL OF UPPER GASTROINTESTINAL FUNCTION

The principal nutritive functions of the GI tract, the digestion, absorption and propulsion of nutrients, and the maintenance of proper fluid balance, are critically dependent upon a hierarchy of enteric, parasympathetic and sympathetic neural control.

The enteric nervous system (ENS) provides powerful control over the smooth musculature, secretory glands and microvasculature of the digestive tract [reviewed in Woods (2004)]. This so-called "mini-brain" of the gut is comprised of primary afferent neurons, interneurons, and efferent neurons that are capable of complete reflex activities and quasi-autonomous control of digestion. The ENS mediates digestion through localized control over the individual reflex systems and by integrating the actions of these effectors along the GI tract into organized patterns of digestion. Without this localized autonomous control, proper digestive processes do not occur (De Giorgio and Camilleri, 2004). However, while the intrinsic reflexes necessary for proper intestinal function are mediated by the ENS, these digestive processes must ultimately integrate with the homeostatic needs of the entire organism through brain-gut connections.

Unlike the small and large intestines, the ENS of the stomach lacks the capacity to independently control the moment-to-moment changes necessary for appropriate ingestive, milling, and emptying reflexes. While vago-vagal reflex circuits modulate digestive processes from the oral cavity to the transverse colon, the level of vagal control diminishes caudally. Gastric function is dominated by extrinsic neural circuits residing within the brainstem that modulate the gastric ENS neurocircuitry. Specifically, the extrinsic brainstem integration of gastric reflex function is centered within circuits of the dorsal vagal complex (DVC), which comprises the area postrema (AP), the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vagus (DMV; **Figure 1**) (Travagli et al., 2006). The integrative activity that occurs within the DVC nuclei is the result of inputs originating from higher central nervous system (CNS) areas (Blevins et al., 2004; Morton et al., 2005; Blevins and Baskin, 2010); from spinosolitary inputs (Menetrey and Basbaum, 1987; Menetrey and de Pommery, 1991; Gamboa-Esteves et al., 2001) as well as from neurohormonal signals from the periphery. This latter-most signaling pathway occurs as a function of the fenestrated capillaries within the DVC that permit diffusion of circulating neuromodulators across a "leaky" blood brain barrier (Gross et al., 1990). All of these signals finely tune the coordinated emptying of nutrients from the stomach.

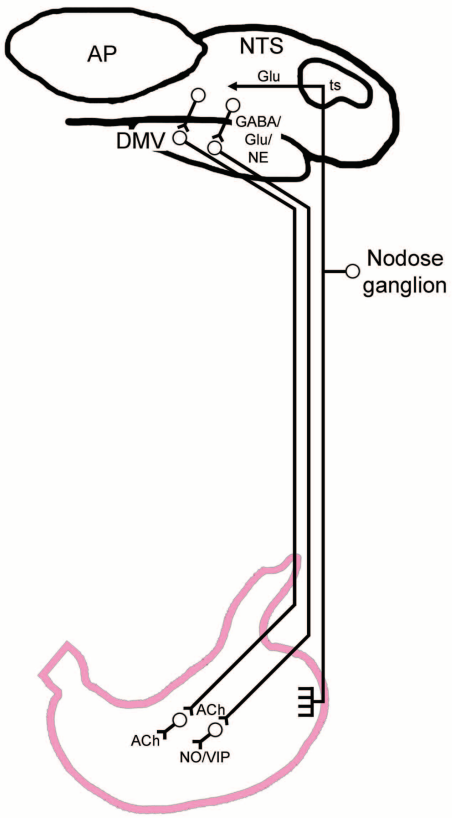

FIGURE 1 | General visceral afferent signals are transmitted to the brainstem by afferent fibers distributed throughout the proximal GI tract that traverse within the vagus nerve. The *dashed line* arbitrarily indicates the transition between the fundus (orally) and the corpus (caudally). The cell bodies for these vagal afferents reside within the nodose ganglion. Vagal afferents enter the brainstem by way of the tractus solitarius (ts) and terminate principally as a glutamatergic (Glu) synapse onto second order neurons within the nucleus tractus solitarius (NTS). At the level of the NTS, converging projections from higher CNS centers (not pictured) are integrated and relayed by NTS neurons to regions which include the parasympathetic preganglionic neurons of the dorsal motor nucleus of the vagus (DMV) using the neurotransmitters GABA, glutamate or norepinephrine (NE). Together with the area postrema (AP) the NTS and DMV form the region of the dorsal vagal complex. Preganglionic DMV motor neurons innervate gastric enteric neurons by way of two competing pathways. Activation of one pathway initiates the cholinergic (Ach) mediated excitation of gastric smooth muscle which is necessary for gastric tone and motility. Alternatively, activation of a non-adrenergic, non-cholinergic (NANC) pathway exerts a profound gastric relaxation through the release of nitric oxide (NO) or vasoactive intestinal polypeptide (VIP). Reduction in gastric tone and motility, therefore, can be produced by either the withdrawal of excitatory cholinergic drive, or activation of NANC-mediated inhibition.

VAGAL AFFERENT SIGNALING

Details regarding the sensory innervation of the gut have been reviewed previously (Beyak et al., 2006). Briefly, the cell bodies of vagal afferent (sensory) fibers, including those that innervate the proximal GI tract, are located within the nodose ganglion (Browning and Mendelowitz, 2003). Afferent information originating in the gut terminates directly upon second order NTS neurons by way of a glutamatergic synapse (Hornby, 2001). These GI afferents can be categorized based upon two essential receptor qualities. Mechanosensitive receptors in the form of intraganglionic laminar endings (IGLEs; Powley and Phillips, 2002) and, possibly, intramuscular arrays (IMAs) (Berthoud and Powley, 1992) innervate the muscle layers in a manner consistent for

the transduction of contractile and shearing forces (Powley and Phillips, 2002). Vagal IGLE innervation is densest in the esophagus and proximal-most portions of the GI tract (Berthoud et al., 1997; Neuhuber et al., 1998; Wang and Powley, 2000) and viseral sensory afferents terminate topographically within the subnuclei of the NTS (Altschuler et al., 1992). Specifically, esophageal sensory receptors that include IGLE's project exclusively to the subnucleus centralis (NTSc) (Cunningham and Sawchenko, 1990; Sengupta, 2000). As will be seen later, these exclusive esophageal projections to the NTSc provide a very unique model of a pure vago-vagal gastric reflex.

The second principal receptor classes are chemosensitive vagal afferents, particularly those within the lamina propria and distributed throughout the villi, which have been described throughout the GI mucosa (Berthoud et al., 1995). The specific response characteristics, ligands, and signal transduction pathways utilized by these vagal afferents are too extensive to be reviewed herein. However, two particular examples of luminal signaling have received considerable attention. One of the best characterized peptides controlling gastric and digestive functions is cholecystokinin (CCK), which is released from so-called I-cells within the proximal small intestine in response to fat or protein content of a meal (Dockray, 2006). CCK release has profound inhibitory effects on GI functions (Ritter et al., 1994; Moran and Kinzig, 2004; Woods, 2004), and its vagal mechanisms of action commonly ascribed to include a paracrine activation of vagal afferent fibers along the gut wall [reviewed in Raybould (2007)].

The GI neurohormone ghrelin, secreted from oxyntic cells within the gastric mucosa (Date et al., 2000; Grönberg et al., 2008), is up-regulated during periods of negative energy balance, such as before meals, and is down-regulated after feeding (Cummings et al., 2001). In animals and humans, ghrelin and ghrelin agonists exert profound stimulatory effects upon gastric motility and acid secretion as well as food intake and energy metabolism (Masuda et al., 2000; Levin et al., 2006; Tack et al., 2006; Ariga et al., 2007, 2008; Wang et al., 2008; Ejskjaer et al., 2009; Kobashi et al., 2009) though an inhibitory effect has been reported for fundic tone (Kobashi et al., 2009). Ghrelin has received considerable clinical interest as an endogenous stimulant of gastric motility (Nass et al., 2011; Stengel and Taché, 2012). Peripherally, ghrelin is considered to exert its gastroexcitatory effect on a vagally-mediated pathway which involves growth hormone secretagogue receptors that originate in the nodose ganglion and are transported to vagal afferent terminals (Date et al., 2002). The role of afferent fibers of the gastric vagal circuit was confirmed physiologically in that peripherally administered (i.e., circulating) ghrelin diminishes vagal afferent activity while vagotomy, midbrain transection, or perivagal capsaicin abolishes ghrelin-mediated facilitation of feeding, GH secretion, as well as activation of neuropeptide Y (NPY)- and growth hormone-releasing hormone (GHRH)-producing neurons (Date et al., 2002, 2006).

Many of the effects of endogenous gut peptides are mediated via a paracrine activation of the peripheral endings of vagal afferent fibers as described above. However, based upon a large body of work with CCK, mounting evidence has shown that GI peptides exert physiologically relevant actions when applied to central GI neurocircuitry (Talman et al., 1991; Branchereau et al., 1992, 1993; Blevins et al., 2000, 2004; Lin et al., 2004; Appleyard et al., 2005; Baptista et al., 2005b, 2006; Wan et al., 2007; Kobashi et al., 2009) including direct actions upon nodose ganglion (Blackshaw and Grundy, 1990; Simasko and Ritter, 2003), and DVC neurons (Baptista et al., 2005b; Zheng et al., 2005; Holmes et al., 2009a,b). Similarly, ghrelin receptor expression has been reported within the medullary brainstem and brainstem application of ghrelin exerts behavioral (Faulconbridge et al., 2003), gastric (Kobashi et al., 2009) and cardiovascular (Lin et al., 2004) responses. These studies suggest that functional CCK and ghrelin receptors are present on the vagal afferent terminals, the neuronal membrane and nerve terminals of subgroups of the NTS as well as on gastric-projecting DMV neurons. The source of feeding-related peptides acting centrally upon DVC neurocircuitry may be through local neuronal release or through the circulation, since the DVC has a leaky blood-brain barrier (Gross et al., 1990; Cottrell and Ferguson, 2004). The exact mechanism of gut peptides activating these neural circuits within the DVC remains to be elucidated. However, the GI effects of peptides such as CCK-8s (the sulfhated, octapeptide variant of CCK) may not be limited to a paracrine activation of the peripheral terminal of vagal afferent fibers but through direct effects on brainstem circuits which must be considered in physiological studies of neurally intact as well as injured preparations.

VAGAL EFFERENT SIGNALING

With regard to the stomach, motor output originating in the DMV regulates a complex interplay between two separate postganglionic circuits. While a small percentage of visceral afferent inputs modulate gastric reflex function by directly synapsing onto DMV neurons (Renehan et al., 1995), the majority of afferent signaling is directed to second order neurons within the NTS through a glutamatergic synapse (see Browning and Travagli, 2010). In turn, three distinct neurochemical phenotypes (glutamatergic, GABAergic and noradrenergic) of NTS neurons synapse onto DMV neurons. Emerging evidence following application of GABAergic antagonists to the DVC, strongly suggests that NTS GABAergic inputs onto DMV neurons tonically regulate the basal motor outflow to the stomach (Sivarao et al., 1998; Browning and Travagli, 2001; Herman et al., 2009, 2010; Babic et al., 2011), whereas glutamatergic and noradrenergic antagonism has little effect (Saltzstein et al., 1995; Soret et al., 2010).

As with all parasympathetic preganglionic neurons, DMV neurons are cholinergic and activate postganglionic neurons via actions at a nicotinic receptor. Gastric projecting neurons within the DMV exhibit a basal rate of spontaneous action potentials (1–2 Hz); (Travagli et al., 1991; Marks et al., 1993; Browning et al., 1999) which is modulated, though not generated, by synaptic inputs beyond those described for the NTS. Modulation of this spontaneous, low frequency DMV firing regulates an excitatory (cholinergic) circuit that is ultimately important to the antral milling of ingested solids and the delivery of reduced particles to the duodenum (Malagelada and Azpiroz, 1989). Gastric relaxation can occur as a consequence of inhibiting this

tonically firing excitatory pathway (Abrahamsson and Jansson, 1969; Abrahamsson, 1973; Gillis et al., 1989; McCann and Rogers, 1992, 1994). However, activation of vagal afferents produces a potent gastroinhibition by also activating a non-adrenergic non-cholinergic (NANC) inhibitory vagal projection to the stomach mainly by the release of nitric oxide (NO) (Jansson, 1969; Abrahamsson, 1973; Takahashi and Owyang, 1997; Krowicki et al., 1999) though purinergic, and vasoactive intestinal polypeptide mechanisms have also been identified [reviewed in Chang et al. (2003)].

UPPER GI DYSFUNCTION AFTER HUMAN SCI

ESOPHAGEAL FUNCTION

The principal functions of the esophagus are centered on (1) the propulsion of ingesta to the stomach; and (2) prevention of gastroesophageal reflux of stomach contents. Esophageal motor control is a complex interplay of proximal striated musculature which is a combination of voluntary and reflexive control proximally that progressively yields to involuntary smooth muscle contractions distally.

Despite the clinical implications regarding the risk of aspiration that accompanies esophagogastric reflux, such that intensive management of the airway is also required (Kirshblum et al., 2002), there are relatively few reports addressing esophageal function following SCI. Based upon barium contrast imaging, early reports noted that the incidence of gastroesophageal reflux and hiatal hernia were limited to persons with SCI greater than 5 years duration (Gore et al., 1981). Later evidence, based upon subject questionnaire and endoscopic follow-up, reported a higher incidence of heartburn and esophageal chest pain in SCI subjects which was accompanied by endoscopic and histological evidence of esophagitis as well as diminished esophageal contractility (Stinneford et al., 1993). Diagnoses of dysphagia in cervical SCI population confirmed these earlier reports, but also identified the potential causal relationship between dysphagia and both artificial ventilation techniques (including tracheotomy) as well as anterior versus posterior approaches during spinal stabilization (Wolf and Meiners, 2003). Finally, to test the role of diaphragmatic crura upon gastroesophageal reflux containment, comparisons have been made between quadriplegic and paraplegic subjects. While cervical injury did significantly increase subjective reflux ratings, high-level injury did not predispose subjects to differences in endoscopic, manometric, or histological indications of esophagogastric abnormalities (Silva et al., 2008). Thus, the prevalence and potential mechanism of esophageal dysfunction after human SCI remains largely unresolved.

GASTRIC FUNCTION

The principal functions of the stomach are centered on (1) a reservoir component for ingested solids and liquids; (2) reduction of the size of food particles through both digestive secretions and the mechanical milling evoked by gastric contraction and relaxation; and (3) the feed-back mediated propulsion of ingesta into the duodenum. The gastric compartment can be subdivided into the fundus, which serves as reservoir and regulates intragastric pressure, and the more muscular corpus where food is churned until reduced in size in order for contraction of the antrum to facilitate passage through the pylorus leading to the duodenum.

Peptic ulceration has been reported following SCI (Tanaka et al., 1979) and in other traumatic injuries requiring intensive care. The prophylactic administration of proton pump inhibitors or histamine-2 receptor antagonists is widely employed in the ICU and may minimize this particular co-morbidity, though debate remains whether such practices are justified (Jung and MacLaren, 2002; Stollman and Metz, 2005). Less well characterized, and managed, are alterations in the motor components of gastric reflex function. Despite utilizing a variety of technical approaches, the majority of reports in the clinical literature describe derangements in upper GI reflex emptying and motility, especially after SCI occurring above the mid-thoracic spinal cord (Kewalramani, 1979; Berlly and Wilmot, 1984; Fealey et al., 1984; Nino-Murcia and Friedland, 1991; Rajendran et al., 1992; Stinneford et al., 1993; Segal et al., 1995; Kao et al., 1999; Williams et al., 2012). In extreme cases, the high degree of gastric feeding intolerance demonstrated by these patients necessitates aggressive total parenteral nutrition and occasionally invasive GI surgical intervention in order to maintain positive energy and nitrogen balance (Dwyer et al., 2002).

The GI neurohormone ghrelin, previously described as being secreted from oxyntic cells within the gastric mucosa (Date et al., 2000; Grönberg et al., 2008), is up-regulated during periods of negative energy balance, such as before meals, and is down-regulated after feeding (Cummings et al., 2001). Recently, a study of uninjured, paraplegic and quadriplegic individuals reported no differences in levels of serum ghrelin across all groups following an overnight fast (Wang et al., 2005). Unfortunately, with the exception of a study on eating attitudes (Levin et al., 2006), clinical studies are lacking regarding the dietary behaviors of the SCI population. In contrast to the able-bodied population, data regarding the orexigenic and pro-motility responses to exogenous ghrelin following SCI is non-existent.

PROXIMAL DUODENAL FUNCTION

The principal functions of the proximal duodenum include (1) neutralization of acid in the chyme delivered from the stomach; and (2) enzymatic reduction of particles to simple molecules for absorption. Considerable feedback mechanisms exist between the antrum, pylorus, and duodenum for the delivery of chyme in a manner that does not exceed the digestive capacity of the small intestine (Ueno et al., 2005; Schulze, 2006). As a result of normal exposure to both an appropriate composition of reduced food particles and the rate of trans-pyloric delivery of chyme, the duodenum releases GI peptides and hormones that are integral to these feedback mechanisms (Dockray, 2006; Englander and Greeley, 2008; Dockray and Burdyga, 2011).

Data regarding gut hormone levels in the SCI population are scarce. Motilin, a 22-amino acid peptide released from the upper intestine, stimulates gastric and intestinal myoelectric activity during phase III contractions of the migrating myoelectric complex of the interdigestive phase. Comparisons of serum motilin levels in a limited sample of uninjured, paraplegic and quadriplegic subjects revealed that motilin levels were

largely similar across all three groups, though there was a trend toward elevated motilin levels in the paraplegic group (Saltzstein et al., 1995). Peptide YY (PYY) is a 36-amino acid peptide hormone that is similar to GI peptides PP and NPY that is released from epithelial cells within the ileum and colon. However, the actions of circulating PYY target the upper GI as an "ileal brake" whereby PYY potently diminishes gastric acid secretion, gastric emptying, intestinal propulsion and pancreatic exocrine secretion (Englander and Greeley, 2008). In this same study described above, levels of PYY in chronic SCI individuals were similar in the fasted state but were significantly elevated in the early postprandial state of quadriplegic subjects (Saltzstein et al., 1995). The limited sample size and the absence of essential physiological parameters, such as gastric emptying rates during the postprandial serum measurements, limit the interpretation of these findings and merit re-evaluation in a larger sample from the SCI-population.

UPPER GASTROINTESTINAL DYSFUNCTION IN EXPERIMENTAL MODELS OF SCI

Over the past 20 years, the SCI research community has gained a clearer understanding of the interrelated cellular and biochemical processes which comprise the aftermath of SCI; identified the challenges for successful regeneration of damaged tissue; and expended considerable intellectual capital upon the recovery of stepping and standing after SCI. Unfortunately, post-SCI changes to GI autonomic reflexes remain inadequately explored, however, recent attention has been directed at derangements in GI function in animal models of experimental SCI.

Published reports and our own preliminary observations in an animal model of SCI have demonstrated striking similarities to the clinical presentation of profound GI dysmotility in humans. Expanding upon the initial reports that high thoracic spinal transection delayed the emptying of a phenol red liquid test meal (Gondim et al., 1999, 2001), we reported that T3-SCI animals show a diminished food intake (Primeaux et al., 2007). Subsequent studies utilized an Inactin-anesthetized preparation in which sub-miniature, dual-element, strain gauges were sutured to the serosal surface of the gastric corpus, thus recording circular smooth muscle contractions. We reported a significant reduction in gastric motility that was not altered by sympathectomy (Tong and Holmes, 2009). Our results were in agreement with earlier conclusions by Gondim et al. who concluded that post-SCI dysmotility was vagally mediated (Gondim et al., 2001). Furthermore, utilizing the [^{13}C]-octanoate breath test see Ghoos et al. (1993) as an indirect measure of gastric emptying in intact rats, we concluded that diminished gastric function after SCI was not likely due to the effects of Inactin anesthesia (Qualls-Creekmore et al., 2010b).

Canon and Lieb were the first to describe the gastric accommodation reflex (Canon and Lieb, 1911). This vagally-mediated reflex is elicited either by physiological (low pressure) distension of the esophagus (Saltzstein et al., 1995)or direct filling of the isolated stomach (Takahashi and Owyang, 1997)and permits the stomach to relax in response to large volumes of ingesta, thus maintaining low levels of intragastric pressure (Wilbur and Kelly, 1973). In our T3-SCI model, physiological distension of the esophagus failed to elicit a reflex relaxation of the stomach (Tong and Holmes, 2009). Expanding upon the study by Takahashi and Owyang (Takahashi and Owyang, 1997), retrograde gastric filling in T3 surgical control rats elicited marked increases in intragastric pressure as well as intragastric pressure waves similar to

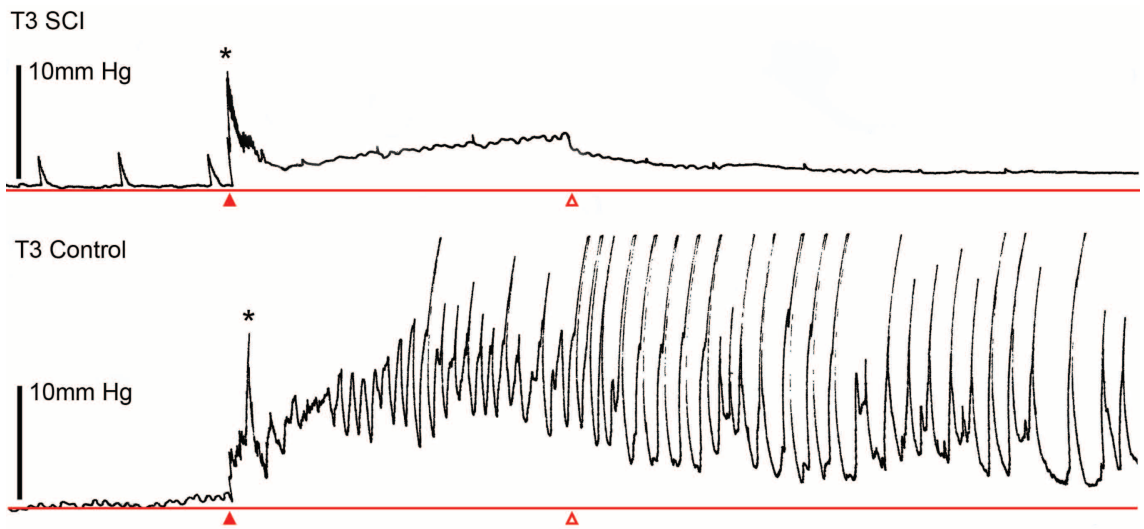

FIGURE 2 | Spinal cord injury diminishes mechanical sensitivity of the stomach to fluid distension. Representative gastric pressure traces in high thoracic spinal cord injured (T3 SCI, *upper trace*) and surgical (laminectomy only) controls (T3 Control, *lower trace*) demonstrating that during 6 min of continuous filling (at a rate of 1 ml/min, starting at closed arrowhead and terminating at open arrowhead). T3 SCI rats exhibited a smaller increase in gastric pressure and that pressure-evoked motility waves were less pronounced. Initial pressure peak (denoted by asterisks) was an artifact of initiating the filling cycle. Gastric distension was maintained at the termination of the filling cycle. Distension was performed by passing a saline-filled catheter via an incision in the proximal duodenum and through the occluded pylorus. The lower esophageal sphincter was untouched and maintained closure at the gastric cardia.

those observed in conscious, freely moving rats fed a test meal (Janssen et al., 2008). In T3-SCI rats, both intragastric pressure, as well as intragastric pressure waves, are substantially diminished (**Figure 2**; Holmes et al., 2008).

Further studies using [^{13}C]-octanoate tagged solid meals in awake animals confirmed that gastric dysmotility is accompanied by a delay in gastric emptying and that dysmotility persists up to 6 weeks after T3-SCI, (Qualls-Creekmore et al., 2010a). These persistent deficits led us to conclude that delayed gastric emptying is unlikely to be due to "spinal shock" as gastric dysmotility persists long after SCI animals are generally considered to have stabilized (ca. 3–6 weeks post-SCI).

Finally, with the understanding that the reflex control of the stomach is under considerable modulation by gut hormones, like CCK and ghrelin, we have begun to test the sensitivity of T3-SCI rats to GI peptides which evoke vagally-mediated gastric reflexes (Ueno et al., 2005). It is well accepted that peripheral CCK-8s activates C-type vagal afferent fibers and increases *c-Fos* immunoreactivity in NTS cells of neurally intact animals (Renehan et al., 1995; Zittel et al., 1999; Sullivan et al., 2007). Our study was particularly revealing in that peripheral CCK-8s administration 3 days after injury induced significantly less *c-Fos* expression in the NTS than in uninjured control rats (Tong et al., 2011). In the same experimental animals, *c-Fos* expression within the adjacent AP was similar in both groups, suggesting that gastric neurocircuitry involving the NTS was selectively impaired. Previous experimental studies suggest that CCK acts both peripherally and directly upon brainstem vagal circuits (Raybould and Tache, 1988; Fraser and Davison, 1992; Li and Rowland, 1995; Sayegh and Ritter, 2000; Baptista et al., 2005a,b, 2006; Holmes et al., 2009b). However, T3-SCI rats did not demonstrate a gastric efferent vagal response to central microinjection of CCK-8s into the DVC and the reduced sensitivity to centrally administered CCK-8s in the DVC persisted at 3 weeks after injury. Furthermore, whole-cell patch clamp recordings of NTS neurons from T3-SCI rats suggested a reduced activity of CCK-8s on synaptic inputs onto NTS neurons. Our preliminary data with peripheral and central administration of the prokinetic gut hormone, ghrelin, also reveals a reduced sensitivity within the NTS of SCI rats (Holmes et al., 2009a; Browning et al., 2010).

In summary, the reduction in vagal afferent responsiveness to mechanical and chemical stimuli, as well as the reduction in presynaptic glutamatergic inputs onto NTS neurons, suggests a generalized hyposensitivity of vagal afferent neurotransmission to the brainstem following SCI. Evidence of vagal afferent hyposensitivity has been identified in other GI pathobiological states (Hatanaka et al., 1997; Xue et al., 2009). In particular, Xue and colleagues suggest that part of the diminished visceral afferent sensitivity in an inflammation-induced model of functional dysmotility is mediated through an inducible NOS (iNOS) mechanism (Xue et al., 2009). However, this observation was limited only to afferents within the mesenteric arcade, and did not include the vagus. While levels of neuronal NOS are chronically diminished following experimental T9-SCI (Kabatas et al., 2008), preliminary data in T3-SCI rats suggests that GI iNOS levels are elevated in the days after SCI (Holmes, unpublished observation).

IS DIMINISHED VAGAL SENSORY PROCESSING ONE CULPRIT?

Whether these preliminary observations reflect a mechanism of SCI-mediated GI dysfunction requires further testing. The fact that SCI affects a neural circuit as spatially removed from the site of injury as vago-vagal control of the stomach presents an intriguing paradox. Since the main neural circuitry controlling the stomach remains physically intact after human and experimental SCI, why is gastric function compromised so persistently? Our evidence points to a persistent inhibition of vagally-mediated gastric reflexes which do not appear to involve sympathetic input to the stomach. While the neural mechanisms responsible for this post-SCI gastroinhibition remain obscure, recent reports provide a potential explanation.

NEUROPLASTICITY IN THE BRAIN-GUT AXIS

The spontaneous firing property of DMV neurons, mentioned previously, implies that alterations in firing rate can produce profound changes in gastric vago-vagal reflexes. Activation of NMDA and non-NMDA receptors in response to high levels of glutamate release is essential for the rapid transmission of feeding-relevant stimuli (Berthoud et al., 2001; Hornby, 2001). However, the visceral organ or function-specific wiring of brainstem neurocircuitry is complex, and the available permutations in synaptic organization confers a substantial degree of functional plasticity within GABAergic and glutamatergic synapses (see Babic et al., 2011). Recently an elegant model for vago-vagal plasticity has been put forward (Browning and Travagli, 2010). Essentially, these authors propose that cAMP levels within nerve terminals of GABAergic NTS neurons modulate receptor trafficking to the neuronal membrane, and hence regulate the ability of neurotransmitters of this synapse to be modulated (Browning et al., 2006; Browning and Travagli, 2009). Furthermore, cAMP levels may be regulated by vagal afferent input via group II metabotropic glutamate receptors (mGluR) known to be expressed within the DVC (Hay et al., 1999). Unlike glutamate release in response to digestion-relevant stimuli, tonic low level release of glutamate from vagal afferents keeps cAMP expression low due to a higher affinity of mGluR for glutamate. This activation of mGluR by low-level glutamate release confers greater NTS release of GABA, thus inhibiting gastric function, in addition to rendering the GABAergic neuron resistant to modulation (Browning et al., 2006; Browning and Travagli, 2007).

The NTS and DMV neurons are not the only point in the gut-brain axis where significant neuroplasticity occurs. ENS plasticity has been described in response to luminal contents (Soret et al., 2010) and disease processes (Huizinga et al., 2009; Mawe et al., 2009). As mentioned previously, vagal afferent fibers and cell bodies are sensitive to feeding related peptides such as CCK) (Appleyard et al., 2005; Baptista et al., 2005b). These vagal afferent neurons also demonstrate considerable plasticity to trauma (Zhang et al., 1996) as well as physiological stimuli (Burdyga et al., 2004, 2006a, 2008; de Lartigue et al., 2007). These physiological stimuli are proposed to switch vagal afferents between "fed" and "fasted" states (see Dockray and Burdyga, 2011) and provide one mechanism ultimately elevating cAMP levels in GABAergic neurons (Browning and Travagli, 2009).

FUTURE DIRECTIONS

Upon revisiting the vagal neurocircuitry controlling gastric function (presented in **Figure 1**), it is evident that there are several points where this pathophysiological cascade may occur. At the level of the GI lumen, diminished synthesis and/or release of GI peptides may represent a signaling failure following SCI (**Figure 3.1**). In addition to a persistent (up to 6 week post-SCI) reduction of gastric reflex responses to mechanical and peptidergic signaling, our observations demonstrate that *in vivo* CCK release following a mixed nutrient meal is diminished following SCI while iNOS mRNA expression is elevated (Holmes—unpublished observations). NO^- serves as an important regulator of CCK release in STC-1 cells (Mangel et al., 1996) as well as an endogenous modulator of vagal afferent sensitivity (Page et al., 2009). This suggests that in addition to smooth muscle relaxation, iNOS-derived NO^- may be implicated in the diminished release of GI peptides following SCI while also initiating a pathophysiological reduction of vagal afferent signaling at the level of the receptive fields.

Diminished CCK release entails additional pathobiological sequelae beyond failure to promote nutrient-mediated vagal afferent signaling. For example, gut permeability is a hallmark of numerous disease states and spinal transection has been shown to induce bacterial translocation within the gut (Liu et al., 2004). In a hemorrhagic shock model of inflammation and bacterial translocation, CCK-1 receptor activation of the

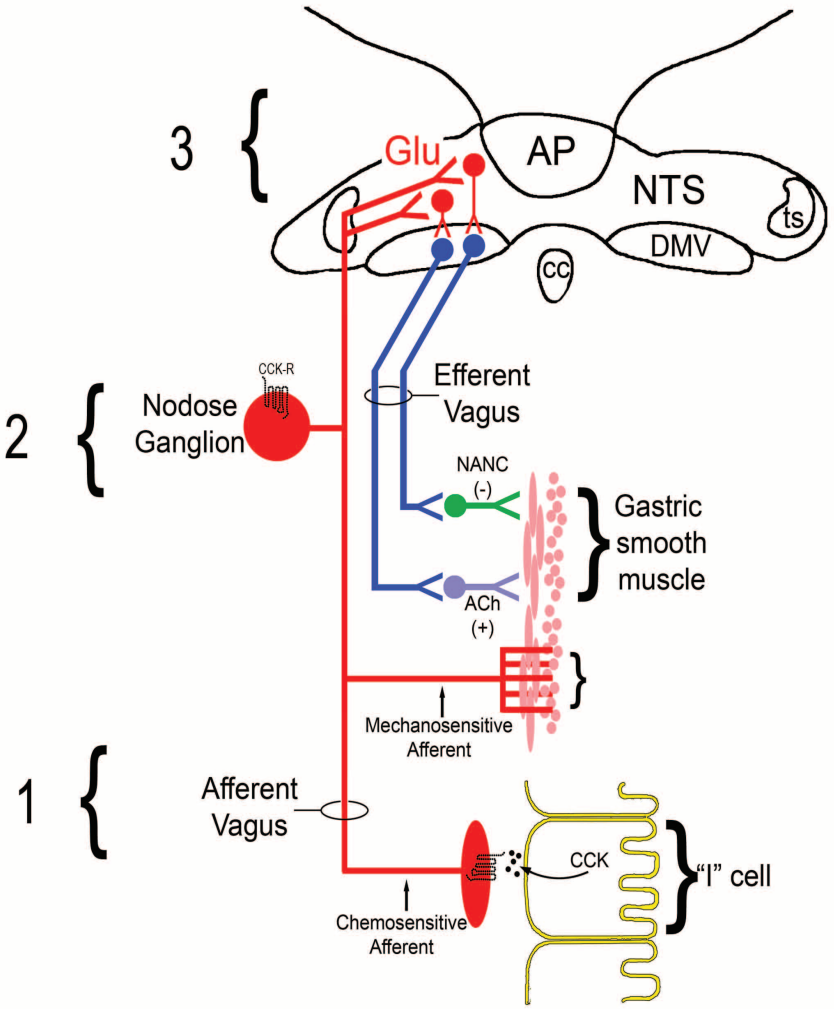

FIGURE 3 | Hypothesized schematic of diminished vagal sensory processing following T3-SCI. (1) Impairments within GI enterocytes (for example CCK-secreting "I" cells) leading to a reduction in the synthesis or release of feeding-related GI peptides such as CCK may reflect a failure in transduction mechanisms at the level of the primary afferent. (2) Downregulation of appropriate receptors, which most likely occurs at multiple levels of vagal afferent neurons residing within the nodose ganglion [reviewed in Dockray and Burdyga (2011)], to feeding-related GI peptides diminishes glutamatergic signaling in the NTS-DMV leading to failed signaling at first synapse. Additionally, derangements in the transmission of gastric distension signals by mechanosensitive vagal afferents may also promote a parallel reduction in visceral stimuli which is independent of GI peptide release. (3) Ultimately, reduced signaling to NTS neurons permits unmodulated GABAergic inhibition of DMV motor outflow to the stomach that represents a failure of reflex integration at the second synapse in the vago–vagal circuit. Deficits at any one, or across all, of these levels will lead to gastric dysmotility and may limit the efficacy of potential therapeutic mechanisms.

vagus maintained intestinal integrity (Lubbers et al., 2009, 2010), presumably through activation of an α7-nicotinic receptor (α7-nAChR) anti-inflammatory mechanism (Luyer et al., 2005).

Independent of whether normal GI release of either CCK or ghrelin occurs after SCI, our observation that exogenous CCK fails to increase NTS *c-Fos* expression would indicate that the mechanism of gastric dysmotility may occur, at least in part, through reduced receptor expression and/or reduced excitability of vagal afferents. Neurophysiological changes in gastric vagal afferent fibers may form the second level of vagal afferent dysfunction in the development of post-SCI dysmotility (**Figure 3.2**). For example, vagal afferent reorganization occurs in response to surgically-induced gastric trauma (Phillips and Powley, 2005) and extends to alterations in the spontaneous firing rate of the afferent vagus (Miranda et al., 2009). The role of capsaicin-sensitive C-type fibers has long been recognized in GI reflex function, particularly CCK-mediated reflexes (Raybould and Tache, 1988; Sivarao et al., 1998). Additionally, vagal afferent neurons, which reside within the nodose ganglion, express CCK-1 receptors [aka. CCK type "A" receptors (Zhao et al., 2010)]. Identifying changes in vagal afferent responsiveness would provide a logical mechanistic explanation for our reported observation that exogenous peripheral administration of CCK induces diminished *c-Fos* expression following SCI (Tong et al., 2011). Similar observations have been provided for the action of ghrelin (Page et al., 2007) and expression of ghrelin receptors (Burdyga et al., 2006b; Page et al., 2007) and would extend to our observations after T3-SCI (Holmes et al., 2009a; Browning et al., 2010). These GI peptide-mediated changes are not the only mechanism by which visceral sensations fail to reach the brainstem as our data illustrates an impairment of mechanosensitive neurotransmission which also occurs following SCI.

Based upon the available data, our present research is further aimed at addressing the hypothesis that post-SCI reduction in vagal and/or NTS neuronal sensitivity to visceral signals biases GI brainstem circuits toward a tonic GABAergic inhibition of DMV efferent outflow to the stomach (**Figure 3.3**). Whether the mechanism of action is an inability of GI peptides to elevate cAMP levels in GABAergic neurons and/or nerve terminals after SCI or limited glutamatergic input that does little more than drive mGluR-mediated dampening of cAMP levels, it is the inability to modulate the inhibitory effects of this NTS-DMV GABAergic synapse that is most likely responsible for triggering gastric dysmotility following SCI.

TRANSLATIONAL PERSPECTIVE

Despite considerable evidence of upper GI dysmotility in the SCI population, the mechanisms remain poorly understood and require further study in order to develop evidence-based therapeutic strategies individuals with SCI. The conclusions of sympathetic involvement in the clinical report by (Fealey et al., 1984) still merits further investigation. However, there is growing evidence that derangements in gastric vagal neurocircuitry contribute to functional GI motility disorders in neutrally intact patients (Holtmann et al., 1998; Lunding et al., 2008; Manabe et al., 2011). Chronic changes in gut hormone levels, as well as the long term changes in vago–vagal gastric reflexes signaling are two universal targets for research in both intact and neurotrauma patients.

Ultimately, the failure within the brain-gut axis to respond to GI signaling pathways presents a clinical dilemma. Promising therapeutic strategies for other functional motility disorders may not necessarily translate to the SCI population or even across different regions of the GI tract (Holmes et al., 2009a; Browning et al., 2010; Ferens et al., 2011). Furthermore, while enteral feeding is associated with improved outcome in the critical care patient, conditions of enteral intolerance exist which may lead to increased mortality. Increasing gastric motility by pharmacological agents such as ghrelin mimetics offers considerable therapeutic potential. However, failure to understand the mechanisms which result in feeding intolerance may render therapeutic interventions ineffective if not detrimental. The unique sequelae of secondary injuries and pathologies spatially remote from the injury site, which are not limited to GI dysfunction, underscores the knowledge gaps which remain in our understanding of SCI.

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Nociceptors as chronic drivers of pain and hyperreflexia after spinal cord injury: an adaptive-maladaptive hyperfunctional state hypothesis

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Spinal cord injury (SCI) causes chronic peripheral sensitization of nociceptors and persistent generation of spontaneous action potentials (SA) in peripheral branches and the somata of hyperexcitable nociceptors within dorsal root ganglia (DRG). Here it is proposed that SCI triggers in numerous nociceptors a persistent hyperfunctional state (peripheral, synaptic, and somal) that originally evolved as an adaptive response to compensate for loss of sensory terminals after severe but survivable peripheral injury. In this hypothesis, nociceptor somata monitor the status of their own receptive field and the rest of the body by integrating signals received by their peripheral and central branches and the soma itself. A nociceptor switches into a potentially permanent hyperfunctional state when central neural, glial, and inflammatory signal combinations are detected that indicate extensive peripheral injury. Similar signal combinations are produced by SCI and disseminated widely to uninjured as well as injured nociceptors. This paper focuses on the uninjured nociceptors that are altered by SCI. Enhanced activity generated in below-level nociceptors promotes below-level central sensitization, somatic and autonomic hyperreflexia, and visceral dysfunction. If sufficient ascending fibers survive, enhanced activity in below-level nociceptors contributes to below-level pain. Nociceptor activity generated above the injury level contributes to at- and above-level sensitization and pain (evoked and spontaneous). Thus, SCI triggers a potent nociceptor state that may have been adaptive (from an evolutionary perspective) after severe peripheral injury but is maladaptive after SCI. Evidence that hyperfunctional nociceptors make large contributions to behavioral hypersensitivity after SCI suggests that nociceptor-specific ion channels required for nociceptor SA and hypersensitivity offer promising targets for treating chronic pain and hyperreflexia after SCI.

Keywords: inflammatory pain, neuropathic pain, primary afferent neuron, hyperexcitability, sensitization, memory of injury, evolution

INTRODUCTION

Neuropathic pain in general and spinal cord injury (SCI) pain in particular are usually viewed as maladaptive consequences of neural injury (Costigan et al., 2009; Yezierski, 2009; Gwak and Hulsebosch, 2011). Chronic neuropathic pain is certainly maladaptive for patients because of the suffering and disability that can occur. A broader view is that SCI results in the pathological recruitment of mechanisms that under other conditions can be biologically adaptive. I will argue that SCI presents a complex set of signals to primary nociceptors that induces a persistent hyperfunctional state in these neurons that can be triggered by extrinsic *central signals* that are associated with severe but survivable *peripheral injury*. My focus is on the many uninjured nociceptors that are chronically altered by SCI and positioned to help drive below-level and at-level pain.

CENTRAL NEUROPATHIC PAIN INVOLVES MANY MECHANISMS, SOME OF WHICH MAY INVOLVE ENHANCED ACTIVITY IN PRIMARY AFFERENT NEURONS

Before considering the role of primary nociceptors in driving chronic SCI pain, I note that numerous mechanisms at many different central loci may contribute to central neuropathic pain, and different forms of central pain are likely to differ in their dependence upon alterations in primary sensory neurons. Injury to any part of a pain pathway could result in chronic pain if the injury persistently enhances excitatory activity within the pathway. For example, small strokes in the thalamus and other parts of the brain can produce chronic pain (Finnerup, 2008), and these injuries and their sequelae may have little impact on primary afferent neuron function.

Some injuries to the spinal cord might also produce chronic pain with relatively little involvement of primary afferent neurons. For example, a small electrolytic lesion encompassing part of the spinothalamic tract in the cervical spinal cord causes persistent behavioral hypersensitivity (Masri et al., 2009) and tonic pain (Davoody et al., 2011), possibly by disinhibition of thalamic pathways (Masri et al., 2009). More extensive spinal injuries that are more likely to impact primary afferents produce many central alterations that, in principle, can underlie chronic pain. For example, SCI causes disinhibitory effects within the dorsal horn by severing descending inhibitory pathways (Bruce et al., 2002; You et al., 2008), killing inhibitory interneurons (Meisner et al., 2010), reducing spinal GABA levels (Gwak et al., 2008), and changing the Cl^- equilibrium potential in projection neurons receiving inhibitory input (Lu et al., 2008). In addition, long-lasting intrinsic hyperexcitability of central neurons within pain pathways can occur after SCI. Notably, hyperexcitability is expected from the upregulation of Na^+ channels described in spinal dorsal horn neurons and thalamic neurons (Hains et al., 2003, 2005). There is also morphological evidence for synaptic potentiation in dorsal horn neurons after SCI (Tan and Waxman, 2012). Finally, SCI causes long-lasting alterations in spinal microglia and astroglia that are reported to promote pain-related behavior (e.g., Hains and Waxman, 2006; Zhao et al., 2007; Detloff et al., 2008; Carlton et al., 2009; Marchand et al., 2009; Tan et al., 2009; Gwak et al., 2012).

Finding long-lasting alterations in central pain pathways after SCI does not demonstrate that alterations within the CNS are exclusively responsible for chronic pain. Central alterations might be driven in large part by chronic increases in the activity of primary sensory neurons. Primary afferents are clearly capable of driving chronic pain; for example, their persistent activity plays a major role in maintaining pain in some peripheral neuropathy models (e.g., Gracely et al., 1992; Zhang et al., 2000; Sukhotinsky et al., 2004; Xie et al., 2005; Pitcher and Henry, 2008).

SCI ALTERS CENTRAL AND PERIPHERAL BRANCHES OF PRIMARY NOCICEPTORS

The first evidence for SCI-induced alterations of primary sensory neurons came from observations suggesting that primary afferents, and especially those immunoreactive for CGRP (a marker for "peptidergic nociceptors"), sprout new branches within the dorsal horn after SCI, which potentially could lead to more extensive nociceptive input to dorsal horn neurons (Helgren and Goldberger, 1993; Christensen and Hulsebosch, 1997; Krenz and Weaver, 1998; Weaver et al., 2001; Ondarza et al., 2003; Zinck et al., 2007; Hou et al., 2009). Although a failure to find evidence of sprouting of CGRP-positive fibers has been reported in some SCI models (Kalous et al., 2007, 2009), the possibility of nociceptor sprouting after spinal contusion injury is supported by the finding that SCI triggers an intrinsic growth-promoting state that further enhances the growth in culture of dissociated small and medium-sized DRG neurons, including CGRP-positive neurons, sampled from spinal segments close to and distant from an injured segment (Bedi et al., 2012).

Physiological evidence for persistent hyperexcitability of C-fiber neurons after SCI first came from observations of increases in TTX-sensitive Na^+ currents in dissociated somata of primary afferents innervating the bladder (Yoshimura and de Groat, 1997; de Groat and Yoshimura, 2010). An association of persistent hyperexcitability in nociceptors with pain-related behavior was not reported until twelve years later. A contusion injury at T10 that produced mechanical and thermal hypersensitivity of forelimb withdrawal responses tested 35 days later increased the sensitivity of the peripheral terminals of functionally identified nociceptors to mechanical and thermal stimulation in an isolated forepaw skin-nerve preparation, as well as producing low-frequency spontaneous activity (SA) in the terminals of the nociceptors (Carlton et al., 2009). This important discovery suggested that hyperexcitability and SA in peripheral branches of primary nociceptors might help to drive chronic pain after SCI, although it did not show whether this hyperexcitability was an intrinsic property of the nociceptors.

SCI INDUCES PERSISTENT SPONTANEOUS ACTIVITY (SA) IN NOCICEPTOR SOMATA

SCI has surprisingly strong effects on the excitability of nociceptor somata (cell bodies) (Bedi et al., 2010). *In vivo* recordings from nociceptive C and Aδ fibers in anesthetized rats showed that contusive SCI dramatically increased the incidence of SA generated within L4 and L5 DRG 1 to 3 months after injury. SCI-induced SA in nociceptor somata was also expressed *in vitro*, 1 day after dissociation in low-density cultures lacking serum or growth factors. The incidence of *in vitro* SA was remarkably high (35–70%) 3 days to 8 months after SCI compared to what has been reported (0–20%) for dissociated small DRG neurons sampled from rats with peripheral neuropathy (Petersen et al., 1996; Abdulla and Smith, 2001; Ma and LaMotte, 2005; Zheng et al., 2007). All of the spontaneously active neurons sampled *in vitro* after SCI were small, ∼80% were capsaicin sensitive, and ∼33% bound isolectin B4 (a positive marker for "non-peptidergic" nociceptors; Wang et al., 1994), indicating that most of the neurons exhibiting SA were nociceptors (Bedi et al., 2010). The incidence of SA after SCI was highest in neurons dissociated from DRG far below the injury level (L4/L5, 50–70%) and high just below the injury (T11/T12, 35–50%) compared to the incidence in dissociated neurons from sham-operated animals (16%) and naive animals (15%). Somally generated SA was not elevated significantly in neurons from DRG far above the injury (C6/C7, 10–25%), even though SCI enhanced peripherally generated SA in nociceptors at this level (Carlton et al., 2009). Another difference between nociceptor effects above and below the injury level was the absence of elevated SA just above the injury (T8/T9, 10%) 3 days after SCI, whereas 1 month or later 50% of T8/T9 neurons exhibited SA. Thus, contusive SCI can persistently increase the prevalence of somally generated SA close to and far below the injury site. It is not yet known if peripherally generated SA occurs in nociceptors outside the cervical region. Finding the highest incidence of SA in small, C-type neurons distant from the injury site shows that axotomy of these neurons by SCI is not required to trigger the chronic SA (see below). Enhancement of the incidence of nociceptor SA by SCI is not restricted to spinal contusion injury; in preliminary studies 70% of below-level DRG neurons dissociated 1–3 months after spinal hemisection injury

at T10 showed SA (Carlton et al., 2011), and 80% showed SA 1 month after unilateral avulsion of T13/L1 dorsal roots (Du et al., 2011). In both cases there was similar incidence of SA in dissociated neurons from DRG ipsilateral and contralateral to the injury, which again shows that direct injury of nociceptor axons is not an important trigger for chronic SA after SCI. Note that unilateral dorsal root avulsion, like most forms of SCI, causes chronic pain (Wieseler et al., 2010) and widespread, bilateral inflammatory and glial responses in the spinal cord (Chew et al., 2011).

SCI INDUCES A DISCRETE HYPEREXCITABLE/SPONTANEOUSLY ACTIVE (HSA) STATE IN NOCICEPTOR SOMATA

Differences in various somal properties related to hyperexcitability were found in nociceptors dissociated from SCI animals compared to those from sham-operated and naïve animals (Bedi et al., 2010). Surprisingly, all the observed differences in excitability properties could be accounted for by the existence of a single population of hyperexcitable/SA (HSA) nociceptors, which was large in SCI animals and much smaller in naive and sham-treated animals. Compared to silent neurons, SA neurons from any of the treatment groups were depolarized at rest by nearly 5 mV, exhibited more repetitive firing during depolarizing test pulses, required less depolarizing current to evoke action potentials, and had much lower membrane conductance when tested within the range of membrane potentials where SA occurred. The stronger correlation among all the hyperexcitability properties and SA than between any of these properties and SCI indicates that SCI promotes entry of nociceptor somata into a discrete HSA state. The expression of the HSA state 1 day after dissociation and 5 or more months after SCI shows that the intrinsic HSA state is persistent. The persistence could come from a *stable HSA state* that, once induced, is expressed for very long periods in similar fashion *in vivo* and *in vitro*, even in the absence of inflammatory signals and growth factors. This possibility is supported by the high incidence of SA recorded in dissociated DRG neurons and *in vivo* from axons in dorsal roots connected to the DRG but disconnected from the periphery and spinal cord, and the similarity of the spontaneous firing rates (∼1 Hz) under these quite different conditions (Bedi et al., 2010). Persistence could also come from a *stable hypersensitive state* in which a nociceptor's predisposition to enter a more transient HSA mode is enhanced, but remains latent unless inflammatory mediators or other injury-related stimuli are present. Such stimuli might include signals generated during the injurious process of dissociation, which by itself can modestly promote entry into an HSA-like state, increasing the incidence of nociceptor SA from ∼0 to ∼13% (Zheng et al., 2007)—much lower than the >50% incidence of SA in dissociated nociceptors sampled from DRG after SCI (Bedi et al., 2010). A very long-term, SCI-induced intrinsic hypersensitive state that enhances a nociceptor's responding with an intermediate-term HSA mode to inflammation- and injury-related signals would be consistent with the phenomenon of "hyperalgesic priming" that has been extensively studied in nociceptors (Reichling and Levine, 2009) and is discussed below.

SA IN NOCICEPTOR SOMATA IS CORRELATED WITH AND MAY HELP DRIVE PAIN-RELATED BEHAVIOR AFTER SCI

The incidence of somal SA after SCI was significantly correlated with behavioral hypersensitivity tested 1 and 3–5 months after injury; the animals showing the greatest sensitivity to mechanical and thermal test stimuli applied to all four paws also had the highest incidence of nociceptor SA recorded *in vitro* (Bedi et al., 2010). Significant correlations between mechanical or thermal hypersensitivity and incidence of nociceptor SA were found for hindlimb responses, which were correlated with SA in neurons from L4/L5 DRG. Furthermore, forelimb responses were correlated with SA in the above-level neurons sampled from T8, T9, C6, and C7 DRG. Particularly interesting effects of SCI were found on vocalization elicited by mechanical stimuli delivered to an array of test sites on the back. SCI dramatically reduced the incidence of vocalization evoked by below-level test stimuli, suggesting substantial interruption of spinal pathways traversing the injury site. Conversely, SCI increased the incidence of vocalization to above-level stimuli, and the above-level vocalization was correlated with SA in neurons sampled from at- and above-level DRG. Surprisingly, relatively little chronic SA was observed in somata of neurons from C6/C7 DRG; influences of nociceptor SA on supraspinal responses and forelimb responsiveness may come from wide-ranging effects of active nociceptors in above-level DRG closer to the injury site, or from nociceptor SA generated in the periphery (Carlton et al., 2009).

SA generated within somata and peripheral branches of nociceptors is likely to drive central sensitization (Carlton et al., 2009; Bedi et al., 2010). If this SA also drives pain-related behavioral alterations, then manipulations that selectively block the SA should reduce the behavior. The nociceptor-specific Na^+ channel, Nav1.8 is important for the expression of nociceptor SA and other sensitizing effects in other pain models (Lai et al., 2002; Roza et al., 2003; Jarvis et al., 2007; Abrahamsen et al., 2008). Importantly, knocking down the expression of Nav1.8 largely eliminates SCI-induced nociceptor SA *in vitro* and greatly reduces behavioral hypersensitivity to mechanical and thermal test stimulation applied *in vivo* (Yang et al., 2012). This finding indicates that SA and hyperexcitability in primary nociceptors plays a major part in driving chronic hypersensitivity and possibly pain after SCI.

PERSISTENT NOCICEPTOR ALTERATIONS ARE HYPOTHESIZED TO BE TRIGGERED BY SOMAL INTEGRATION OF CENTRAL AND LOCAL INFORMATION THAT INDICATES SEVERE INJURY

My central hypothesis is that a long-lasting hyperfunctional state (which includes the somal HSA state) can be triggered in nociceptors by a combination of events after SCI that mimic patterns of signals used by nociceptors to recognize particularly severe *peripheral* injury. In the next sections I discuss the types of signals that this hypothesis suggests are integrated by the nociceptor soma to induce and maintain a persistent hyperfunctional state (**Figure 1**). I then present adaptive arguments for this hypothesis, and discuss the nature of the hyperfunctional state and some of its pathological consequences after SCI.

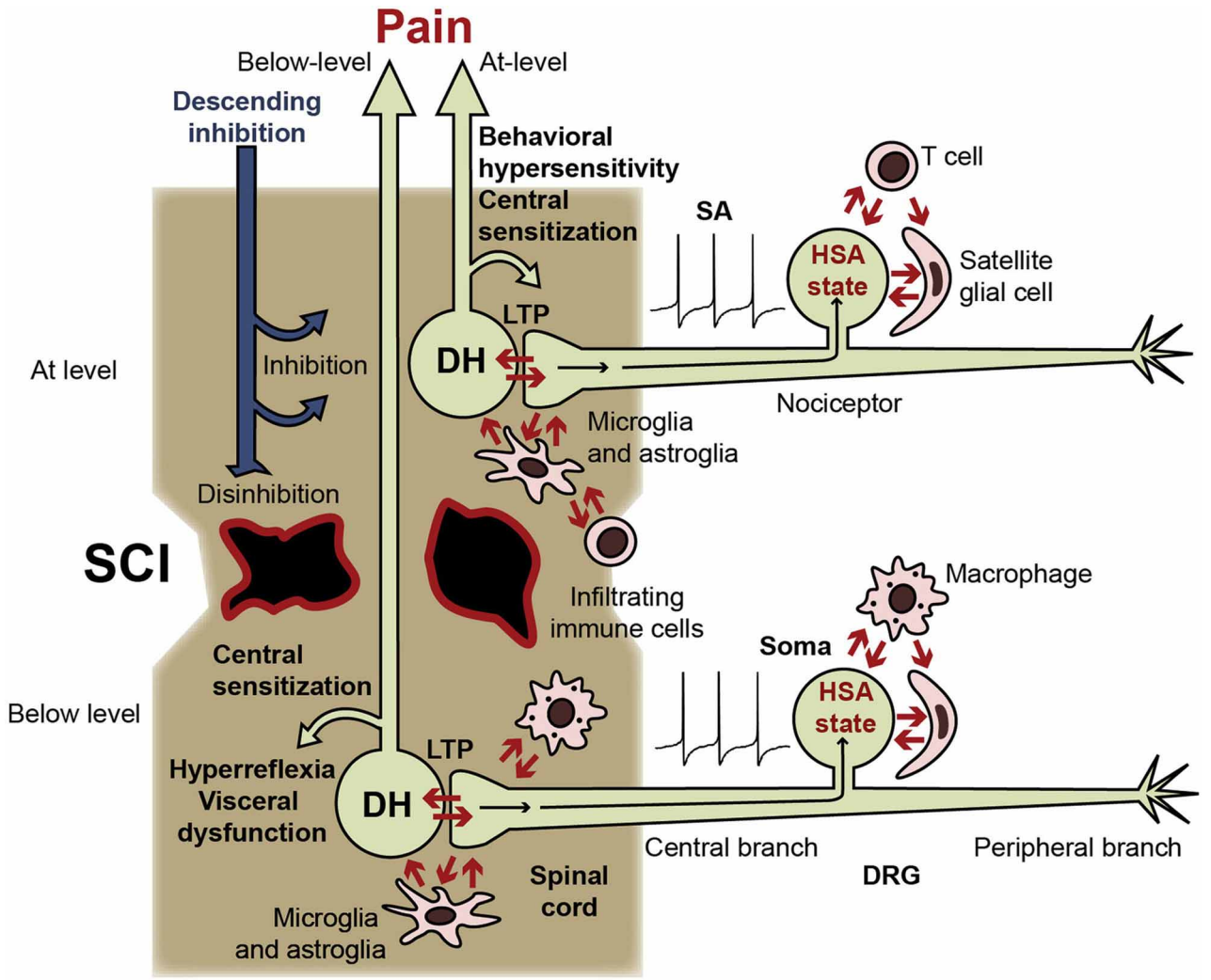

FIGURE 1 | Central and somal signals received by nociceptors during SCI, and consequences of switching nociceptors into a persistent hyperfunctional state. Nociceptors receive injury-related signals within the spinal cord (from intensely activated postsynaptic dorsal horn neurons [DH], activated glia, and infiltrating immune cells) and within the DRG (from other DRG neurons, satellite glial cells, and the blood). Nociceptors have potent excitatory effects on pain pathways (indicated by DH neurons) and on circuits subserving somatic and visceral functions (not shown). LTP at DH synapses can be produced by somal and peripheral SA and afterdischarge, facilitated by the nociceptor hyperfunctional state (including the somal hyperexcitable/SA [HSA] state). Nociceptor activity produces central sensitization, promotes spontaneous and evoked pain, and enhances somatic and visceral reflexes. Nociceptor activity also leads to positive feedback interactions with postsynaptic neurons, other DRG somata, inflammatory cells (microglia, infiltrating macrophages, and T cells), astrocytes, and satellite glial cells. Similar interactions of peripheral branches with surrounding cells are possible, but are less likely after central than peripheral injury and are not indicated here. Because SCI severs or demyelinates many ascending fibers, much of the activity in pain pathways generated below the injury level may be blocked, although residual pathways (illustrated) are likely to contribute to the sensation of below-level pain in cases of incomplete SCI. Conversely, interruption of descending inhibitory pathways enhances spinal excitability, promoting entry of nociceptors into the hyperfunctional state and further increasing the somatic and autonomic hyperreflexia and visceral dysfunction driven by SA in below-level nociceptors. Nociceptor SA generated immediately above the injury level should have ready access to intact spinal circuits and projection neurons in pain pathways, contributing to central sensitization, behavioral hypersensitivity, and at-level pain. At-level nociceptor alterations may involve additional signals generated by direct damage to the nociceptor (axotomy) and to nearby cells.

Long-lasting changes in intrinsic functional properties of primary sensory neurons are likely to depend upon changes in gene expression in these neurons (e.g., Lewin and Walters, 1999; Woolf and Costigan, 1999; Thompson et al., 2004; Lee et al., 2008; von Schack et al., 2011), although continuing exposure to extrinsic signals might also maintain functional changes (see below). Assuming that changes in nociceptor gene transcription are necessary for a persistent hyperfunctional state, what types of information does a nociceptor nucleus use to decide whether to enter this state?

One potential set of signals that will *not* be emphasized here is produced by direct injury (axotomy) to the nociceptor's own branches. This includes immediate injury discharge and both positive injury signals (e.g., activated protein kinases), and

negative injury signals (e.g., a decrease in target-derived growth factors) transported from damaged axons to the nucleus (Ambron and Walters, 1996). Persistent functional changes in primary afferent neurons associated with changes in gene expression are prominent in axotomizing peripheral nerve injury models (e.g., Obata et al., 2003; Persson et al., 2009). Axotomy-induced and demyelination-dependent signals, especially in nociceptors and other primary afferent neurons close to the injury level, may also contribute to chronic pain after SCI, as may other effects at the injury site, such as glial scarring. The constellation of injury signals expressed solely at the spinal injury level is likely to contribute to at-level pain and may distinguish SCI pain from other forms of chronic pain. These at-level injurious effects may not contribute directly to below-level or above-level pain.

In contrast, the present hypothesis focuses on signals causing alterations in nociceptors that have *not* been injured. SA and hyperexcitability or sensitization are prominent in nociceptors distant from a T10 contusion site (in neurons from lumbar and cervical DRG), which are unlikely to have axons projecting close enough to the lesion to experience axotomy or other forms of direct damage (Carlton et al., 2009; Bedi et al., 2010; see also Chung et al., 1979; Traub et al., 1990; Huang et al., 2006). Furthermore, uninjured nociceptors after SCI (Bedi et al., 2010) display much higher incidence of SA than reported for peripherally axotomized nociceptors (Liu et al., 2000; Djouhri et al., 2006). A functional consideration is that signals of injury to a nociceptor's own branches (axotomy signals) provide little information to the soma about the severity of a bodily injury because the natural signal source is restricted to a single, small receptive field. As outlined in the following sections, I propose that nociceptors assess the severity of peripheral injury by integrating nociceptive information coming from the spinal cord, other cells within the DRG, and blood, and that SCI happens to produce similar combinations of signals, leading to a maladaptive induction of the nociceptor hyperfunctional state.

CENTRAL INFLAMMATORY SIGNALS ARE HYPOTHESIZED TO PROVIDE INJURY-RELATED INFORMATION TO NOCICEPTORS

In the periphery, prolonged or repeated inflammation in the absence of axotomy often causes generation of nociceptor SA near a site of inflammation in peripheral terminals (Djouhri et al., 2006; Xiao and Bennett, 2007), along uninjured axons adjacent to degenerating axons (Campbell, 2001; Wu et al., 2001), and within the DRG (Xie et al., 2006; Huang et al., 2011). Inflammation makes major contributions to chronic pain in some peripheral neuropathy models that lack extensive axotomy (e.g., Clatworthy et al., 1995; Miller et al., 2009; Bastos et al., 2012). Importantly, peripheral injury or inflammation produces central inflammatory effects, including pronounced activation of microglia in the dorsal horn (e.g., Fu et al., 1999; Xie et al., 2009; Kim and Moalem-Taylor, 2011). After SCI (**Figure 1**), the central branches of nociceptors are exposed for long periods to inflammatory mediators released by resident microglia and infiltrating immune cells (Alexander and Popovich, 2009; Byrnes et al., 2011), as well as cytokines and chemokines released from astroglia and even from nociceptors themselves (see Miller et al., 2009). Enhanced expression of cytokines is found near the injury site and also in distant spinal segments months after SCI (Detloff et al., 2008; Hulsebosch, 2008; Sandhir et al., 2011). Although little is yet known about effects of central inflammation on nociceptors, peripheral inflammation or treatment with inflammatory mediators causes an upregulation in DRG neurons of numerous molecules that can increase nociceptor excitability, including TRPV1 (Ji et al., 2002; Yu et al., 2008), TRPA1 (Katsura et al., 2006), Nav1.7 (Strickland et al., 2008), and Nav1.8 (Coggeshall et al., 2004; Villarreal et al., 2005; Strickland et al., 2008). In addition, inflammation can downregulate K^+ channels (La and Gebhart, 2011; Marsh et al., 2012). Whereas alterations of axotomized nociceptors after nerve injury involve reduced access to target-derived trophic factors, which can result in conflicting effects on nociceptive function (e.g., decreased expression of both Na^+ and K^+ channels) (Costigan et al., 2009), alterations of uninjured nociceptors after inflammation typically result in increased levels of trophic factors and other inflammatory mediators, enhancing nociceptive function (e.g., by increasing activity and expression of Na^+ channels and decreasing activity and expression of K^+ channels) (Gold and Gebhart, 2010; Linley et al., 2010; Marsh et al., 2012). Although little is known about the molecular alterations underlying SCI-induced hyperexcitability and SA in nociceptors, the effects observed thus far resemble those accompanying persistent peripheral inflammation: increased sensitivity to capsaicin and upregulation of TRPV1 channels (Wu et al., 2011), and a dependence upon Nav1.8 channels (Yang et al., 2012), suggesting that the hyperfunctional state depends upon prolonged exposure of a nociceptor's central terminals (and perhaps its soma) to inflammatory signals.

RETROGRADE SIGNALS FROM INTENSELY ACTIVATED POSTSYNAPTIC NEURONS IN THE DORSAL HORN ARE HYPOTHESIZED TO PROVIDE INJURY-RELATED INFORMATION TO NOCICEPTORS

Summation within dorsal horn neurons of the synaptic effects of numerous nociceptors activated by severe peripheral injury and/or inflammation will lead to synaptic LTP, which is likely to be accompanied by the generation of a series of retrograde synaptic and axonal signals that are transported back to the nucleus of presynaptic nociceptors (Parada et al., 2003; Perry and Fainzilber, 2009; Ho et al., 2011). As one example of several potential retrograde synaptic signals that might also be activated after SCI, bidirectional ephrinB-EphB signaling between nociceptors and dorsal horn neurons promotes long-lasting behavioral hypersensitivity, LTP, and upregulation of ephrinB1 in nociceptor somata after peripheral nerve injury (Song et al., 2008a,b). After SCI (**Figure 1**) intense activation of postsynaptic neurons in the dorsal horn will be produced by direct excitation from active nociceptors and from activated microglia and astroglia, and will be amplified by interruption of descending neural inhibition (Hains and Waxman, 2006; Hulsebosch, 2008; Carlton et al., 2009; Marchand et al., 2009; Miller et al., 2009; Gwak et al., 2012). Signaling from neurons and glia might also lead to initiation of action potentials within the central terminals of nociceptors (Lin et al., 2000; Price et al., 2009) that could signal retrogradely to

the soma. Interestingly, continuing LTP of nociceptor synapses might also be a direct consequence of SA in these cells. C-fiber LTP *in vivo* occurs at very low firing frequencies (e.g., 2 Hz) (Drdla and Sandkuhler, 2008), well within the range of SA firing rates observed after SCI (Bedi et al., 2010). SA-driven LTP and retrograde signals arising during LTP might contribute to positive feedback interactions between nociceptors and postsynaptic targets after SCI.

EXTRACELLULAR CHEMICAL SIGNALS WITHIN THE DRG ARE HYPOTHESIZED TO PROVIDE INJURY-RELATED INFORMATION TO NOCICEPTORS

Extracellular signals (in addition to the axonally transported intracellular signals just described) are conveyed directly to cells within the DRG after peripheral injury/inflammation and after SCI (**Figure 1**). Peripheral injury and inflammation will activate numerous nociceptors and this electrical activity will lead to release of neurotransmitters, including neuropeptides and chemokines (Miller et al., 2009), not only from nociceptor terminals in the dorsal horn, where they can activate neurons and glia (Wen et al., 2007), but also from the somata of active nociceptors within the DRG (Huang and Neher, 1996; Zhang and Zhou, 2002; Zhang et al., 2007; Jung et al., 2008), leading to the stimulation of satellite glial cells (Zhang et al., 2007), and probably other DRG neurons (Devor and Wall, 1990). Peripheral nerve injury can lead to the infiltration of hematogenous immune cells into the DRG (Kim and Moalem-Taylor, 2011) and spinal cord (Grace et al., 2011). Similarly, spinal transection causes infiltration of macrophages and T cells into DRG close to and distant from the injury site (McKay and McLachlan, 2004), where they might stimulate nociceptor somata. Severe peripheral or central injury will also cause the release of numerous extracellular signaling molecules into the blood. For example, SCI causes systemic inflammation (Gris et al., 2008) and long-term elevation of circulating cytokines (Davies et al., 2007). Because the DRG, unlike the CNS or nerves, lacks an effective vascular permeability barrier (Abram et al., 2006; Jimenez-Andrade et al., 2008), nociceptor somata and satellite glial cells will be fully exposed to systemic, blood-borne signals of injury, and inflammation.

SEVERAL MECHANISMS ARE HYPOTHESIZED TO MAINTAIN THE NOCICEPTOR HYPERFUNCTIONAL STATE

Three general possibilities exist for maintaining the hyperfunctional state: (1) continuing release of extrinsic signals, such as inflammatory mediators, that continuously refresh the hyperfunctional state, (2) positive feedback loops between nociceptor activity and inflammatory and retrograde synaptic effects, and (3) switching of the nociceptor into a potentially permanent intrinsic hyperfunctional state that remains after the extrinsic induction signals fade ("nociceptor memory"). First, widespread inflammation may persist chronically after SCI (Byrnes et al., 2011; Pajoohesh-Ganji and Byrnes, 2011). Moreover, macrophages and microglia can show priming that may outlast the apparent resolution of inflammation for weeks (Hains et al., 2010), suggesting a form of cellular memory in these inflammatory cells. Second, because activity in nociceptors leads to the release or activation of signals from interacting cells (postsynaptic neurons, glia, immune cells, neighboring DRG neurons, and peripheral cells) that can stimulate the nociceptors, ongoing positive feedback between nociceptors and various target cells may contribute to the persistence of the nociceptor hyperfunctional state (Miller et al., 2009; Xie et al., 2009). Third, cellular memory within nociceptors may be particularly important. The SCI-dependent somal HSA state was found to persist for at least 1 day after isolation of nociceptors (Bedi et al., 2010), but the full duration of this intrinsic state is unknown. Peripheral models of chronic inflammatory pain show that nociceptor alterations can persist for weeks in the absence of obvious continuing inflammation. Notably, hyperalgesic priming is produced by a single episode of acute cutaneous inflammation in a rat hindpaw (e.g., from carrageenan injection) or brief injection of an inflammatory signal (e.g., TNFα, NGF, and GDNF), which is followed days or weeks after abatement of the resulting acute pain by dramatically increased sensitivity of nociceptors to a subsequent inflammatory challenge (usually injection of PGE_2) (e.g., Aley et al., 2000; Reichling and Levine, 2009; Alvarez et al., 2012; Bogen et al., 2012). Moreover, repeated daily injections of PGE_2 into a hindpaw under a condition (concurrent indomethacin application) that avoids apparent tissue inflammation produce behavioral hypersensitivity for at least 1 month afterwards, which is accompanied by Nav1.8 upregulation and is dependent upon Nav1.8 expression, suggesting a key role for intrinsically altered nociceptors (Villarreal et al., 2005). A number of memory-like modifications involving hyperexcitability and synaptic facilitation have been described in various nociceptors (e.g., Walters et al., 1991; Kandel, 2001; Weragoda et al., 2004; Bogen et al., 2012; Zhang et al., 2012) and some of these modifications might be triggered by signals associated with SCI.

SCI IS HYPOTHESIZED TO CAUSE MALADAPTIVE ACTIVATION OF A NOCICEPTOR HYPERFUNCTIONAL STATE THAT MAY BE BIOLOGICALLY ADAPTIVE AFTER SEVERE PERIPHERAL BUT NOT CENTRAL INJURY

My central hypothesis is that severe peripheral injury (**Figure 2A**) generates a complex set of central signals that triggers a biologically adaptive and highly persistent nociceptor hyperfunctional state, and that many of these signals are also generated by SCI, resulting in maladaptive chronic pain and hyperreflexia in SCI patients (**Figure 2B**). It is evident that chronic pain, like other clinical consequences of SCI, is maladaptive for patients. Furthermore, a long-lasting hyperfunctional state induced by SCI should not be adaptive in the evolutionary sense of increasing survival and reproductive success because SCI, like all major CNS trauma, is almost always fatal to mammals in the absence of medical intervention (Branco et al., 2007; Weil et al., 2008). Any adaptiveness of this state must be for conditions other than SCI—although SCI does provide an extremely favorable setting for pathological recruitment of the nociceptor hyperfunctional state (see below). The proposal that human SCI pain involves the maladaptive recruitment of a nociceptor state that has adaptive functions under other conditions may seem questionable for at least two reasons. First, the SCI-induced HSA state found in isolated nociceptor somata might be a purely pathological effect, perhaps amplified by the abnormal conditions of dissociated cell

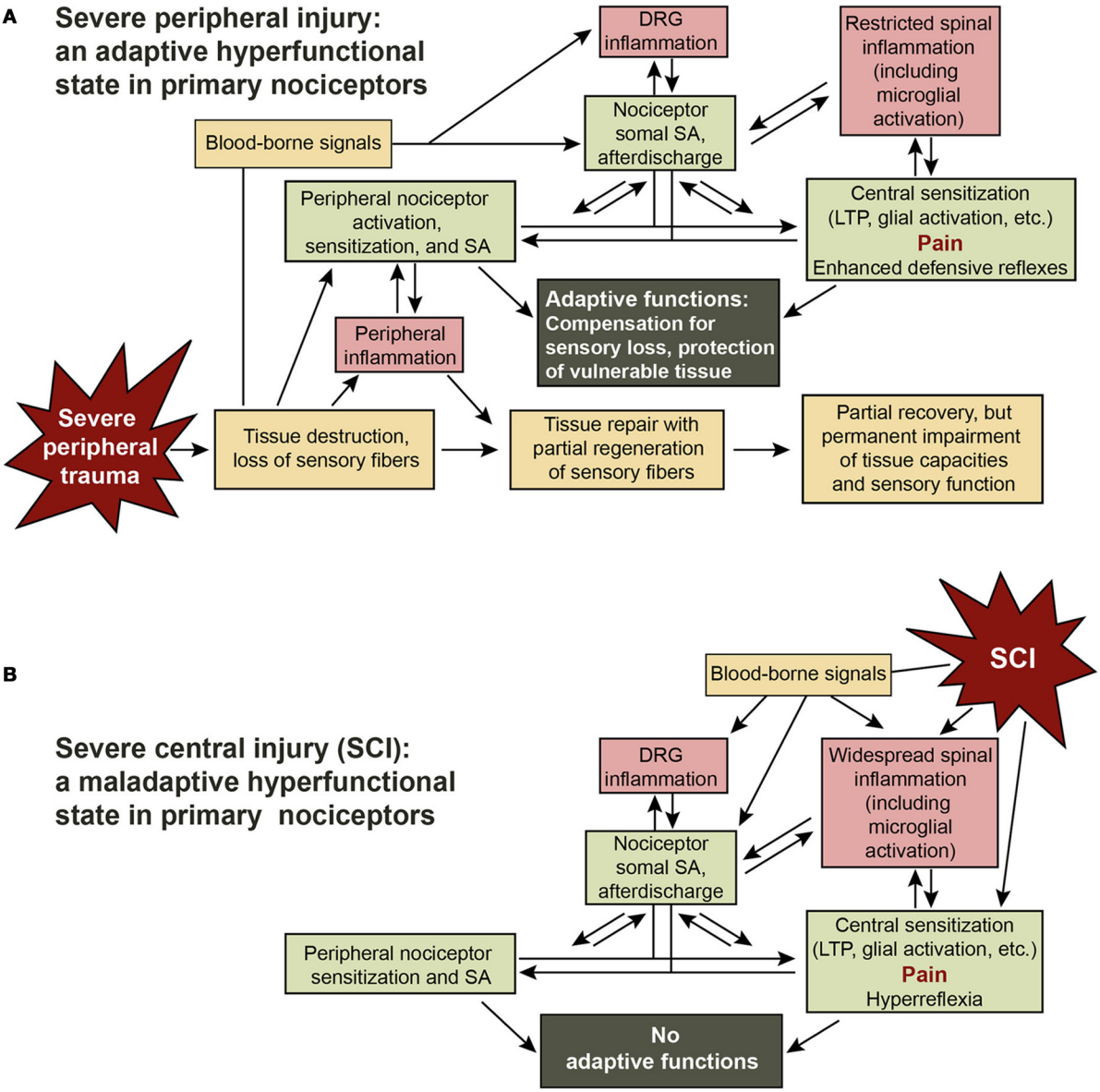

FIGURE 2 | Adaptive-maladaptive nociceptor hyperfunctional state hypothesis. (A) Adaptive nociceptor-driven sensitization and pain after severe peripheral injury. Compensation for permanent impairment of peripheral sensory function and protection of weakened tissue are achieved by enhancing the function of surviving nociceptors that innervate the region of injury via peripheral sensitization and by peripheral and somal hyperexcitability that produce SA, afterdischarge, LTP, and activation of central neurons and glia. Some degree of localized chronic pain can be useful for enhancing awareness of and protecting a chronically weakened body part. The decision to enter a persistent hyperfunctional state requires integration by the nociceptor soma of injury-related information from the peripheral receptive field, other cells in the DRG, cells in the spinal cord, and signals in the blood. **(B)** Maladaptive triggering of the nociceptor hyperfunctional state by SCI. SCI leads to the generation of many of the same signals in the spinal cord, DRG, and blood that are produced during severe peripheral injury, switching numerous nociceptors into a persistent hyperfunctional state. In this case the consequent sensitization, SA, hyperreflexia, and pain have no adaptive functions.

culture (Zheng et al., 2007). However, the finding of SA generated within DRG *in vivo* months after SCI (Bedi et al., 2010) suggests that the nociceptor hyperfunctional state is not an artifact of cell culture. Second, it has not yet been established in mammals that severe peripheral injury induces a chronic hyperfunctional state in nociceptors, or that such a state is adaptive in that context. These assumptions are supported by the following arguments.

A shared function of nociceptors and the innate immune system, which mediates most of the inflammatory responses to trauma, is to protect the body during and after survivable injury. This function is served by bidirectional communication between nociceptors and the innate immune system, including granulocytes and macrophages in the periphery, and resident microglia in the spinal cord (Miller et al., 2009). The adaptive

immune system also appears to play a role after peripheral injury, with T cells crossing the blood-spinal cord barrier and promoting pain by interactions with microglia (Grace et al., 2011). More severe injuries are associated with greater inflammation and longer periods of repair and recovery. Whereas severe CNS injuries are fatal in the absence of medical care, many animals can sometimes survive quite severe peripheral injuries, especially to appendages, without medical treatment. Examples include accounts of wolves surviving after loss of a limb in a trap, and humans surviving the amputation of digits or even limbs without medical care. A common consequence of severe peripheral injury is incomplete recovery of tissue function (**Figure 2A**), especially if muscle, bone, and nerves are seriously damaged. Resulting sensory loss can be a life-threatening problem because damaged tissue is especially vulnerable, both to inadvertent self-inflicted injury during movement and to attacks from predators and parasites that are attracted to signs of bodily injury (Walters, 1994).

Sensitization to mechanical and other types of stimuli, as well as additional hyperfunctional changes in surviving sensory fibers in and around injured tissue can both compensate for loss of sensory function and help protect the vulnerable region by promoting protective behavior (Walters, 1994; Smith and Lewin, 2009). Hyperfunctional alterations in nociceptors include peripheral sensitization and hyperexcitability (Gold and Gebhart, 2010), with the latter expressed not only as lower action potential threshold but as afterdischarge (Clatworthy and Walters, 1993; Gasull et al., 2005; Weng et al., 2012) and SA. Sensory function can also be enhanced by increasing the synaptic effectiveness of surviving nociceptors, which occurs after peripheral inflammation and nerve injury (Walters et al., 1991; Woolf and Costigan, 1999; Ikeda et al., 2006; Song et al., 2008a,b; Zhao et al., 2010), and is likely after SCI (Tan and Waxman, 2012). These effects should be coordinated with restoration of sensory function by regenerative and compensatory growth (sprouting) in peripheral and potentially central compartments of the nociceptor (Kinnman and Aldskogius, 1986; Doucette and Diamond, 1987; Billy and Walters, 1989; Steffensen et al., 1995; Belyantseva and Lewin, 1999; Hill et al., 2010; Bedi et al., 2012). Hyperfunctional alterations might, in addition, include enhanced sensitivity of nociceptors to chemical signals associated with injury (Song et al., 2003b), such as endogenous activators of TRPV1 channels (Patapoutian et al., 2009; Patwardhan et al., 2009; Diogenes et al., 2011; Wu et al., 2011). Traumatic injuries accompanied by extensive tissue disruption are likely to interrupt pathways that permit successful sensory regeneration or compensatory sprouting, so that substantial amounts of tissue may remain deprived of sensory innervation after tissue repairs are completed and inflammation subsides. In such cases it could be adaptive for compensatory alterations to persist for very long periods, perhaps for the remainder of the injured animal's reproductive life. Indeed, selection for persistent compensatory alterations in phylogenetically ancient sensory neurons after severe peripheral injury has been suggested as an early stage in the evolution of mechanisms that later found uses in some forms of long-term memory (Walters and Moroz, 2009).

Hyperfunctional alterations in low-threshold primary afferent neurons that detect innocuous stimuli and can elicit rapid defensive responses to potentially threatening but not tissue-damaging stimuli could also be adaptive after severe peripheral trauma. Even though Aβ-type neurons exhibit SA and other alterations in some chronic pain models (e.g., Gracely et al., 1992; Devor, 2009; Song et al., 2003a, 2006, 2012; Xie et al., 2012), it is not yet known if low-threshold mechanosensory neurons express a persistent, intrinsic hyperfunctional state after injury or inflammation.

THE NOCICEPTOR SOMA CAN BE A SITE FOR THE GENERATION OF ADAPTIVE ELECTRICAL ACTIVITY

In principle, somal as well as peripheral and synaptic alterations in nociceptors are likely to help compensate for loss of peripheral sensory branches after severe peripheral injury (and to contribute to pain after SCI, and perhaps to phantom pains after amputation). Somata of nociceptors identified thus far in vertebrates, molluscs, and annelids are often located centrally, distant from their peripheral receptive fields, which ensures that these cells can survive sublethal injury that destroys their peripheral branches. In the mollusc, *Aplysia*, peripheral injury induces somal hyperexcitability lasting months that amplifies brief bursts of action potentials arriving from the periphery by generating high-frequency afterdischarge (Clatworthy and Walters, 1993; Gasull et al., 2005), and similar afterdischarge has been described in the somata of mammalian Aβ afferent neurons (Song et al., 2012). This possibility has not yet been tested in the somata of vertebrate nociceptors, although nociceptor afterdischarge is generated peripherally (Weng et al., 2012). SA and enhanced discharge generated anywhere within a nociceptor would be expected to sensitize defensive responses, and could also stimulate conscious, protective attention to the injured region (pain). In the case of nociceptors that have lost peripheral branches in an injured region, somally generated SA could achieve this result. Moreover, even in nociceptors with surviving peripheral branches, somally generated SA and afterdischarge may reach the CNS more reliably than activity generated peripherally. Under normal conditions nociceptor action potentials are subject to significant conduction block where distal branches join the main axon (Sun et al., 2012), and this conduction block should be greatly enhanced by inflammatory conditions (e.g., via inactivating depolarization of axons) and by structural changes associated with tissue disruption and the small diameters of regenerating fibers. Thus, somally generated SA in a nociceptor innervating a severely injured region may be an effective way to ensure maintained sensitization of appropriate defensive responses and awareness of a vulnerable part of the body that requires extra attention (**Figure 2A**) even if regeneration of injured sensory fibers is limited.

Why haven't chronic, somally expressed HSA states in nociceptors been reported previously in mammalian models of long-lasting pain? Few chronic studies have tested somal excitability in nociceptors, or recorded under conditions where nociceptor SA generated in the DRG could be distinguished from SA generated peripherally. Moreover, most studies of dissociated nociceptors have examined ionic currents under voltage clamp,

without testing for somal hyperexcitability or SA. In addition, common models of long-lasting pain, including models based on cutaneous injection of inflammogens, surgical incision models (which produce relatively little inflammation or nerve damage), and various nerve injury models may not mimic adequately severe peripheral injuries that involve extensive amounts of tissue destruction (including neural damage) combined with prolonged inflammation—i.e., the traumatic conditions most likely to result in chronic pain in humans. Thus, while standard pain models produce many sensitizing effects on nociceptors (Gold and Gebhart, 2010), these pain models may not be severe enough to induce the persistent somal HSA state in sufficient numbers of nociceptors for it to be detected readily.

NOCICEPTOR SA MAY BE A USEFUL TARGET FOR TREATING PAIN AND HYPERREFLEXIA AFTER SCI

Nociceptor activity has extremely powerful effects on sensation, behavior, emotion, and autonomic function, so chronic activity and hyperexcitability occurring in numerous nociceptors after SCI would be expected to be clinically significant (**Figure 2B**). Chronic SCI pain in humans can be felt in many parts of the body, but is most commonly experienced in segments near the injury (at-level pain) and below the injury (below-level pain) (Siddall et al., 2003; Finnerup and Jensen, 2004). Spontaneous and evoked activity in below-level nociceptors (**Figure 1**) should help excite and sensitize local circuits mediating segmental responses such as hindlimb withdrawal reflexes, and help drive below-level spontaneous pain, allodynia, and hyperalgesia. By definition, pain includes an emotional, subjective component, which in mammals involves cortical processing (e.g., Melzack, 2005; Neugebauer et al., 2009). Thus, below-level pain caused by below-level nociceptor activity would require that a sufficient number of ascending fibers remain functional (intact and still myelinated), and this probably occurs in many incomplete spinal injuries (Wasner et al., 2008; Densmore et al., 2010). However, observations that moderate contusive injury in rats can eliminate supraspinally mediated responses to below-level test stimuli (Baastrup et al., 2010; Bedi et al., 2010), raise questions about the extent to which below-level pain is driven by below-level neural activity in many rodent SCI models. In addition to potentially promoting below-level pain after incomplete SCI, below-level central sensitization driven by nociceptor activity is likely to contribute to below-level somatic and autonomic hyperreflexia, such as sensitized responses of hindlimbs to mechanical and thermal test stimuli (Waxman and Hains, 2006; Hulsebosch et al., 2009; Yezierski, 2009) or hypertensive autonomic dysreflexia (Rabchevsky, 2006). Some DRG neurons innervate the viscera and most of these meet the functional definition of nociceptor (Christianson and Davis, 2010; Gold and Gebhart, 2010). Enhanced activity occurring in visceral nociceptors after SCI might contribute to visceral pain (Siddall et al., 2003; Kogos et al., 2005) and other visceral problems, such as bladder and gastrointestinal dysfunction (de Groat and Yoshimura, 2011; Fynne et al., 2012). Below-level somatic, autonomic, and visceral effects of nociceptor activity are likely to be enhanced by hyperexcitability of spinal circuits resulting from disinhibition consequent to interruption of descending inhibitory tracts by SCI (Lu et al., 2008) (**Figure 1**).

At-level alterations of nociceptors (**Figure 1**) are well situated for driving spontaneous and evoked at-level pain by direct excitation of pain pathways and promotion of at- and above-level central sensitization. Direct and indirect effects of injury on many other cell types within the damaged region of the cord are also likely to contribute to at-level pain (e.g., Yezierski, 2009). Above-level neuropathic pain in human patients is much less common than at- and below-level pain, although above-level reflex hypersensitivity is readily produced in rodents (Carlton et al., 2009; Densmore et al., 2010). An interesting possibility is that somal SA in nociceptors, which in rats has been observed at and below but not far above the injury level (Bedi et al., 2010), is more important for driving conscious pain than is peripherally generated SA, which occurs in nociceptors far above the injury level (Carlton et al., 2009). Consistent with this possibility, firing rates observed in nociceptors after SCI are substantially higher for somal SA (∼1 Hz) (Bedi et al., 2010) than peripheral SA (<0.1 Hz) (Carlton et al., 2009). This would suggest that forelimb hypersensitivity may occur with relatively little ongoing pain in the forelimb, consistent with growing evidence that hyperreflexia and conscious pain in rodents, as in humans, can be poorly correlated after SCI (Baastrup et al., 2010). It will be important to see if somal SA in nociceptors is correlated with operant measures of SCI pain associated with different spinal levels in rats. The correlation Bedi et al. (2010) found between SA in nociceptors sampled at but not below a contusion site with vocalization—a supraspinal response that is sometimes linked to aversive behavior in rodents (e.g., Meagher et al., 2001)—suggests that at-level somal SA might help to drive conscious pain in SCI rats.

If SA (somally and peripherally generated) and other hyperfunctional alterations in nociceptors contribute significantly to human at- and below level pain, as well as to hyperreflexia and visceral problems after SCI, pharmacological agents that selectively target nociceptor hyperexcitability should be clinically useful. A number of genes important for excitability are preferentially expressed in nociceptors, raising the possibility that blocking their function could ameliorate some of the suffering caused by SCI while producing minimal side effects. Indeed, preliminary results show that SA in dissociated nociceptors depends upon nociceptor-specific Nav1.8 channels, and indicate that knocking down the expression of these channels can attenuate behavioral hypersensitivity after SCI (Yang et al., 2012). As efforts to develop effective *in vivo* blockers of Nav1.8 channels and other nociceptor-specific ion channels proceed, such drugs may become useful for treating chronic pain in SCI and other conditions involving hyperfunctional nociceptors.

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Estradiol treatment prevents injury induced enhancement in spinal cord dynorphin expression

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Administration of the ovarian steroid estradiol in male and female animals has been shown to have neuromodulatory and neuroprotective effects in a variety of experimental models. In the present study, spinal tissues from dermatomes just above (T5–T7, at level) a severe chronic spinal cord injury (SCI) at T8 were analyzed for expression levels of prodynorphin (PRDN) and phospho-(serine 369) κ-opioid receptor (KOR-P) in 17 β estradiol (EB)- and placebo-treated adult male rats. Dynorphin was targeted since (1) it has previously been shown to be elevated post-SCI, (2) intrathecal injection of dynorphin produces several of the same adverse effects seen with a SCI, and (3) its increased expression is known to occur in a variety of different experimental models of central neuropathic pain. A significant elevation of extracellular levels of both PRDN and KOR-P in the placebo-treated SCI group relative to uninjured surgical sham controls was found in spinal tissues above the injury level, indicating increased dynorphin levels. Importantly, the EB-treated SCI group did not show elevations of PRDN levels at 6 weeks post-injury. Immunohistochemical analysis of at level tissues revealed that EB treatment significantly prevented a post-SCI increase in expression of PRDN puncta co-labeling synapsin I, a nerve terminal marker. The dynorphin-containing terminals co-labeled vesicular glutamate receptor-2 (a marker of glutamatergic terminals), a finding consistent with a non-opioid basis for the adverse effects of dynorphin. These results support a beneficial role for EB treatment post-SCI through a reduction in excessive spinal cord levels of dynorphin. Studies manipulating the timing of the EB treatment post-injury along with specific functional assessments will address whether the beneficial effects are due to EB's potential neuromodulatory or neuroprotective action.

Keywords: spinal cord injury, neuropathic pain, dynorphin, estradiol

INTRODUCTION

The effect of circulating blood levels of 17β-estradiol on the development of central neuropathic pain in a model of severe chronic spinal cord injury (SCI) at T8 has been a focus of recent studies in our laboratory. We have previously reported that the proportion of ovariectomized (ovx) SCI female rats and placebo-treated SCI males displaying pain-like behaviors (allodynia) evoked by light touch or pressure applied to sensitive dermatomes (T5–T8) up to 6 weeks post-injury (67 and 75%, respectively; Hubscher et al., 2010) was similar to our previous studies on SCI males (69%; Hubscher and Johnson, 1999, 2006). In contrast, significantly fewer estrous cycling SCI female rats and 17β-estradiol (EB) treated SCI male rats showed sensitivity to touch at level (26 and 30%, respectively) up to 6 weeks post-SCI (Hubscher et al., 2010). Thus, our prior studies demonstrate a positive outcome on the development and/or maintenance of at level (T5–T8) allodynia after treatment with subcutaneous EB pellets that were implanted prior to the injury. Note that several different research groups have shown beneficial functional outcome of EB treatment in SCI rodent models for locomotor function, which several studies have shown is due in part to a reduction in apoptotic cell death (Yune et al., 2004; Chaovipoch et al., 2006; Sribnick et al., 2006; Ritz and Hausmann, 2008; Kachadroka et al., 2010).

The purpose of the present study is to begin investigating whether EB acts to diminish the observed sensory disturbances at level post-SCI by acting upon some of the adverse neuromodulatory changes that disrupt normal homeostatic mechanisms in the tissues just above the level of lesion (region of cord supplying the hypersensitive dermatomes). Dynorphin, an endogenous κ opioid agonist (Chen et al., 2007), has been chosen for the study of adverse at level neuromodulatory changes because high tissue levels of dynorphin in spinal cord has been shown in different experimental models of neuropathic pain, including nerve injuries by spinal nerve ligation (SNL) at L5, L6, and S2 (Malan et al., 2000; Lai et al., 2006), inflammatory pain models in rats induced by injections of the complete Freund adjuvant (CFA; Millan et al., 1985; Calza et al., 1998; Zhang et al., 1998; Lin et al., 2010) or collagen II (Persson et al., 1994), cancer pain produced by intramedullary injection of sarcoma cells in the femur of mice (Honore et al., 2000), and excitotoxic injury of spinal cord by intraspinal injection of quisqualic acid (Abraham et al., 2001). Several studies to-date have also reported increased tissue levels of dynorphin, just above, at and below the level of SCI relative to intact controls during the acute/sub-acute phase post-lesion (Faden et al., 1985; Przewlocki et al., 1988; Sharma et al., 1992; Tachibana et al., 1998; Abraham et al., 2001). Moreover, neuropathic pain

lasting 70 days was induced with a single intrathecal injection of dynorphin in spinally intact animals, suggesting that high tissue levels of dynorphin can result in long-lasting post-injury changes (Vanderah et al., 1996; Laughlin et al., 1997). In addition, the pronociceptive role of increased dynorphin levels in spinal tissues in post-SNL pathophysiology is confirmed when paw withdrawal latencies and response thresholds in prodynorphin (PRDN) gene knockout mice return to normal by day 10 (1) after L5/6 SNL, which coincides with the upregulation of PRDN, and (2) after the intrathecal application of dynorphin antiserum also in a late phase in wild type mice with SNL (Wang et al., 2001). In a study using an inflammatory pain model induced by CFA injection, intrathecal administration of MK-801, a non-competitive blocker of *N*-methyl-D-aspartate receptors (NMDAR) suppressed the upregulation of PRDN mRNA as well as the hyperalgesic response (Zhang et al., 1998) supporting the argument that dynorphin upregulation is needed for induction of the hyperalgesic response.

Accordingly, we hypothesize that (a) an increase in the expression of spinal cord PRDN will also be present during the chronic phase of the current SCI (T8) model of at level (T5–T8) allodynia in male rats and (b) this increased expression of PRDN will be reversed in male rats which receive systemic EB treatment (slow release pellets at serum levels that simulate proestrus) prior to injury. Hence, the effect of the injury and the EB treatment on PRDN levels 6 weeks following a contusion at T8 spinal cord was investigated first by Western blot using the T5–T6 at level spinal tissue and subsequently with immunohistochemistry using the T7 at level spinal tissue. To determine the levels of extracellular dynorphin in spinal tissues, the phosphorylation of κ-opioid receptor (KOR) at serine 369 was measured. Phosphorylation has been shown to occur in response to prolonged activation by dynorphin, in a concentration dependent manner in the presence of exogenous κ-specific agonist, U50,488 (Appleyard et al., 1997; McLaughlin et al., 2003). To gain additional insights into potential mechanisms involved, the phenotypes and connectivity of dynorphin-containing neurons were examined using immunohistochemistry, including PRDN expression in neuronal soma and nerve terminals in laminae I and II, which contain important nociceptive circuits (Todd, 2002).

MATERIALS AND METHODS

Twenty male Wistar rats (four surgical shams, eight placebo-treated SCI, and eight EB-treated SCI) approximately 60–70 days old and weighing 200–250 g, were housed individually on a 12-h light–dark cycle and handled daily. One additional non-injured male rat was used for obtaining spinal cord tissues for electron microscopy. All animal procedures were reviewed and approved by the Institutional Animal Use and Care Committee at the University of Louisville, School of Medicine.

EB TREATMENT

One week prior to SCI (to mirror the procedures in our preceding behavioral study – Hubscher et al., 2010), hormone and placebo pellet insertion was done via a small incision in the skin of the neck just behind the right ear under brief isofluorane anesthesia. Sixteen animals were given subcutaneous implants of either EB (60-day release, 0.25 mg/pellet; IRA, Sarasota, FL, USA; eight rats) or placebo (eight rats) pellets as previously described (Reed et al., 2009). All experimenters were blinded to the content of the implant. In four sham animals, which did not receive any implants, laminectomy was done without any contusion to the spinal cord. The EB pellets are expected to yield continuous serum hormone levels that are reached during proestrus (Kalra and Kalra, 1974; Nequin et al., 1979; Samuel et al., 1998; Dantas et al., 2002). Furthermore, a previous study from this laboratory showed that the use of these subcutaneous placebo and EB implants in ovariectomized female rats had resulted in the presence of cells in the vaginal smears that resembled the diestrus and proestrus stages, respectively (Reed et al., 2009).

CONTUSION INJURY

Spinal cord contusions were carried out using a protocol described previously (Hall et al., 2010). Briefly, rats were anesthetized with a mixture of ketamine (80 mg/kg of body weight) and xylazine (10 mg/kg of body weight) injected intraperitoneally. The Infinite Horizon Impactor device (Precision Systems and Instrumentation, LLC, Fairfax, VA, USA; Scheff et al., 2003) was used to produce a severe injury at T8 spinal cord exposed after T7 laminectomy, with a pre-set force of 225 kdyn and a 1-s dwell time. During the post-operative period, all animals were monitored three times daily and were given an analgesic (Ketoprofen, 2.5 mg/kg, sc, twice daily) for 2 days, and Gentamicin (5 mg/kg, sc) for 4 days to prevent bladder infections.

BEHAVIORAL MEASUREMENTS

Behavioral testing of all rats for sensitivity to normally innocuous stimuli (light touch and gentle squeeze/pressure) to monitor allodynia was done in a dedicated facility as previously described (Hall et al., 2010). Baseline measurements were obtained pre-injury for comparison with those obtained during the 6-week post-SCI period. For each testing session, a total of 10 stimuli were applied to the dorsolateral trunk at and just above the level of lesion (T5–T8) alternating between the right and left sides (five each). After each stroking with a number 5 paintbrush (a 17 g stimulus force), the presence or absence of any one of three types of pain-like behavior (freeze, escape, or grab/push probe with their forepaws) was documented (Hubscher and Johnson, 2006; Hubscher et al., 2008; Hall et al., 2010). Note that for an animal to be considered responsive to a stimulus, the animal has to show a pain-like response during at least 60% of the trials in a given session (Hubscher and Johnson, 2006; Hubscher et al., 2008). For those animals not responsive to the brush stroke, a gentle squeeze/pressure (a 60-g mechanical, normally innocuous stimulus) was tested using Adson tissue forceps applied to T5–T8 dermatomes on the dorsolateral trunk five times each side using the 60% criteria.

A 10-point scale (Hall et al., 2010) was used for calculating the allodynia score for each session. The criterion for allodynia is a score higher than 0 on 3–5 weeks, two of which must be on consecutive weeks (to ensure that the response was consistent, and not a one-time occurrence). Scores of 1, 2, and 3 were assigned respectively for freeze, escape, or grab/push probe responses to gentle squeeze. An additional three points are added if the same response occurred with the brush stroke, a gentler stimulus. Whenever

responses to brush strokes occurred, we did further testing with Semmes–Weinstein monofilaments. Points were added for lower thresholds thereby accounting for sensitivity differences. A mean allodynia score is then assigned to each SCI rat, based on the values of five scores obtained from the second week (when allodynia first develops) through the sixth week post-SCI.

TISSUE PROCESSING

After completion of the chronic 6 weeks post-injury period, each animal was euthanized with a lethal dose of urethane (2.0 g urethane/kg of body weight). Enough serum was collected for radioimmunoassay of duplicate samples for serum EB levels, which were then coded and sent to the Ligand Assay and Analysis Core Laboratory at the Center for Research in Reproduction at the University of Virginia Health Sciences Center. Spinal cord tissues were removed for Western blot analysis by cutting a block containing two vertebrae (T4/5) and extruding the cord segments (T5/6) by injecting ice-cold saline, which was then immediately frozen in liquid nitrogen and stored at −80˚C. The remainder of the spinal cord from T7 to T13 was removed with intact vertebral column and placed promptly in 4% paraformaldehyde for 2–4 days at 4˚C. The spinal cord was dissected out after laminectomy, cut into segments for immunohistochemistry (T7) and the lesion epicenter reconstruction (T8), transferred into 30% sucrose solution for at least 48 h at 4˚C for cryoprotection, sectioned at 30 μm thickness on a cryostat, mounted on positively charged slides (Fisher Scientific, Pittsburgh, PA, USA) and stored at −20˚C.

For calculating white and gray matter sparing, the slides containing sections from the epicenter (T8) were stained using the Klüver–Barrera method to separately visualize white and gray matter as previously described (Hall et al., 2010). The areas of white and gray matter were estimated using Spot Imaging software (Spot Imaging Solutions, Sterling Heights, MI, USA). The percent sparing was calculated by dividing the total areas of gray and white matters at the epicenter by the total areas of relatively normal gray and white matters at 1 mm rostral to the epicenter.

WESTERN BLOT

A modification of previously described protocol was used (Dogra et al., 2006). Each spinal cord segment (T5–T6) from sham and chronic lesioned rats with and without EB treatment, representing 20 animals, was suspended (1 mg weight per 20 μl) into ice-cold lysis buffer (50 mM Tris–Cl (pH 7.4), 200 mM NaCl, 0.3% NP-40, 2 mg/ml leupeptin, 2 mg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride, 0.5 mg/ml benzamidine, 1 mM dithiothreitol, and 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM β-glycerophosphate) by mechanical grinding in a glass homogenizer. A clear extract was then obtained for Western blot after centrifugation at 4˚C at 14,000 RPM. The protein concentration of each sample was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each sample containing 50 μg of protein was then loaded and electrophoresed with a 10% SDS gel with 20 lanes using the Protean® II xi Cell system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The proteins in gel were transferred to nitrocellulose paper. Only those blots showing uniform transfer were later incubated overnight in primary antibodies at appropriate dilutions (**Table 1**). Horseradish peroxidase linked secondary antibodies (1:1000), goat anti-rabbit IgG and goat anti-mouse IgG (Cell Signaling Technology, Beverly, MA,

Table 1 | List of primary antibodies.

Antigen	Immunogen	Manufacturer, species, type, catalog number	Dilution used	Application
α-ER	KLH conjugated synthetic peptide (last 15 amino acids of rat α-ER)	Upstate, rabbit antiserum, 06-935	1:1000	I.H.
Dynorphin	C-terminus of proenkephalin B of human origin.	Santa Cruz Biotechnology, Inc., goat polyclonal, sc-46313	1:500	W.B.
KOR-P	Synthetic polypeptide around residue S369 in rat KOR	Abcam, rabbit polyclonal, ab63511	1:500	W.B./I.H.
β-tubulin	N-terminus of human tubulin	Abcam, rabbit polyclonal, ab18587	1:1000	W.B.
PRDN	Synthetic sequence (amino acids 245-258 of rat PRDN)	Neuromics, guinea pig polyclonal, GP10110	1:500	I.H.
NeuN	Purified cell nuclei from mouse brain	Millipore, mouse monoclonal, MAB377	1:1000	I.H.
CGRP	Full length rat protein conjugated to thyroglobin	Abcam, rabbit polyclonal, ab49873	1:2000	I.H.
VGLUT1	C-terminus 17 residue synthetic peptide of rat VGLUT1	Novus Biologicals, rabbit antiserum, NB 100-1837	1:2000	I.H.
VGLUT2	Recombinant protein from rat VGLUT2	Millipore, mouse monoclonal, MAB5504	1:500	I.H.
GAD 67/GAD 65	Synthetic peptide (identical in GAD67 and GAD65) conjugated to KLH	Abcam, rabbit polyclonal, ab49832	1:1000	I.H.
Synapsin I	Full length native protein purified (cow)	Abcam, rabbit polyclonal, ab8	1:1000	I.H.
MAP-2	Bovine Microtubule associated protein	Sigma, mouse monoclonal, M1406	1:1000	I.H.

USA), and bovine anti-goat IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used.

After incubation with secondary antibodies and washes with phosphate buffered saline (PBS, pH 7.4; 10× PBS contained 1.37 M NaCl, 14.7 mM KH_2PO_4, 78.1 mM Na_2HPO_4, and 2 g KCl), the membranes were treated with chemilumiscent horseradish peroxidase detection reagent (HyGLO™; Denville Scientific, Inc., Metuchen, NJ, USA) and exposed to autoradiography film (Denville Scientific, Inc., Metuchen, NJ, USA). After every Western blot with a specific antibody against a specific antigen, the blots were washed with a stripping buffer (0.2 M Glycine, 10% Tween 20, 0.1% SDS, pH 2.2) to remove bound antibodies, and re-probed with an anti-rabbit β-tubulin antibody as a control. β-tubulin bands were seen at 50 kDa as expected. ImageJ 1.40 (ver. 1.40, NIH) was used to quantify proteins in the Western Blot. The protein levels were expressed as the total optical density (OD) units in a band, which were normalized by dividing with the total OD units in the corresponding β-tubulin bands.

IMMUNOHISTOCHEMISTRY

For immunohistochemistry, sufficient T7 at level tissues (based upon close proximity to T8 contusion) were available from four sham, five SCI animals with placebo treatment and four SCI rats with EB treatment. The experimenter was blinded to the type of implant. A modification of our previously published protocol was used (Cothron et al., 2008). Each slide contained five 30 μm sections, which were at least 120 μm apart. All slides were heated at 80˚C in 10 mM sodium citrate buffer for 35 min for antigen retrieval (Jiao et al., 1999) before each immunohistochemistry. After cooling, slides were washed with 0.1 M Tris buffered saline (TBS containing 150 mM NaCl, 50 mM Tris, pH 7.4) three times, for 5 min and warmed on a slide warmer for 60 min at 37˚C. To block the non-specific antigenic sites, slides were incubated for 2 h in 0.1 M TBS with 5% donkey and 5% goat sera. For double labeling studies, sections were incubated in a mixture of two primary antibodies (**Table 1**) from different species at 4˚C for 48 h, and washed with 0.1 M TBS with detergent (0.25% Triton-x, TBS-T) for 30 min, six times. Slides were then incubated in a mixture of secondary antibodies for 2 h at room temperature followed by six washes for 30 min with TBS-T. Finally, the slides were coverslipped with Fluoromount G (SouthernBiotech, Birmingham, AL, USA), and stored in the dark at 4˚C.

Twenty 1 μm thick optical slices of confocal images in *z*-axis were collected with a Nikon Eclipse® microscope, which were rendered to produce a final XY image. Cells and puncta were counted using NIH software ImageJ which was upgraded with a plugin (Cell Counter) downloaded from NIH (http://rsbweb.nih.gov/ij/plugins/). For the purpose of counting, a single optical section having 1 μm thickness from each Z-stack was used to avoid confusion between co-localization and overlap of structures. Each image was digitally magnified for a clear visualization before counting by increasing the pixel sizes of *X* and *Y* dimensions by 7- to 10-fold with Adobe Photoshop®. Up to three sections were used to generate a mean count from each animal. Each section on a given slide was at least 120 μm apart from the others to avoid double counting the same structures. Counting was done separately in two regions, superficial laminae (I and II) and the remaining laminae (III–X) using boundaries as illustrated in Paxinos' Rat Brain Atlas (Paxinos and Watson, 1998). The settings for exposure, brightness and contrast were adjusted before each image acquisition to get a crisp and clear boundary of histological structures. Negative controls in all studies consisted of replacing the antiserum with the non-immune serum of the host animals. In addition, PRDN antiserum, after blocking in the presence of 1 μg/ml of blocking dynorphin peptide (Neuromics, Edina, MN, USA, Cat# P10116) in final diluted antibody, was used as an additional negative control for testing the specificity of PRDN antibody. There was an absence of specific staining in all negative controls. To prevent the non-specific labeling, the final concentrations of all antibodies were maintained between 0.5 and 5 μg/ml. The secondary antibodies used were DyLight 488, 594 conjugated F(ab′)2 fragments of goat or donkey antibodies against IgG of different species (Jackson ImmunoResearch, Inc.). F(ab′)2 fragments was used in place of whole antibodies containing Fc portions, which bind in a non-specific manner to the Fc receptors expressed by the macrophages (Diamond and Yelton, 1981) that commonly migrate to the injury sites. The use of better performance flurochromes, DyLight 488 and Dylight 594, was adopted to minimize the photobleaching.

ELECTRON MICROSCOPY

A single male rat with intact spinal cord was perfused transcardially with 100 ml of 4% formaldehyde and 0.25% glutaraldehyde in phosphate buffer under urethane anesthesia. After perfusion, the spinal cord (T6–T7) was immediately removed and post-fixed overnight in 4% formaldehyde, 0.25% glutaraldehyde at 4˚C. For nickel-enhanced 3,3′diaminobenzidine (Ni-DAB) immunohistochemistry, 50 μm sections were pre-incubated in 10% normal goat serum for 30 min. Sections were then incubated overnight with antibodies against PRDN or VGLUT2 (**Table 1**) contained in 5% normal goat serum. The next day after three, 10 min washes in PBS, the sections were incubated in appropriate biotinylated secondary antibodies (200× dilution). Following 1 h of incubation, sections were washed again three times for 10 min in PBS, and later incubated in ABC solution (Elite Standard ABC kit, Vector Laboratories, Burlingame, CA, USA). The sections were then washed once in 0.1 M sodium acetate (pH 6.0) and twice with PBS for 10 min each. Equal volumes of 0.1% DAB and 3% nickel ammonium sulfate in 0.1 M sodium acetate were mixed and 3 μl/10 ml of hydrogen peroxide was added. Sections were immersed in Nickel/DAB solution containing hydrogen peroxide for 5 min and then washed once with 0.1 M sodium acetate (pH 6.0) and twice with PBS for 10 min each. The sections were then stored in PBS at 4˚C before embedding them in LX112 epon resin for electron microscopy. Ultrathin histological sections (800 Å) were cut and stained with uranyl acetate. Stained sections were mounted on a 200 mesh copper grid and viewed under Philips CM-12 transmission electron microscope with an operating voltage of 80 kV. Electron micrographs were obtained under 10,000× to 19,500× magnifications.

STATISTICAL ANALYSIS

A two-way ANOVA followed by Tukey HSD *post hoc* pairwise *t*-tests using SPSS software (SPSS Inc., Chicago, IL, USA) was used to

analyze the effect of the injury and the EB treatment on the expression levels of various proteins obtained by the Western blot study. The confocal data was analyzed by the GraphPad Prism 5.0 (La Jolla, CA, USA). Pearson Correlation test was done to study different correlations. The value of p for rejection of null hypothesis was set at 0.05 for all analyses.

RESULTS

SEVERITY OF INJURY

The actual force of compression of the spinal cord at T8, measured at the tip of the impounder, averaged 227 ± 3.7 kdyn with an average displacement of 1.2 ± 0.1 mm in placebo-treated animals and 225 ± 2.1 kdyn with an average displacement of 1.4 ± 0.1 mm in EB-treated animals. The number of animals used initially in this study including 4 shams was 20; however, 1 animal in the EB-treated group was excluded from the data analysis as the values of initial parameters of injury from Infinite Horizon Impactor did not meet our minimum criteria for the desired injury severity (displacement above 1.0 mm which is necessary to obtain a "severe" SCI and thus allodynia). The average percent white matter sparing at the lesion epicenter (T8) was 4.2 ± 1.7 and 5.5 ± 0.8%, respectively in placebo ($n = 8$) and EB-treated ($n = 7$) rats. The mean percent gray matter sparing was 0.21 ± 0.21 and 0.24 ± 0.17% in placebo and EB-treated SCI rats, respectively. The low percent sparing of gray and white matters indicates the presence of severe injuries in these animals, which is expected from an injury force of 225 kdyn with a dwell time of 1 s.

SYSTEMIC EB LEVELS AFTER SUBCUTANEOUS PELLET TREATMENT

The mean serum EB level in male rats receiving subcutaneous EB pellets (7 weeks post-implant; 6 weeks post-SCI) was 50.5 ± 13.1 pg/ml, which is significantly ($p < 0.01$) higher than the mean level of 9.4 ± 4.0 pg/ml observed in the rats that had received subcutaneous pellets containing vehicle alone. The values of EB levels in the treated group were similar to those seen during the proestrus phase in female rats (Nequin et al., 1979; Kramer and Bellinger, 2009; Santmyire et al., 2010).

BENEFICIAL EFFECT OF SYSTEMIC EB TREATMENT ON PAIN-LIKE BEHAVIOR IN SCI RATS

At level (T5–T8) allodynia developed in seven out of eight male animals in the placebo-treated group. The high incidence of allodynia in adult male rats with a severe T8 contusion in the placebo-treated group is consistent with our previous studies (Hubscher and Johnson, 1999, 2006; Hall et al., 2010; Hubscher et al., 2010). In contrast, only three from the group of seven EB-treated male animals (with proestrus-like EB levels for proof of principle) showed allodynia. A binomial proportion test corrected for small sample sizes (Siegel and Castellano, 1988) showed significant differences between the incidence of allodynia in the two groups ($p < 0.01$).

THE WESTERN BLOT STUDY OF PRDN AND KOR-P EXPRESSION AT LEVEL

A two-way ANOVA revealed significant main effects of injury [$F(1,16) = 16.35$, $p = 0.001$] and the EB treatment on the spinal cord (T5–T6) levels of PRDN [$F(1,16) = 12.53$, $p < 0.005$; **Figure 1A**]. Tukey HSD *post hoc* test further revealed that injury significantly increased tissue levels of PRDN, which was significantly reduced by EB pretreatment ($p < 0.01$, both).

A significant main effect of injury on tissue levels of KOR-P in the spinal cord [$F(1,16) = 8.73$, $p < 0.01$] was also seen (**Figure 1B**). However, a main effect of EB treatment on KOR-P levels was not detectable, which may be attributed to the relatively small sample size of the sham group of animals. Significantly, higher spinal cord (T5–T6) levels of KOR-P were observed in SCI rats with placebo treatment in comparison to the sham animals ($p < 0.01$). The Pearson correlation analysis revealed a significant positive correlation between the tissue levels of KOR-P and PRDN ($p = 0.001$, $r = 0.72$, **Figure 1C**), indicating that the changes in KOR-P levels are paralleled by changes in PRDN levels in the spinal cord tissues after the SCI.

THE IMMUNOHISTOCHEMICAL STUDY OF PRDN EXPRESSION AT LEVEL

Figure 2 shows PRDN expression at T7 (the typical example shown is from SCI rats with placebo treatment). The magnified image (**Figure 2C**) of the superficial laminae shows co-expression of PRDN in NeuN labeled neurons. Note the puncta-like profile of PRDN expression (**Figure 2C**), which is consistent with its expression within nerve terminals, dendrites, and axons. No significant co-expression with dendritic marker microtubule associated protein-2 was seen (data not shown). However, PRDN co-expression with the nerve terminal marker synapsin I (De Camilli et al., 1983) was seen (**Figure 2E**). In total, 887 (mean: 177 ± 17 per 1 μm thick optical section) out of 1421(mean: 285 ± 49 per section) PRDN-IR puncta (∼62%) in all laminae ($n = 5$; one section counted per placebo-treated SCI animal) showed co-expression with synapsin I.

To study the phenotype of dynorphin terminals, double labeling was done with antibodies against vesicular glutamate transporter1 (VGLUT1) and vesicular glutamate transporter2 (VGLUT2), which are markers for glutamatergic terminals (Fremeau et al., 2004), and glutamate decarboxylase 67/glutamate decaboxylase 65 (GAD67/GAD65), which is the marker for the inhibitory GABAergic terminals (McLaughlin et al., 1975). Only VGLUT2 and PRDN co-expression was seen (**Figure 2F**). A total of 467 (mean: 93 ± 8 per section) PRDN-/VGLUT2-double labeled puncta were present among 1263 (mean: 253 ± 18 per section) PRDN-IR puncta, (∼37%), which is based on counts from all laminae ($n = 5$; one section per animal). When considered in combination with our finding that ∼62% of PRDN-IR puncta co-label synapsin I, it is estimated that ∼60% (37 of 62%) of dynorphin terminals at level are glutamatergic. Since dynorphin expression in spinal cord is mostly within the interneurons, which is indicated by previous studies showing the lack of effect of bilateral dorsal rhizotomy (Botticelli et al., 1981) or complete mid-thoracic transection (Goldstein and Ghazarossian, 1980) on the dynorphin contents of the spinal cord, it is contended that VGLUT2/PRDN-IR puncta in spinal cord most likely represent the terminals from interneurons.

The lack of PRDN co-expression with GAD67/GAD65 (data not shown) indicates the absence of a significant number of inhibitory dynorphin terminals. A significant number of GAD67/GAD65-IR puncta were however found to be juxtaposed to PRDN-IR cell bodies and puncta, consistent with inhibitory

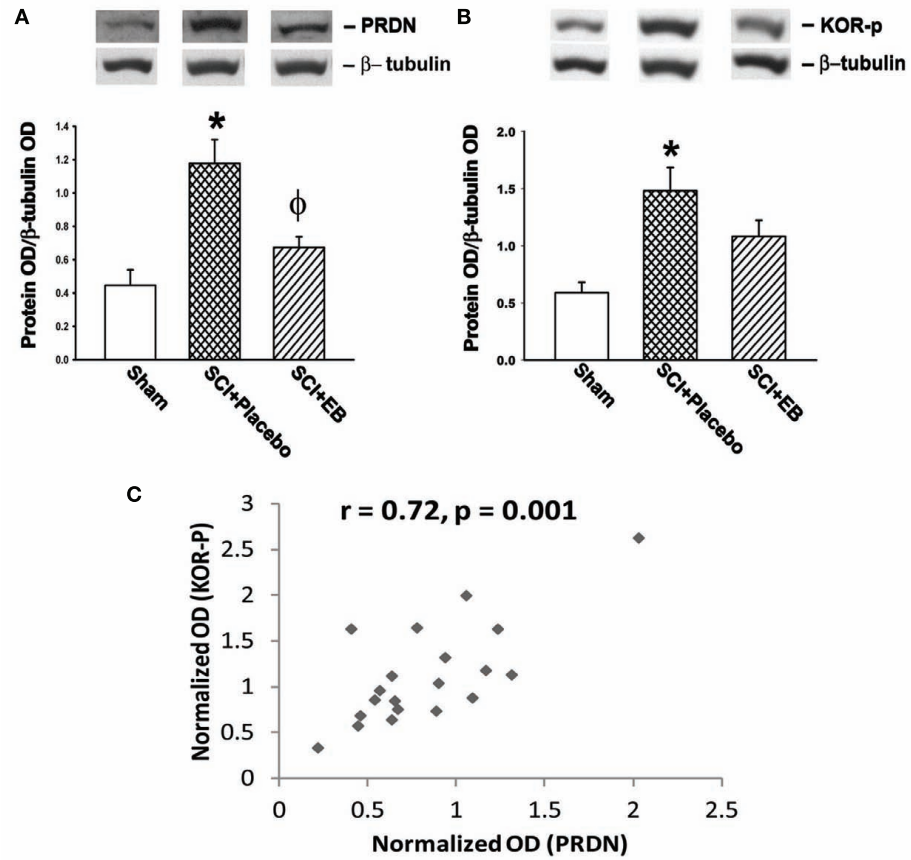

FIGURE 1 | (A,B) Western blot analysis of at level (T5–T6) spinal cord 6 weeks following spinal cord injury (SCI) in male rats shows prodynorphin (PRDN) band at ∼26 kDa and the phospho-(serine 369)-κ-opioid receptor (KOR-P) band at ∼43 kDa. **(A)** The tissue levels represented by PRDN band shows a significant elevation (∼164%) post-SCI in rats with placebo treatment (*$p < 0.01$). The increased PRDN levels in SCI rats was significantly reversed in the group of male rats having proestrus-like serum levels of 17β estradiol (EB; Φ, $p < 0.01$). **(B)** Spinal cord level of KOR-P was significantly elevated (∼151%) in the placebo-treated SCI animals in comparison to the shams (*$p < 0.01$). The KOR-P levels after EB treatment were not found to be significantly different from those in either the sham or the placebo-treated group. **(C)** Depicts the presence of a positive correlation between the normalized OD (optical density) units representing the tissue levels of PRDN and KOR-P in the at level spinal cord. The total OD units of PRDN and KOR-P bands were normalized after division with the respective OD levels of β-tubulin (50 kDa band). All data are represented as mean ± SEM of the normalized OD values, and were analyzed by ANOVA. $n = 4$, Sham; $n = 8$, SCI + placebo; $n = 7$, SCI + EB.

inputs to dynorphin neurons. A similar conclusion was reached previously based on an ultrastructural analysis (Cho and Basbaum, 1989).

Double labeling with antibodies for different estrogen receptors (ERs), ERα, ERβ, and G-protein coupled estrogen receptor-1 (GPER-1)/G-protein coupled receptor 30 (GPR30) antibodies in T7 spinal tissues revealed only PRDN/ERα co-expression, which was seen predominantly in laminae I and II (**Figure 2D**; data is not shown for other antibodies). PRDN/ERα co-expression was shown previously in the intact lumbar and sacral spinal cord segments of female rats (Gintzler et al., 2008). The co-expression of PRDN and ERα provides a morphological basis for the effect of EB treatment on the tissue levels of PRDN in the at level spinal cord.

ELECTRON MICROSCOPIC STUDY OF PRDN-, VGLUT2-IR EXPRESSION

The electron microscopic images confirm PRDN labeling of vesicles in the nerve terminals, endoplasmic reticulum, and myelinated axons (**Figures 3A–D**). The expression within nerve terminals and axons is consistent with the puncta- and fiber-like profiles seen in the confocal images (**Figure 2C**). In a magnified view of a synaptic nerve terminal labeled by PRDN (**Figure 3D**), the synaptic cleft is seen filled with DAB-Ni precipitate (white arrowheads), which may represent diffusion of dynorphin after its release. The presence of post-synaptic density is consistent with the excitatory phenotype of dynorphin nerve terminals. Notably, PRDN labeling is absent from the dendrites, which is consistent with our immunohistochemical analysis. VGLUT2 specific DAB-Ni labeling is seen within dendrites, axons, and vesicles inside the nerve terminals (**Figures 3E,F**).

PRDN EXPRESSION IN SUPERFICIAL (I–II) VS. DEEPER LAMINAE (III–X)

The frequent co-expression of ERα/PRDN in superficial laminae (typical examples in **Figure 2**) indicates the possibility that EB treatment may affect the dynorphin expression of neurons in laminae I and II, which contains important nociceptive circuits. To study the effect of systemic EB treatment on PRDN expression

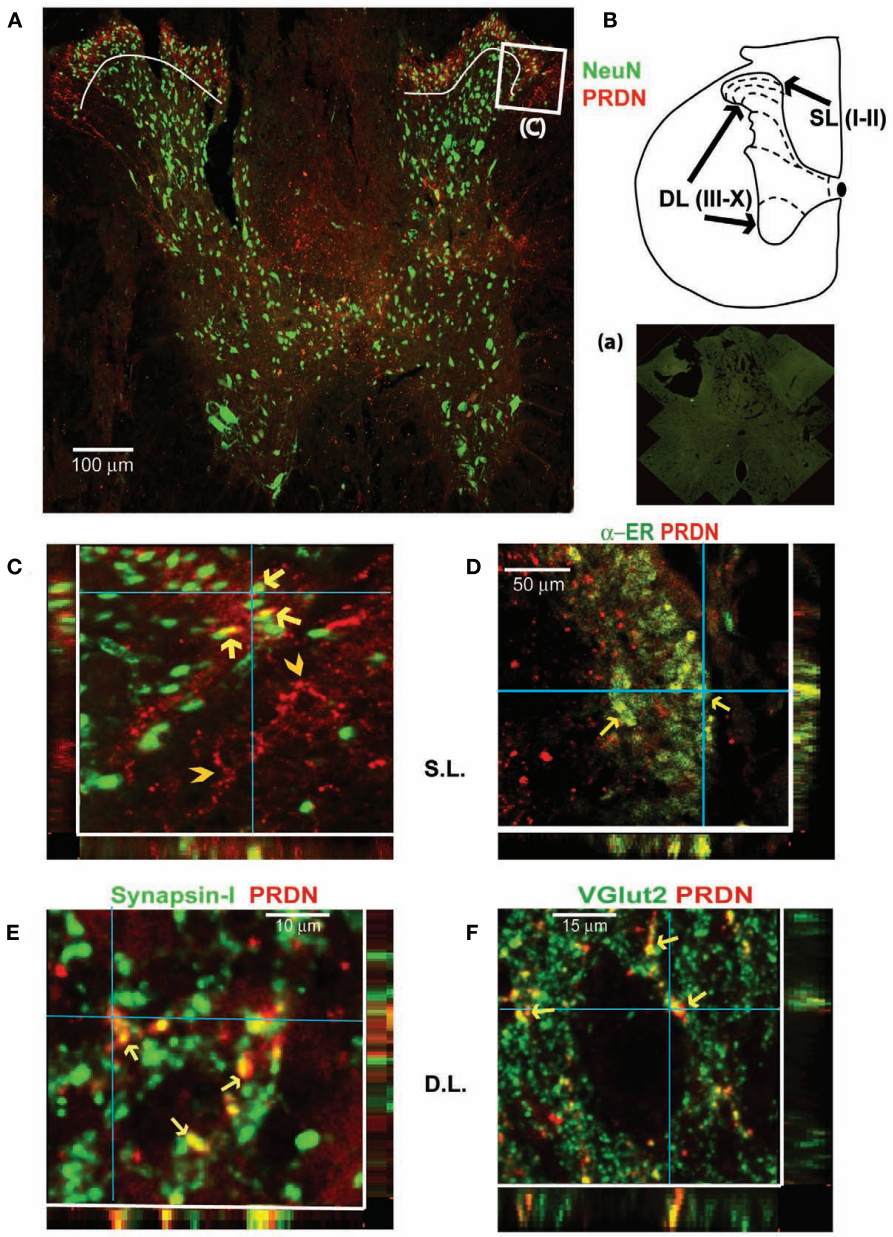

FIGURE 2 | PRDN expression immediately above the level of injury at T7 (at level) is seen in neuronal cell bodies and nerve terminals (C,E,F). The confocal image **(A)** shows a typical spinal cord section from SCI rat with placebo treatment, double labeled with antibodies against neuronal marker NeuN and PRDN at T7. The schematic in **(B)** shows organization of different mid-thoracic laminae in rats, based on the "The Rat Brain in Stereotaxic Co-ordinates" by Paxinos and Watson (1998). The white curved lines in **(A)** demarcates superficial laminae I and II from the deeper laminae. The magnified image **(C)** from the boxed portion from the superficial laminae (SL) in image **(A)** reveals PRDN expression within neuronal soma (yellow arrows). Magnified image **(C)** also reveals that a substantial amount of PRDN immunoreactivity at level has a puncta-like profile. A portion of PRDN-IR puncta is arranged in a fiber-like fashion (yellow arrowheads) suggestive of expression in axons. **(D)** Shows the co-localization of PRDN and estrogen receptor α (ERα) in SL (yellow arrows). Image **(E)** shows the co-expression of nerve terminal marker, synapsin I within the PRDN-IR puncta (shown in deep laminae (DL), yellow arrows). **(F)** PRDN-IR puncta is shown to co-express glutamatergic marker vesicular glutamate transporter 2 (VGLUT2). Image in **(a)** shows a typical absence of specific labeling in a section at T7 from SCI animals with placebo, when primary antibodies (PRDN and NeuN) were substituted with non-immune sera from the respective host animals. Images shown are typical of sham, SCI + placebo and SCI + EB rats ($n = 4$–5 each). For consistency, only images from the SCI + placebo group of male rats are shown. Co-localizations [yellow arrows in **(C,F)**] are also seen in XZ and YZ planes passing through the blue lines. Abbreviations are as in **Figure 1**.

within neurons in laminae I and II, counts of PRDN-/NeuN-IR neurons were compared between the superficial laminae (laminae I–II) and the rest of the gray matter (laminae III–X; **Figure 4A**). A two-way ANOVA revealed significant main effects of the region

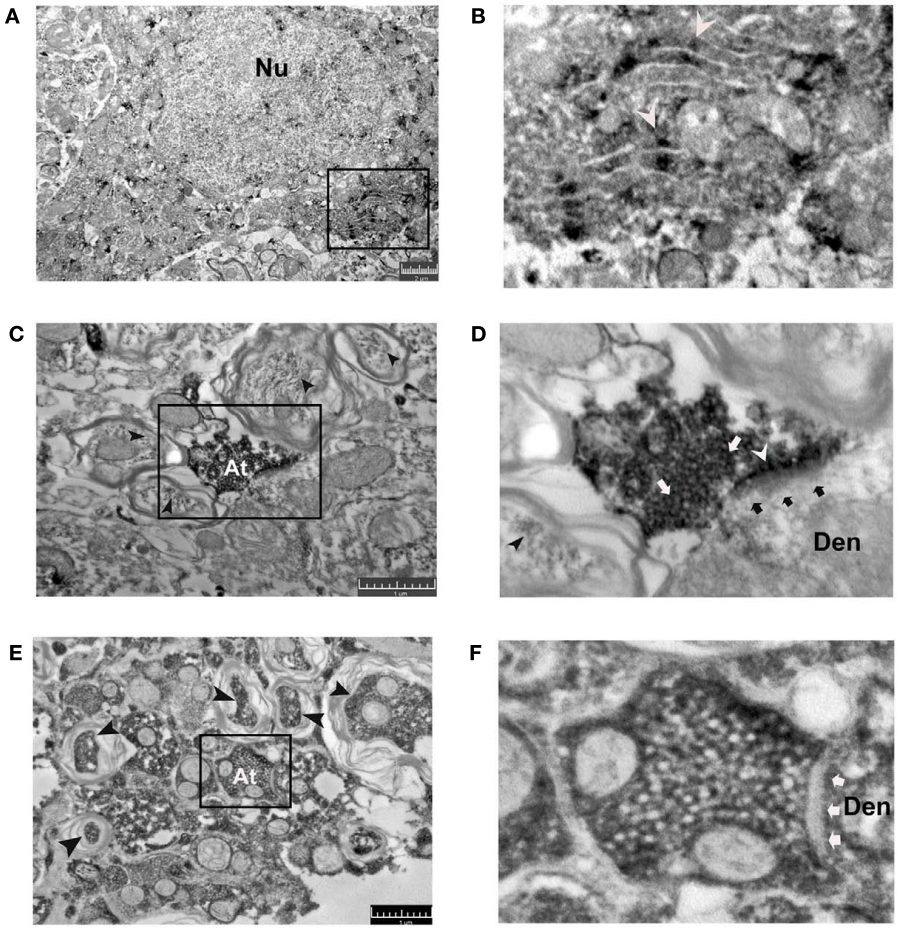

FIGURE 3 | Representative electron microscopic images of T7 spinal cord sections from an uninjured animal illustrate immunocytochemical staining of PRDN (A–D) and VGLUT2 (E,F) visualized with the nickel-enhanced diaminobenzidine (DAB-Ni) reaction. Images in **(B,D,F)** illustrate at higher magnification the boxed portions indicated in **(A,C,E)**, respectively. PRDN labeling is seen in the cytoplasm of neurons [**(A)** nucleus indicated, Nu] where DAB-Ni is associated with endoplasmic reticulum [white arrowheads **(B)**]. PRDN is also seen in axon terminals (At) where DAB-Ni surrounds synaptic vesicles [white arrows **(D)**]. PRDN-labeled terminals contact the unlabeled dendrites [Den, black arrows point to the post-synaptic density, white arrowhead indicates synaptic cleft filled with more intense, DAB reaction from released dynorphin **(D)**]. **(D)** PRDN-IR rounded vesicles (white arrows) are seen. Light PRDN labeling of myelinated axons is also seen [black arrowheads **(C,D)**]. **(E,F)** VGLUT2 labeling is seen in myelinated axons (black arrowheads), axon terminals (At), and dendrites (Den). **(F)** VGLUT2 labeled terminals contact VGLUT2 labeled dendrites (black arrows indicate the synaptic cleft). The scale bars measure 2 μm in **(A)** and 1 μm in **(C,E)**. Abbreviations are as in **Figures 1** and **2**.

[laminae I and II vs. the rest of the gray matter, laminae III–X; $F(1,14) = 29.7$, $p < 0.01$] and the treatment groups [placebo vs. EB pellets; $F(1,14) = 6.56$, $p < 0.03$]. There was a significantly higher number of PRDN-/NeuN-IR neurons in laminae I to II in comparison to the rest of the gray matter (laminae III–X) in both groups of SCI rats ($p < 0.05$ for both comparisons; 4.5 times higher in laminae I and II in the placebo group). Comparisons of specific groups using *t*-tests showed a significant drop in the number of PRDN-/NeuN-IR neurons by 48% in laminae I to II in SCI rats after EB treatment ($p < 0.05$). Note that we did not include the sham animals in this analysis because the neuronal cell loss, which affects the two SCI groups (those with placebo and EB, but not the sham group), could confound the analysis. Moreover, based on Western blot expression, an increase in the counts of PRDN positive NeuN neurons and/or immunoreactivity intensity was anticipated after the injury in the placebo treatment group. A two tailed *t*-test, with the assumption of equal variance, showed an absence of any significant differences in counts observed in the sham group (31/section) vs. the SCI placebo treatment group (27/section; $p > 0.05$).

The effect of EB treatment on dynorphin expression in nerve terminals was examined by analyzing counts of synapsin I-/PRDN-IR puncta (**Figure 4B**). The two-way ANOVA revealed significant main effects of the region [$F(1,14) = 11.3$, $p < 0.01$] and the treatment group [$F(1,14) = 23.5$, $p < 0.01$] on the counts of synapsin I-/PRDN-IR puncta. Further *post hoc* comparisons showed significantly lower counts of synapsin I-/PRDN-IR puncta in laminae I–II ($p < 0.05$) and laminae III–X ($p < 0.01$) in animals with the subcutaneous EB pellets vs. the placebo pellets. Decreased counts of synapsin I-/PRDN-IR puncta following EB treatment is

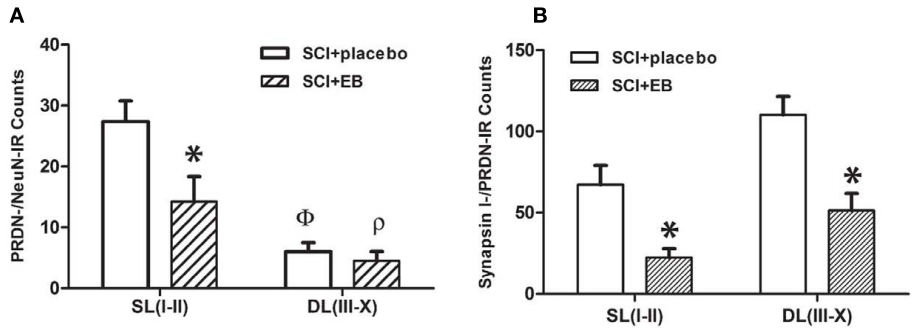

FIGURE 4 | (A) In the superficial laminae I–II (SLI-II), significantly higher counts (*y*-axis) of PRDN-/NeuN double labeled neuronal soma are present in the placebo-treated vs. the EB-treated SCI animals (* $p < 0.05$). The number of PRDN-/NeuN double labeled cells is significantly more in SLI-II as compared to the deeper laminae, III–X (DLIII-X) in placebo (ϕ, $p < 0.01$) and EB-treated (ρ, $p < 0.01$) groups of SCI animals. **(B)** Significantly higher counts of synapsin I-/PRDN-IR puncta-like profile, representing dynorphin nerve terminals, are present in the placebo-treated SCI group as compared to the EB-treated SCI group in both regions, the SLI-II and DLIII-X (* $p < 0.05$, for both). Before counting, the gray matter was divided into SL I-II and DL III-X based on the "The Rat Brain in Stereotaxic Co-ordinates" by Paxinos and Watson (1998), as shown in the schematic in **Figure 2B**. A complete optical section (1 μm) taken from the center of stacks of confocal images was counted. Counts shown on the *x*-axis are mean ± SEM per section ($n = 4$–5), and was analyzed by a two-way ANOVA. Abbreviations are as in the **Figure 1**.

consistent with reduced dynorphin content in the nerve terminals indicating a potential reduction in the dynorphin release.

DISCUSSION

The current study reveals a beneficial outcome following subcutaneous treatment with EB that resulted in significantly lowered incidence of pain-like behavior that was detected in male rats after a chronic SCI. Various favorable outcomes have been also reported for systemic EB treatment in other studies (Yune et al., 2004; Webb et al., 2006; Hubscher et al., 2010; Kachadroka et al., 2010). Our study suggests that beneficial outcomes may result, at least partly, by preventing the post-injury toxicity resulting from increased dynorphin expression in spinal cord. Several types of evidence presented in the current study indicate reduced dynorphin expression after treatment with subcutaneous EB pellets. These include reduced tissue levels of PRDN, reduced count of PRDN-labeled NeuN positive neurons, a positive correlation between the tissue levels of PRDN and KOR-P and reduced PRDN labeling of synapsin I-IR puncta, which represent nerve terminals. In addition, others have shown previously that subcutaneous EB replacement can reduce the expression of PRDN in the central nervous system. For example, EB replacement suppressed the post-ovariectomy increase in PRDN gene expression in the adenohypophysis in rats (Spampinato et al., 1995). EB replacement mechanism in ovariectomized mice also reduced the PRDN expression in the arcuate nucleus by an ERα mediated (Gottsch et al., 2009).

Note that the present study was limited to male rats since males constitute 80.7% of the total SCI population (National Spinal Cord Injury Statistical Center, Birmingham, AL, USA – February 2011). However, the current use of estrogens for therapeutic purposes in males is limited because of the serious side effects, such as thromboembolism (Sayed and Taxel, 2003). There are current clinical trials of promising new therapeutic agents for potential use in males known as selective ER modulators, which mimic the effects of estrogen without their serious side effects (Pickar et al., 2010). Also note that other indirect effects of systemic EB treatment, such as reduced levels of testosterone as well as luteinizing and follicular stimulating hormone, which are observed during EB infusion (Bagatell et al., 1994), may also contribute to the decreased dynorphin levels and/or its beneficial effects. However, the present data, showing the reduction in the count of PRDN-/NeuN-IR neurons induced by systemic EB treatment in the superficial laminae where ERα and PRDN co-expression is seen, supports the direct involvement of EB on dynorphin expression.

EXPRESSION OF PRDN AND KOR-P

The Western blot analysis in this study demonstrates an increase of PRDN levels in the at level (T5, T6) spinal cord tissues of male rats with severe chronic (6 weeks) SCI at T8, which is reversed in animals given subcutaneous EB implants prior to the injury (shown to result in proestrus-like blood levels of EB upon measurement at the terminal stage). An increase in the synthesis of PRDN is expected to increase the extracellular levels of the most abundant form of endogenous κ-opioid in the spinal cord, dynorphin A (1–17; Chen et al., 2007), which was studied by measuring the tissue levels of KOR-P, since phosphorylation at serine 369 of KOR is dose dependent in response to activation by κ-agonists (Appleyard et al., 1997; McLaughlin et al., 2003). Our data reveals a significant increase in the KOR-P levels in post-SCI spinal tissues, corresponding to sensitive dermatomes (T5–T8), which is consistent with increased activity of dynorphinergic terminals in spinal cord at level. The tissue KOR-P levels after EB treatment were found not to be significantly different from those after the placebo treatment. In addition, a significant positive correlation between the levels of PRDN and KOR-P in post-SCI spinal cord tissues (**Figure 1C**) is found, which indicates that the PRDN levels positively influence the levels of KOR-P, an indicator of magnitude of dynorphin release.

The decreased release of dynorphin with continuous EB treatment may be due to the diminished stores in nerve terminals, which are indicated by a significant reduction in detectable PRDN

labeling of synapsin I-IR nerve terminals (by ∼67%, as shown in **Figure 4**). The reduced PRDN labeling of the nerve terminals is likely to be associated with the decreased expression of PRDN within the neurons in the superficial laminae after EB treatment. In this context, note that the count of PRDN-/NeuN-IR neurons in superficial laminae I and II, showing frequent PRDN/ERα co-expression, declined by 48% following EB treatment. Another finding worthy of note is the absence of a significant increase in the counts of PRDN positive NeuN neurons after the injury relative to shams. This may be due to the overall loss of neurons following injury. Hence, it is likely that the significantly increased dynorphin levels detected after injury by the Western blot analysis may be due to the increase of its levels in the surviving neurons, axons, and the nerve terminals.

We propose that EB pretreatment, by preventing increased dynorphin expression following SCI, is responsible for the improved behavioral outcome (reduction in the incidence of at level allodynia) previously reported by our group (Hubscher et al., 2010) and observed again with the current group of rats.

PRDN EXPRESSION ENHANCES NEURONAL EXCITABILITY VIA NON-OPIOID MECHANISMS

The immunohistochemical and electron microscopic data shows that dynorphin is expressed in a subpopulation of glutamatergic (VGLUT2) nerve terminals at T7 (consistent with Marvizon et al., 2009), indicating an excitatory phenotype of dynorphin terminals, which is further supported by the electron microscopic finding of the post-synaptic density (**Figure 3D**; Guillery, 2000). This finding is important for understanding the non-opioid basis for the adverse effects of dynorphin, resulting from increased tissue levels post-injury. It has been proposed by Laughlin et al. (2001) that the interaction of dynorphin with NMDARs via multiple binding sites can produce excitatory responses resulting in nociceptive behavior and other adverse effects. The non-opioid basis for the toxic effects of dynorphin is supported by experimental evidence employing the intrathecal injection of fragments lacking the opioid activity, such as dynorphin A (2–17) and dynorphin A (3–17), in addition to dynorphin A (1–17) with full opioid activity, all of which produce persistent adverse effects on sensory, motor and autonomic functions leading to allodynia, hind limb locomotor function loss, and bladder function impairment (Long et al., 1988; Vanderah et al., 1996; Laughlin et al., 1997).

Both the NMDAR dependent and NMDAR independent mechanisms are found to be responsible for the effects of dynorphin in pharmacological and *in vitro* studies (Tang et al., 2000; Laughlin et al., 2001; Woods et al., 2006). NMDAR independent effects of dynorphin include increasing Ca^{++} influx in hybridoma cell lines of embryonic rat dorsal root ganglia cells, mediated by bradykinin receptor B2, which was also responsible for nociceptive behavior after L5, L6 SNL in rats (Lai et al., 2006). The Ca^{++} influx caused by dynorphin may also lead to increased release of neurotransmitters (Perney et al., 1986) at the dynorphin terminals, which are widely distributed. The activation of Ca^{++} dependent protein kinase C, following the Ca^{++} influx produced by dynorphin, can also cause NMDAR activation (Tang et al., 2000). Consistent with this hypothesis, the low pharmacological doses of dynorphin A (1–13), which increased the Ca^{++} influx, were shown to contribute to the post-synaptic enhancement of NMDAR (Skilling et al., 1992).

Dynorphin also directly activates NMDARs via binding to the NR1 subunit, which was shown to be responsible for its toxicity *in vivo* and *in vitro* (Woods et al., 2006). According to an emerging concept, the activity of NMDARs is essential for synaptogenesis, experience-dependent synaptic remodeling and long-lasting changes in synaptic efficacy such as long-term potentiation and long-term depression (Lau and Zukin, 2007), which is likely to play important role in the post-injury toxicity of dynorphin in spinal tissues, since many of the adverse effects of intrathecal dynorphin are persistent in nature (Long et al., 1988; Vanderah et al., 1996; Laughlin et al., 1997).

Evidence for increased neuronal activity includes a study using c-fos, a marker of neuronal activity in gray matter immediately above the level of injury in a SCI model exhibiting at level allodynia (Siddall et al., 1999). The hypersensitivity of neurons in ventral and posterior thalamic nuclei evoked by stimulation of at level (T5–T8) dermatomes in the current model of SCI (Hubscher and Johnson, 2006) suggests increased stimulation of spinothalamic neurons in the spinal cord. Direct recordings of dorsal horn neurons at cervical (Carlton et al., 2009) and at spinal cord levels immediately rostral (T8/9; Crown et al., 2008) to a contusion injury at T10 also showed increased responses to various mechanical stimuli, indicating an injury-induced increase in neuronal activity. The NMDAR activation is seen in animal models exhibiting upregulation of dynorphin in spinal cord. In S2 and L5/6 SNL models, the intrathecal injection of the non-competitive inhibitor of NMDAR, MK-801 suppressed the increased pain response (Malan et al., 2000; Wang et al., 2001). In the neuropathic pain model induced by intrathecal dynorphin injection, intrathecal injection of MK-801 produced transient blockade of the mechanical allodynia (Laughlin et al., 1997). It is important to note that the acute effects of systemic EB can also reduce activity of NMDARs, which is independent of genomic mechanisms, such as reduced expression of dynorphin, as indicated by rapid and reversible inhibition of NMDAR currents in a hippocampal neuron culture (Weaver et al., 1997), and may contribute to the overall beneficial effect of EB in a chronic injury model.

POSSIBLE ATTENUATION OF OPIOID EFFECT IN MODELS WITH DYNORPHIN UPREGULATION

An increased inhibitory κ-opioid tone, responsible for anti-nociceptive effect, is demonstrated in an arthritic pain model, exhibiting increased levels of dynorphin in the lumbar sacral spinal cord, which showed that hyperalgesia was potentiated by a κ specific antagonist, MR2266 (Millan et al., 1985). Note that the current study shows high levels of KOR-P in spinal tissues after severe chronic SCI, which is consistent with the development of desensitization to the effects of KOR activation by dynorphin (McLaughlin et al., 2003). Therefore, we argue that although inhibitory opioid tone may be present as a result of increased tissue levels of dynorphin, promoting anti-nociception as detected in a previous study (Millan et al., 1985), it is likely to undergo significant attenuation as a result of the desensitization. Absence of a significant role of κ-opioid activity is indicated in a L5/L6 SNL model which did not show difference in the stimulation of

γ-[35S]GTP binding by κ receptor agonist U69,593 with or without SNL surgery (Wang et al., 2001).

OTHER BENEFITS OF EB TREATMENT

The maladaptive changes and associated increase in excitability caused by the elevated levels of tissue dynorphin can plausibly contribute to other consequences of SCI, such as locomotor deficits, muscle spasticity, sexual dysfunction, autonomic dysreflexia, and bladder and bowel dysfunction (Levi et al., 1995; Widerstrom-Noga et al., 1999). Note that the potential benefits of EB treatment on locomotor systems has been studied by several groups within the context of neuroprotection, as already discussed. Our future studies will examine the benefits of EB relative to reducing excessive levels of dynorphin, for locomotor as well as other systems (pelvic visceral function). Also note that over-stimulation of motor neurons can cause spasticity. The development of high excitotoxic levels of extracellular dynorphin may contribute to the motor deficits due to the loss of motor neurons. The increased activity and/or the loss of sympathetic and the parasympathetic neurons in thoracic and lumbosacral segments (Jansen et al., 1993; de Groat, 1998) could contribute to the bladder and/or sexual and/or autonomic dysfunctions following the SCI.

It is noteworthy that the incidence of fibromyalgia, a chronic pain condition associated with elevated levels of dynorphin in cerebrospinal fluid (Vaeroy et al., 1991), increases sharply after menopause suggesting that decreased estrogen levels may play a role in its pathogenesis (Waxman and Zatzkis, 1986), which is consistent with the argument that the beneficial outcomes result from estrogen replacement.

Also, since increased microglial activity may produce adverse effects on spinal cord circuitries, which is indicated by the temporal correlation between abnormal pain response and the proliferation of Iba-1 positive microglia in spinal cord dorsal horn in a sciatic nerve injury model of neuropathic pain (Echeverry et al., 2008), the potential beneficial effect of EB may be due to the suppression of adverse microglial activity. This is suggested by the effect of EB pretreatment on a culture of microglia activated by lipopolysaccharide (LPS), which prevents injury produced in the rat mesencephalic primary neuron culture by the conditioned media from LPS treated microglial culture (Liu et al., 2005).

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Predifferentiated GABAergic neural precursor transplants for alleviation of dysesthetic central pain following excitotoxic spinal cord injury

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Intraspinal quisqualic acid (QUIS) injury induce (i) mechanical and thermal hyperalgesia, (ii) progressive self-injurious overgrooming of the affected dermatome. The latter is thought to resemble painful dysesthesia observed in spinal cord injury (SCI) patients. We have reported previously loss of endogenous GABA immunoreactive (IR) cells in the superficial dorsal horn of QUIS rats 2 weeks post injury. Further histological evaluation showed that GABA-, glycine-, and synaptic vesicular transporter VIAAT-IR persisted but were substantially decreased in the injured spinal cord. In this study, partially differentiated GABA-IR embryonic neural precursor cells (NPCs) were transplanted into the spinal cord of QUIS rats to reverse overgrooming by replenishing lost inhibitory circuitry. Rat E14 NPCs were predifferentiated in 0.1 ng/ml FGF-2 for 4 h prior to transplantation. *In vitro* immunocytochemistry of transplant cohort showed large population of GABA-IR NPCs that double labeled with nestin but few colocalized with NeuN, indicating partial maturation. Two weeks following QUIS lesion at T12-L1, and following the onset of overgrooming, NPCs were transplanted into the QUIS lesion sites; bovine adrenal fibroblast cells were used as control. Overgrooming was reduced in >55.5% of NPC grafted animals, with inverse relationship between the number of surviving GABA-IR cells and the size of overgrooming. Fibroblast-control animals showed a progressive worsening of overgrooming. At 3 weeks post-transplantation, numerous GABA-, nestin-, and GFAP-IR cells were present in the lesion site. Surviving grafted GABA-IR NPCs were $NeuN^+$ and $GFAP^-$. These results indicate that partially differentiated NPCs survive and differentiate *in vivo* into neuronal cells following transplantation into an injured spinal cord. GABA-IR NPC transplants can restore lost dorsal horn inhibitory signaling and are useful in alleviating central pain following SCI.

Keywords: cortical progenitor cell, quisqualic acid, spinal cord injury, GABA, transplantation, neuropathic pain, VIAAT, overgrooming

INTRODUCTION

Pain is a major complication in patients with spinal cord injury (SCI) where conventional pharmacological, electrical, or psychological treatments provide only minor and temporary relief (Finnerup et al., 2002; Finnerup and Jensen, 2004; Widerstrom-Noga et al., 2008; Siddall, 2009; Kwon et al., 2010; Mann et al., 2010). Mechanisms involved in the establishment of SCI pain are still not fully understood due to difficulties in dissociating traumatic cascade of events occurring in the spinal cord to those arising from the dorsal root ganglia, sympathetic ganglia, and peripheral nerves (O'Brien et al., 1994; Bethea et al., 1998; Miranda et al., 1999; Springer et al., 1999; Yaksh et al., 1999; Saito et al., 2000; Bruce et al., 2002; Hains et al., 2003b; Hoheisel et al., 2003). Furthermore, biochemical events leading to abnormal firing of spinal neurons (Mills et al., 2001; Yezierski et al., 2004), up-regulation of voltage-gated ion channels (Nashmi and Fehlings, 2001; Edwards et al., 2002; Hains et al., 2003a), recruitment of reactive glia (Carlton et al., 2009), and excessive release of excitatory amino acids (excitotoxicity) in the spinal gray and white matters add to the complexity of neuropathic pain in SCI patients (Mills et al., 2001; Zeilig et al., 2012).

Excitotoxicity-induced SCI model using intraspinal injections of the AMPA- and metabotropic glutamate receptor agonist quisqualic acid (QUIS) have been described by Yezierski et al. (Yezierski et al., 1993, 1998; Gorman et al., 2001). QUIS can produce more controlled-lesion with characteristic neuronal loss, demyelination, cavitation, glial activation, perivascular changes, breakdown of the blood-brain barrier, and inflammation, closely mimicking sequence of events observed in contusion or ischemic injured spinal cords. QUIS injured rats exhibit nociceptive behaviors for mechanical and cold allodynia and self-injurious overgrooming behaviors (Brewer and Yezierski, 1998; Gorman et al., 2001). Overgrooming behavior is thought to be mediated by dysesthetic sensations originating from the affected

at-level dermatomes and/or DRGs ipsilateral to insult (Brewer et al., 2008), and disruption of inhibitory tone maintained by endogenous GABAergic neurons in the superficial dorsal horn (Lee et al., 2008). QUIS injury also up-regulates ERK1/2, TNF-α, and cytokines known to be involved in molecular events leading to development of nociception (Yu and Yezierski, 2005; Brewer and Nolan, 2007).

Loss of endogenous GABA-IR cells following QUIS or CNS/PNS injuries may play an important function in modulating nociception (Zhang et al., 1994; Lee et al., 2008), yet their role remains elusive (Polgar et al., 2003; Polgar and Todd, 2008). Chronic constriction injury in rats induce increased number of picnotic cells, hyperchromatic "dark neurons" possibly indicative of transsynaptic degeneration in the superficial spinal or medullary dorsal horn, in the superficial dorsal horn by one week post injury (Ibuki et al., 1997), and spared nerve injury decreases primary afferent-induced IPSCs in lamina II neurons, presumably due to the loss of GABA resulting from decreased GAD 65 expression (Moore et al., 2002). Such injury-induced nociceptive behaviors can be further exacerbated by pharmacologic blockade of inhibitory neurotransmission (Sugimoto et al., 1987, 1990; Hama and Sagen, 1993). In stereological estimates from EM sections, excitotoxic neuronal cell death in the superficial dorsal horn was also observed in sciatic nerve-lesioned animals following stimulation of A fibers (Coggeshall et al., 2001), and cell death in the superficial dorsal horn (TUNEL labeling) has been observed following CCI and sciatic neurectomy which could be prevented by NMDA antagonists and caspase inhibitors (Azkue et al., 1998; Whiteside and Munglani, 2001; Scholz et al., 2005). Nerve injury-induced nociceptive behaviors can be reversed by the administration of GABA into the spinal cord (Eaton et al., 1999a; Sokal and Chapman, 2003). Intrathecal administration of baclofen or muscimol, GABA receptor agonists, cause dose-dependent analgesia in animals with peripheral nerve injury (Hwang and Yaksh, 1997), and these effects were blocked with the administration of GABA receptor antagonist bicuculline suggesting specificity of GABA on reducing noxious sensation (Malan et al., 2002).

Recently, several reports showed possible therapeutic use of transplanting embryonic neural cells to control central and peripheral nociceptive behaviors. Intraspinal transplantation of mouse embryonic neural stem (ES) cells can reverse QUIS-induced formalin and mechanical nociception (Hendricks et al., 2006). Intraspinal and intrathecal injections of predifferentiated GABAergic embryonic ES cells (human and rodent-derived) can also reverse CCI/SNL-induced nociceptive behaviors (Mukhida et al., 2007; Jergova et al., 2012), possibly by rescuing the endogenous inhibitory neural circuitry (Vaysse et al., 2011). GABAergic neuronal stem cells (hSSC, hNT, and mouse ES) have been used to control pain arising from ischemic paraplegia as well as spinal hemisection-induced nociception (Marsala et al., 2004; Cizkova et al., 2007; Kim et al., 2010). Although it is not clear how the embryonic cells achieve anti-nociception (via local secretion of GABA or rescuing of endogenous GABAergic/inhibitory mechanisms), transplantation of predifferentiated embryonic inhibitory neurons can modulate nerve injury-induced pain. The source of embryonic stem cells also may be of importance, as forebrain-derived cells are reported to yield much higher density of GABAergic cells than spinal cord-derived cells (Watanabe et al., 2004).

The present study examined effects of transplanting partially differentiated GABA-immunoreactive embryonic cortical precursor cells in quisqualic acid-lesioned rat spinal cord to reverse pain behavior. First, we looked at the pre-differentiation of cultured embryonic rat cortical neurospheres into GABAergic phenotype by exposing the neurospheres to varying concentrations of FGF-2. The second part of the experiment focused on transplantation of predifferentiated GABAergic neurospheres into the spinal cord of QUIS-lesioned animals for the alleviation of central pain, indirectly measured by changes in overgrooming area. Results of these experiments show transplantation of predifferentiated GABAergic neural precursor cells (NPCs) into the spinal cord of QUIS-lesioned animals prevents or reduces overgrooming behavior. Portions of this work have been presented previously in abstract form (Lee et al., 2001).

MATERIALS AND METHODS

ANIMALS

Male Sprague Dawley rats (220–250 g, Charles River labs) were housed in a regular light condition (12:12 light and dark cycle) with food and water *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Miami, Miller School of Medicine.

QUISQUALIC ACID LESION

All animals in the study received quisqualic acid lesion (QUIS, 125 mM, Sigma) at T12-L1 spinal level. QUIS was diluted in distilled water and aliquoted into 50 μl/vial and kept frozen (−20°C) until use. Animals were anesthetized using 3% isoflurane/O_2 and a midline dorsal skin incision was made to expose the thoracolumbar vertebrae. Laminectomy was performed to expose the spinal T12-L1 levels. Animals were placed on a spinal stereotaxic unit (David Kopf Inc., CA, USA), and dura was cut and retracted laterally to expose the dorsal root entry zones. QUIS was injected into the dorsal horn halfway between the dorsal vein and the dorsal root entry zone using a glass micropipette attached to a Hamilton syringe (10 μl, Reno, NV, USA). Using a microinjector, three injections of 0.4 μl each of QUIS was injected into the spinal cord gray matter at a depth of 1 mm. Injections were made unilaterally and spaced out at 500 μm rostro-caudal direction (**Figures 1B,C**). Upon completion of each QUIS injection, the glass pipette was left in place for 60 s to prevent backflow. Muscle was closed to cover the injection site and the skin was closed with the wound clips.

OVERGROOMING BEHAVIOR AND OVERGROOMING AREA MEASUREMENTS

To measure changes in pain threshold before and after predifferentiated embryonic cell transplantation, the total skin overgrooming area of each animal was digitally photographed (**Figure 1C**) and then manually recorded by tracing the wound outline onto a semi-transparent plastic film superimposed over the overgrooming site. The outlines on the film were digitally retraced with Neurolucida (MicroBrightField Inc., Williston, VT, USA) and the total overgrooming area was obtained. For each QUIS-lesioned animal, the total overgrooming area was measured in square millimeter

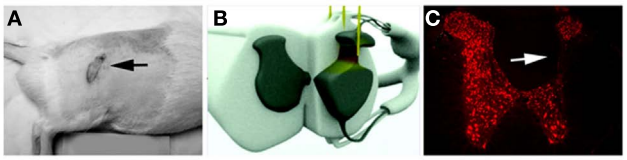

FIGURE 1 | Overgrooming lesions were primarily observed on at-level ipsilateral dermatomes two weeks after lesion [(A): black arrow]. (B) A 3D model of spinal cord showing three injections of approximate 1 mm in depth from the dorsal surface needed to induce overgrooming in QUIS animals. **(C)** Characteristic loss of spinal neurons (Neu-N immunoreactivity) in the neck of dorsal horn 2 weeks after QUIS injection (white arrow). Neurons from the superficial lamina I and II are mostly spared from the excitotoxic cell death.

before transplantation (2 weeks post-lesion) and every week since transplantation for 3 weeks up until the time of perfusion.

ISOLATION OF NEURAL PROGENITOR CELLS

Embryonic cortical neural progenitor cells were isolated from E14 Sprague Dawley rats. Pregnant rats were deeply anesthetized with an overdose of nembutal (Sodium pentobarbital, Abbott, MI, USA) and a midline ventral incision was made to expose the embryos. Lateral ganglionic eminence were dissected out and placed into a 15 ml conical tube containing cold Hank's balanced salt solution (HBSS, pH 7.2; Gibco). Isolated cortical tissues were gently triturated mechanically to dissociate into single cells, resuspended and plated at low density (3.5×10^6 in 7 ml of media) in N2 growth media (DMEM/F12 + N2 supplement, pH 7.2; Gibco) containing standard concentration of FGF-2 (10 ng/ml, R&D) at 37°C. Embryonic precursor cells were grown as small neurospheres and passaged by high flow rate trituration every 3–4 days to maintain initial density. The N2 growth media was changed every 2 days, and additional 6 µl FGF-2 (10 ng/ml) was added into the media in between media changing days.

IN VITRO PARTIAL DIFFERENTIATION OF RAT EMBRYONIC CORTICAL PRECURSOR CELLS INTO GABA-IMMUNOREACTIVE CELL TYPE

Five to ten days old cultured neurospheres were transferred to 15 ml tubes, centrifuged at 700 RPM for 5 min at 4°C, and resuspended in N2 culture media containing either 0.1, 1, or 10 ng/ml FGF-2. Neurospheres were incubated in these media for 4–16 h. Neurospheres were then resuspended in N2 culture media with 10 ng/ml FGF-2 maintaining the initial density of approximately 0.5×10^6 neurospheres/ml. Neurospheres were plated on a poly-L-ornithine/fibronectin (Sigma Aldrich) coated plastic plates for 1 day and fixed with 4% paraformaldehyde to quantitate the number of precursor cells differentiated into GABAergic cells *in vitro* after exposure to different concentrations of FGF-2. Fixed cells were also processed for GABA, NeuN, nestin, GFAP, MAP-2, β-III-tubulin, and BrdU immunofluorescence.

IN VITRO QUANTITATION OF GABA CONCENTRATION SECRETED BY THE EMBRYONIC PRECURSOR NEUROSPHERES

The growth media of cell culture from original harvested cells (P0) and *in vitro* differentiated neurospheres (P1) were sampled to ascertain the concentration of GABA secreted by the precursor neurosphere cells. The neurospheres were not stimulated electrically/physically in any way to induce GABA secretion. Twenty microliters of growth media from both samples were collected every day for 7 days. The passage 0 cells survived up to 4 days post harvest whereas the passage 1 neurospheres were visibly healthy even at 12 days post harvest. High-Pressure Liquid Chromatography (HPLC) was used to measure the concentration of GABA present in 20 µl growth media sample. Samples were analyzed on a chromatograph consisting of a Beckman Model 118 Solvent Module, a Beckman System Gold data system and an ESA Coulochem II electrochemical detector. A 150-mm long, 3-mm-diameter ESA C18 column was used. The mobile phase consisted of 1.5 mM sodium octane sulfonic acid, 75 mM NaH2PO4, triethylamine, and 10% acetonitrile dissolved in water at pH 3.0.

TRANSPLANTATION OF PREDIFFERENTIATED GABA-IMMUNOREACTIVE EMBRYONIC PRECURSOR CELLS OR CONTROL BOVINE FIBROBLAST CELLS IN QUIS RATS

Only rats exhibiting overgrooming behavior 10–14 days post-QUIS lesion received cell transplantation ($n = 34$). Animals were anesthetized and laminectomy was performed to expose the spinal level where the QUIS lesion was made (T12-L1). Using glass micropipette, three injections of 4 µl each of neural progenitor cells (50,000 cell/µl, total 600,000 cells/animal) were injected into the dorsal horn, ipsilateral to QUIS lesion, at a depth of 0.5 mm to 1 mm ($n = 27$). Controls were injected with bovine fibroblast cells ($n = 7$). Glass pipette was left in place for 60 s to prevent backflow of the cells, muscle was closed to cover the injection site, and the skin was closed with wound clips. Rats were immunosuppressed by cyclosporine A (i.p., 10 mg/kg; Bedford Labs, OH, USA) from −1 day until sacrifice.

IN VITRO AND SPINAL CORD IMMUNOHISTOCHEMISTRY

Three weeks after cell transplantation, and after weekly measurements of overgrooming area, animals were anesthetized with an overdose of pentobarbital and perfused transcardially using a peristaltic pump. Animals were perfused with cold 0.9% saline followed by either cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2) or 4% paraformaldehyde plus 1% glutaraldehyde in 0.1 M PB. Spinal cords were removed and post-fixed in the same fixative overnight, then placed in 30% sucrose-PB solution for cryoprotection.

Predifferentiated neurospheres (*in vitro*) and NPC-transplanted QUIS spinal cords were processed for immunohistochemical analysis. The neurospheres (5–7 days old) were fixed with cold 4% paraformaldehyde in Phosphate Buffered saline (PBS; pH 7.4), and were incubated overnight in PBS with 0.4% Triton-X 100 and 5% normal goat serum (PBS-TGS) containing the following primary antibodies: NeuN (1:300, mouse, Chemicon, MA, USA), GABA (1:200, guinea pig, Protos Inc., NY, USA), GFAP (1:200, mouse, Steinberg), nestin (1:10, mouse, rat401, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), and β-III-tubulin (1:200, mouse, Chemicon, MA, USA). Depending on the double labeling schedule, cells were incubated with species-specific secondary antibodies conjugated with either AlexaFluor 488 or AlexFluor 594 (Molecular Probes, NY; 1:200 in PBS-TGS) for 2 h at room temperature. For BrdU labeling, fixed neurospheres were

treated with 2N HCl for 10 min, washed in PBS and incubated in Borate solution for 10 min. After wash, cells were incubated in the blocking solution, primary and secondary antibody according to the protocol above. Some neurospheres were processed for GABA immunostaining using biotinylated secondary antibody, followed by incubation in avidin-biotin solution in PBS for 1h and using DAB as a chromogen.

Spinal cord segments thoracic 10 to lumbar 5 were cut at 30 μm using a freezing microtome (American Optical, MA, USA), rinsed in PBS, and then treated for 1 h at room temperature in PBS-TGS. Sections were incubated overnight in PBS-TGS containing the following primary antibodies: NeuN, GABA, GFAP, nestin, and β-III-tubulin as described above. Spinal sections were washed 3 × 10 min in cold PBS-TGS and incubated with species-specific secondary antibodies conjugated with either AlexaFluor 488 or AlexFluor 594 for 2 h at room temperature. Some spinal sections perfused with 4% paraformaldehyde-1% glutaraldehyde were treated with 1% sodium borohydride in PBS (pH 7.4) for 20 min at room temperature to mitigate background fluorescence. These sections were treated with PBS-TGS as above, and incubated overnight at 4°C in PBS-TGS containing combinations of the following primary antibodies: anti-GABA (1:500, guinea pig, Chemicon, MA, USA), anti-glycine (1:300, rabbit, Chemicon), anti-vesicular inhibitory amino acid transporter (VIAAT; 1:200, rabbit, gift from Dr. Bruno Gasnier, INSERM, Strasbourg-Cedex, France), and anti-synaptobrevin (1:1000, mouse, Synaptic Systems, Gottingen, Germany). Sections were washed 3 × 10 min in cold PBS-TGS, incubated at room temperature for 1 h in secondary antibodies: anti-guinea pig Alexa Fluor 488, anti-rabbit Alexa Fluor 594, and anti-mouse Alexa Fluor 680. All fluorescently labeled sections were washed three times in PBS, mounted on lysine-coated slides, air-dried and coverslipped using Vectashield mounting media containing DAPI (Vector Lab, CA, USA).

MICROSCOPE AND IMAGE CAPTURING

The majority of fluorescent sections were visualized with an Olympus fluorescent microscope. Images were acquired with a color CCD camera and ImagePro plus software (Media cybernetics Inc., Silver Spring, MD, USA) on an Apple Macintosh computer. Glutaraldehyde-treated, double- and triple-labeled sections were imaged on a Carl Zeiss LSM 510 confocal microscopy setup. An Axiovert 100M microscope with a motorized stage was operated using a PC running Zeiss LSM software version 3.2. Fluorescence was excited using Argon (514 nm) and HeNe (543, 633 nm) lasers (Lasos Lasertechnik; Jena, Germany). Images stacks were acquired with 10× dry and 40× oil immersion magnification (4 and 43 optical slices, respectively), and flattened into planar images. Adobe Photoshop was used as a digital layout tool for composition and overlay of acquired images.

STEREOLOGICAL ANALYSIS

For the estimation of GABAergic and NeuN positive profiles within the transplant area in QUIS-injected animals, serial immunostained sections of lumbar spinal cord were analyzed with Neurolucida (MicroBrightField Inc., Williston, VT, USA). The transplant site was identified under 20× magnifications. Cells were counted within 0.015 mm^2 frame positioned over the transplant area at 60× magnification. Stereological estimation was performed in 25–30 sections/spinal cord. Results are presented at average ± SEM.

STATISTICAL ANALYSIS

To analyze the overgrooming area of animals transplanted with control fibroblast cells and GABAergic precursor cells, the data were analyzed by two-way ANOVA with repetitive measurements with group and time post-transplantation as variables followed by Holm–Sidak *post hoc* analysis; level of significance was $p < 0.05$.

RESULTS

Approximately 30% of animals that received quisqualic acid lesion in the spinal cord but did not display overgrooming behavior within two weeks post injury were discarded from the study. In general, overgrooming behavior was confined to ipsilateral at-level dermatomes, but a few animals displayed bilateral overgrooming behavior (**Figure 1A**).

SPINAL CORD HISTOLOGY FOLLOWING QUIS LESION

In the present experiment, micro-injections of 1.2 μl of QUIS (total volume; 125 mM) at a depth of 1 mm from the dorsal surface of the spinal cord (**Figure 1B**) was sufficient to eliminate ipsilateral spinal neurons at the neck of the dorsal horn from laminae III to V/VI (**Figure 1C**). The lesion caused collapse of the gray matter into a thin dorso-ventral neuropil. Most neurons in the superficial laminae I and II, however, were spared from the injury even 3 weeks after QUIS lesion and a large number of NeuN-immunoreactive (NeuN-IR) cells were observed with a minimal gray matter disruption as previously reported (Lee et al., 2008). In almost all cases, neuronal cell losses were confined only to the dorsal gray matter ipsilateral to lesion. In a few animals, there were cell losses in the ipsilateral ventral horn and to the contralateral gray matter due to the spread of QUIS across the central canal, and in some instances, small cavitations in the spinal cord were observed in the ipsilateral lesioned gray matter (Yezierski et al., 1993). These cases were minor and may have been due to the age/weight of animal or proximal to medial placement of the glass pipette tip at the time of QUIS lesion.

CHANGES IN GABA, GLYCINE, AND VIAAT FOLLOWING QUIS LESION

Two weeks after QUIS lesion, there appeared substantially fewer number of endogenous GABA-IR in the superficial laminae I and II (**Figure 2B**) as described previously (Lee et al., 2008). Such a loss was specific to spinal level within the QUIS injury, since endogenous GABA-IR in the contralateral side and spinal levels outside the QUIS lesion area were not affected (**Figure 2A**). Immunostaining for a broader panel of markers for inhibitory neuronal signaling machinery revealed additional injury-induced histological changes. The other major spinal cord inhibitory neurotransmitter, glycine, was decreased in injured gray matter and in spared superficial laminae (**Figure 2D**), but remained unchanged in the contralateral side (**Figure 2C**).

Immunostaining for synaptobrevin was used to mark synaptic vesicles in the spinal cord. Colocalization of synaptobrevin, GABA, and VIAAT was detected in spinal cord dorsal horns as white punctate staining (**Figures 2E–F**). Triple-labeled puncta

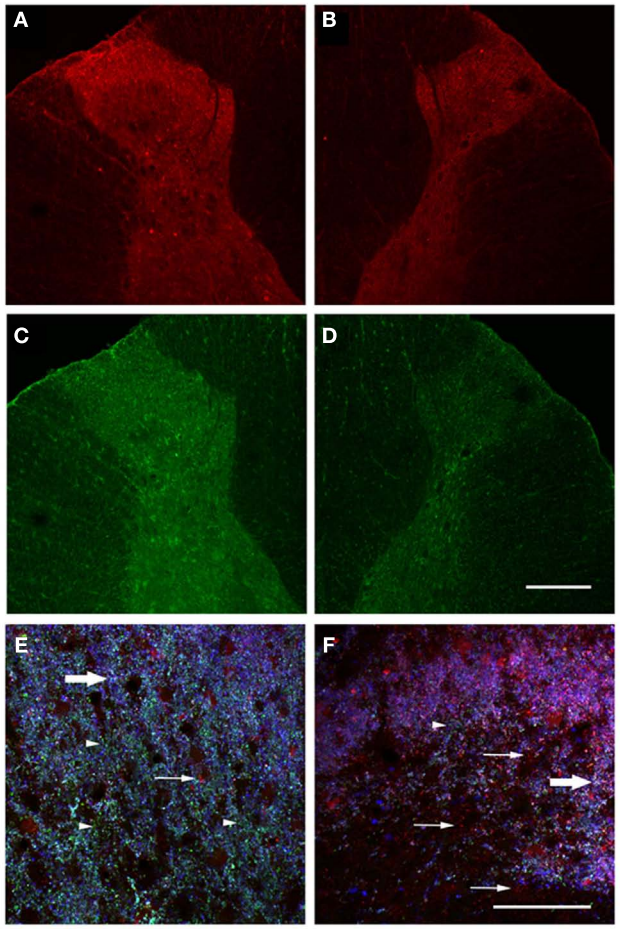

FIGURE 2 | Decreased immunoreactivity for inhibitory signaling components in injured spinal cord gray matter. Spinal cord sections were stained for GABA **(A,B)** and glycine **(C,D)**. Uninjured dorsal horns showed robust staining for both neurotransmitters **(A,C)**, but staining was decreased in injured dorsal horns **(B,D)**. Spinal cord sections were also triple-stained for GABA, VIAAT, and synaptobrevin. A region within the uninjured dorsal horn was enlarged **(E)**, showing extensive VIAAT labeling (arrowheads), few lone GABA puncta (arrows), and numerous triple-labeled white puncta (block arrows). Enlargement of injured dorsal horn **(F)** revealed substantially decreased VIAAT, abundant free GABA, and few triple-labeled puncta. Scale bars = 200 μm **(D)**; 50 μm **(F)**.

were abundant in dorsal horns contralateral to QUIS injection, particularly in superficial laminae (**Figure 2E**). QUIS-injected dorsal horns appear to exhibit reduced triple labeling of inhibitory synaptic vesicle markers (**Figure 2F**). The VIAAT-IR was abundant in gray matter contralateral to QUIS injection (**Figure 2E**) but substantially decreased in the ipsilateral dorsal horn after QUIS (**Figure 2F**) which suggest that inhibitory synaptic vesicles may be depleted in the injured dorsal horn.

TIME COURSE OF CHANGES IN GABA IMMUNOREACTIVITY FROM ISOLATED EMBRYONIC CORTICAL PRECURSOR CELLS

Under standard growth condition with constant 10 ng/ml FGF-2/N2 culture media, the proportion of GABA-IR cells found in freshly isolated primary cortical embryonic tissues was minimal (P0). Presence of low number of GABA-IR cells at this growth stage correlated with the *in vitro* GABA HPLC data (see below).

In order to promote and increase the proportion of cultured cortical precursor cells to differentiate *in vitro* into GABAergic phenotype, 5–10 days post-isolation neurospheres were exposed to 0.1 ng/ml (low), 1 ng/ml (mid), or 10 ng/ml (standard concentration) of FGF-2 for 4–16 h (**Figure 3**). GABA-IR cells were observed in all three conditions, and there were minimal difference between 4 and 16 h exposure in increasing the number of GABA-IR cells. Neurospheres exposed to 0.1 ng/ml FGF-2 contained the highest number of mature-looking GABA-IR cells (**Figures 3A,B,D**). The inhibitory interneurons were mostly found inside neurospheres tightly packed as a cluster composed almost entirely of GABA-IR cells. Individual GABA-IR neuron possessed small and large processes extending radially around and out from the neurosphere. Axonal bouton enlargements were observed in processes of some cells. Neurospheres exposed to 1 and 10 ng/ml FGF-2 did not contain as many GABA-IR cells within the neurospheres as the previous group. In these groups, most of the GABA-IR cells were observed outside the neurospheres, as a single cell, in a migratory pattern dispersing radially outward from the core of neurospheres. These cells possessed short bipolar or multipolar outgrowths from the soma (**Figures 3C,D**). The predifferentiated neurospheres contained numerous dividing cells indicated by presence of BrdU-IR (**Figure 3E**). At this stage none of GABA-IR NPCs colocalized with BrdU-IR, suggesting that these are pre-existing GABA cells.

IN VITRO ANALYSIS OF FGF-2 PREDIFFERENTIATED NEUROSPHERES

Several markers for mature and immature cell types were used to identify different phenotypes present in the predifferentiated neurospheres (**Figure 4**). A large proportion of NPCs expressed GAD65/67-IR where a subpopulation of them also expressed GABA-IR (**Figure 4A**). Many of GAD65/67-IR NPCs also colocalized with NeuN-IR (**Figure 4B**). Most of the embryonic progenitor cells labeled positive for nestin-IR irrespective of FGF-2 pre-differentiation conditions (**Figure 4C**). These NPCs possessed long elongated bipolar projections without any fine dendritic branches coming off from main projections. Large proportions of nestin-IR cells were positively double labeled with GABA (**Figure 4C**), but only a very small subset of GABA-IR NPCs positively colocalized with NeuN (**Figure 4D**), indicating presence of immature GABAergic NPCs in the neurospheres. Beta-III-tubulin-IR was observed in the NPCs, but only very small number of them coexpressed GABA-IR (**Figure 4E**). Many GABA-IR NPCs colocalized with MAP-2, a marker for neural maturation (**Figure 4F**). GFAP-IR was almost not present in the neurospheres (data not shown).

IN VITRO QUANTITATION OF GABA RELEASE FROM PREDIFFERENTIATED EMBRYONIC PRECURSOR CELLS

The level of spontaneously released GABA concentration in the culture media was measured daily for 7 days using HPLC. Establishment of GABA release was critical to prove functional properties of our predifferentiated NPCs. The GABA concentrations from originally harvested (P0) and *in vitro* cultured (P1) neurospheres were measured. There were increased detectable levels

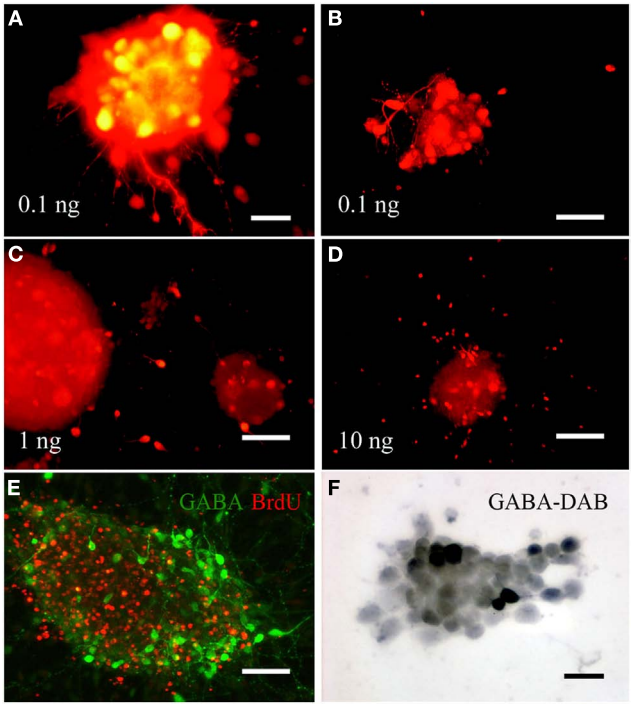

FIGURE 3 | Brief exposure to varying concentrations of FGF-2 induces differentiation of embryonic cortical progenitor cells into GABAergic phenotype in cultured neurospheres. Progenitor cells exposed to low concentration of FGF-2 expressed the largest number of GABA-IR cell **(A,B)**. Majority of these cells were found in or adjacent to the neurospheres and did not show migratory pattern. Progenitor cells exposed to 1 or 10 ng/ml FGF-2 did not display as many GABA-IR cells as those exposed to 0.1 ng/ml. These GABA-IR cells were found predominantly outside the neurospheres radiating outwardly in a migratory pattern **(C,D)**. Large proportion of NPCs can be labeled with BrdU, however, the GABA-IR cells did not co-label with BrdU **(E)**. A high magnification light microscopy image of GABA-IR cells inside a neurosphere labeled with DAB peroxidase reaction **(F)**. Scale bars = 20μm **(A)**; 50μm **(B–E)**.

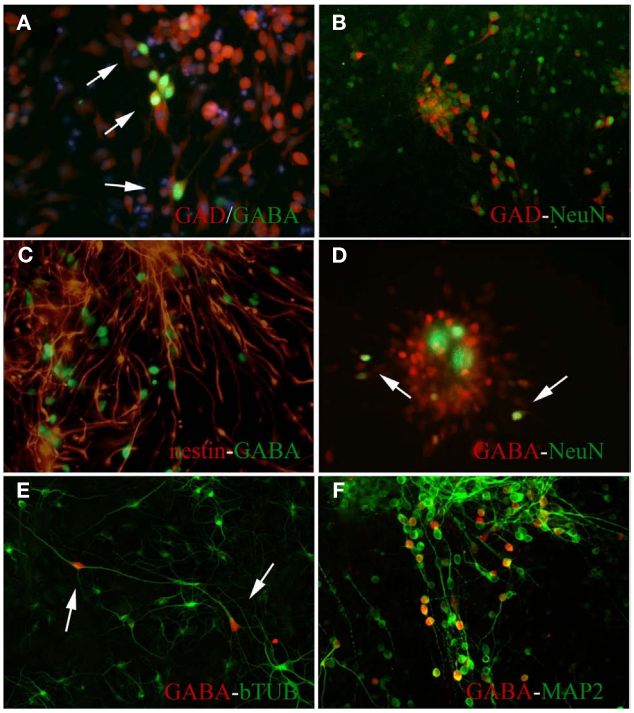

FIGURE 4 | Predifferentiated embryonic precursor neurospheres expressed robust immunoreactivity for GABA and other marker for cell differentiation 5–7 days after harvest. (A) Many of GABA-IR NPCs colocalized with GAD65/67-IR cells (arrows). **(B)** Majority of predifferentiated GAD65/67-IR NPCs also expressed NeuN-IR. **(C)** Majority of predifferentiated GABA-IR NPCs also expressed nestin-IR; Not every nestin-IR cells expressed GABA-IR. Many GABA-IR cells colocalized and expressed NeuN-IR **(D)**, β-III-tubulin-IR **(E)**, and MAP-2-IR **(F)**.

of GABA present in the culture media starting from day 1until the last sample taken at day 7 post-isolation. For P0 cells, the GABA concentration in the media changed very little over time (**Table 2**). Majority of P0 neurospheres did not survive more than 4 days *in vitro*. The GABA concentrations from P0 cells at day 1 and day 4 were 0.3955 and 0.5609 μM/20 μl, respectively. On the other hand, there was a dramatic increase in GABA concentration present in the media of P1 neurospheres. Their initial GABA concentration was very low (day 1 = 0.0941 μM/20 μl) but increased by 10-fold at day 7 (1.0673 μM/20 μl) suggesting massive differentiation of NPCs into GABAergic cells.

CHANGES IN OVERGROOMING AREA FOLLOWING TRANSPLANTATION OF GABAERGIC PRECURSOR CELLS

Excitotoxic QUIS lesion caused a continuous and progressive damage to the spinal cord where the extent of the SCI as well as the size of peripheral overgrooming area increased progressively over time (Brewer and Yezierski, 1998). All of QUIS-lesioned animals that received transplantation of control cells (bovine adrenal fibroblast, $n = 7$) showed a significant increase in overgrooming area over time where the area at pretransplantation (baseline) was $130.2 \pm 28.9\,mm^2$ and increased by three folds to $348.1 \pm 115.08\,mm^2$ at 1 week post-transplantation (**Figures 5A,C** and **6A**). By 2 and 3 weeks, the overgrooming area remained significantly enlarged to $438.5 + 121.0$ and $399.0 + 120.80\,mm^2$, respectively ($p < 0.05$). On the other hand, QUIS-lesioned animals that received transplantation of predifferentiated GABA-IR NPCs ($n = 27$) did not show significant increase in overgrooming area, but remained at near baseline level during 3 weeks of the testing period (**Figures 5B,D**). In these rats, the baseline overgrooming area was $181.5 + 27.3\,mm^2$, and 3 weeks after transplantation the overgrooming area remained at $237.3 \pm 55.8\,mm^2$ (**Figure 6A**). Overall 55.6% of animals ($n = 15/27$) displayed overgrooming areas that were either the same or smaller than at pretransplantation stage; 27% of the animals ($n = 7/27$) displayed a 50% reduction in the overgrooming area by 3 weeks post-transplantation. Seven animals completely recovered from overgrooming injuries. When the percent changes in grooming areas were compared between groups, the overgrooming areas of rats that received GABA-IR NPCs were significantly smaller than fibroblast-transplanted control rats during the experimental period ($p < 0.01$; **Figure 6B**).

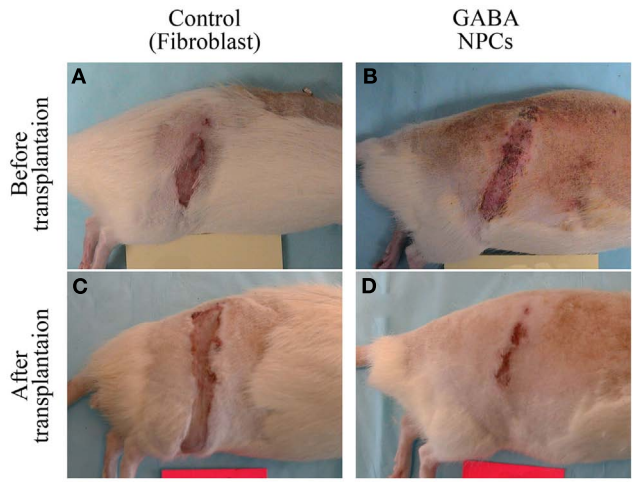

FIGURE 5 | Photomicrographs showing changes in size of overgrooming in QUIS animals transplanted with control bovine fibroblast cells [pretransplantation: (A) 3 weeks post: (C)] and predifferentiated GABA-IR NPCs [pretransplantation: (B), 3 weeks post: (D)].

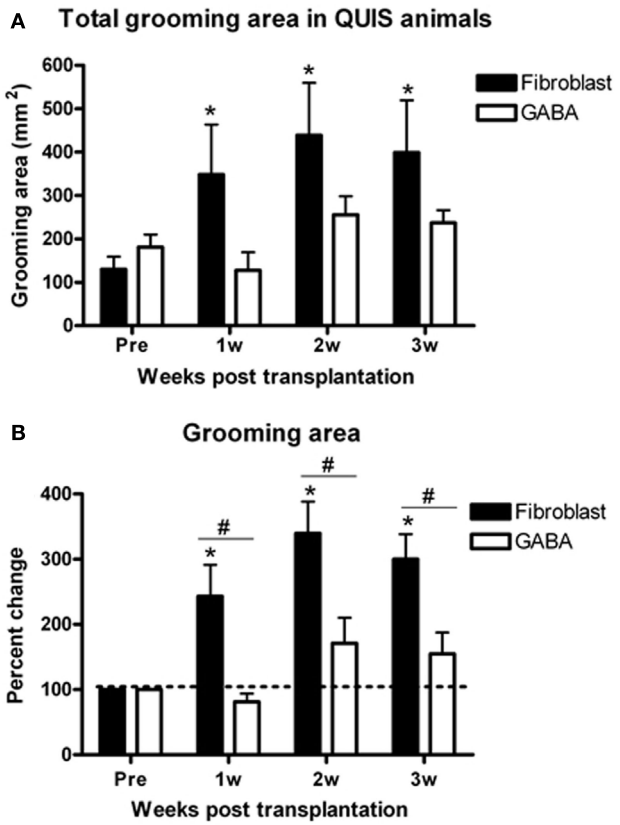

FIGURE 6 | Percent change of the overgrooming area from pretransplantation for GABA and fibroblast-transplanted rats. Control animals displayed significant increases in total overgrooming area **(A)** and percent change of overgrooming area **(B)** post-transplantation vs. pretransplantation and also compared to GABA-transplanted animals. GABA-transplanted rats did not display statistically significant changes in either total or percent change of overgrooming compared to pretransplantation. $^{*}p < 0.05$ vs. pretransplantation, $^{\#}p < 0.05$ vs. GABA group.

IDENTIFICATION OF GABA-IR NPCS IN THE SPINAL CORD OF QUIS-LESIONED ANIMALS

Transplanted NPCs were observed within the spinal level of QUIS-lesioned sites. The immunohistochemical analysis revealed the NPCs were placed adjacent to the midline encompassing the medial aspect of spinal gray matter and the dorsal column (**Figure 7**). Surviving GABA-IR NPCs were observed covering a large extent of the dorsal horn from Rexed's lamina I–VI and as medial as the dorsal column. Based on our previous study (Furmanski et al., 2009), the grafted cells were distinguished from endogenous GABAergic interneurons by their different morphology and location in the spinal cord. Grafted NPCs appeared as large and round cells in superficial and deeper dorsal horn laminae in contrast to much smaller and elongated endogenous interneurons located mainly in superficial laminae. Overall, the spinal cords could be divided into three groups: those containing (i) large, (ii) moderate, or (iii) minimal number of GABA-IR NPCs in the dorsal gray matter. The number of surviving GABA-IR NPCs inversely correlated with the size of overgrooming area. Animals with large number of GABA-IR NPCs in the spinal cord displayed the smallest to no overgrooming lesion area. Animals containing medium to low number of GABA-IR NPCs displayed same or increased overgrooming area compared to pretransplantation state.

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IN VIVO DIFFERENTIATION OF EMBRYONIC GABA-IR NPCs

Surviving NPCs within the injured spinal cord exhibited immunoreactivity for GABA and several other cell markers. The GABA-IR cells ranged in size of a small interneuron to a large spinal motor neuron (**Figure 8** left two columns). The GABA-IR cells double labeled with neuronal marker, NeuN (**Figures 8A–D**), but did not express nestin-IR indicating differentiation into mature neurons. Although GFAP- and β-III-tubulin-IR were present within the transplantation site of NPCs, neither of them colocalized with GABA-IR (**Figures 8I–P**).

STEREOLOGICAL QUANTITATION OF GABA-IR CELLS IN THE QUIS SPINAL CORD

Stereological evaluation was performed on spinal cord sections from these animals in a tissue volume of $0.7 \pm 0.04\,mm^3$. About 30–40% of surviving predifferentiated GABA-IR cells colocalized with NeuN indicating maturity to neuronal phenotype (**Table 1**). The majority of GABA-IR cells ($67.8 \pm 2.7\%$) within the

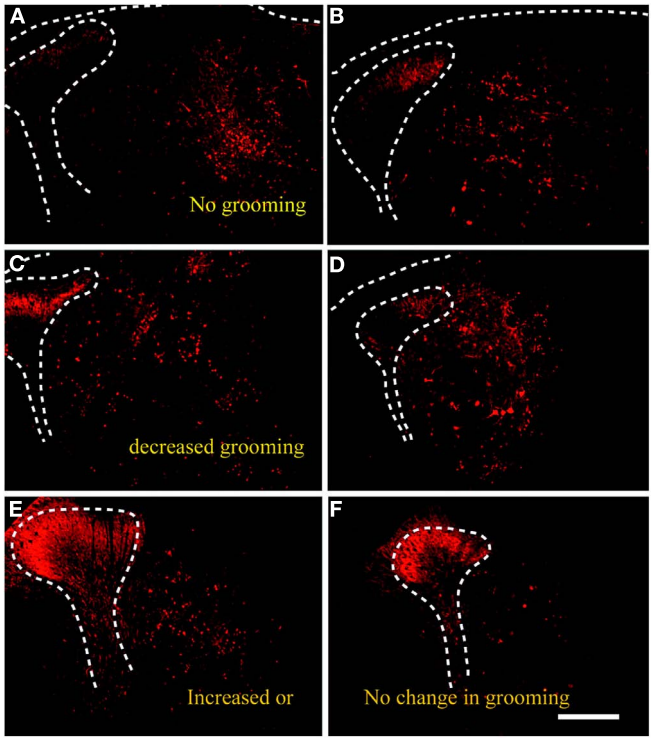

FIGURE 7 | Transplanted predifferentiated GABA-IR NPCs were visible in the spinal cord of QUIS animals three weeks after transplantation, and showed varying densities in the host spinal cord. Animals that stopped overgrooming [healed; **(A,B)**] or displayed reduction in overgrooming **(C,D)** exhibited large number of transplanted GABA-IR NPCs in the spinal gray matter. Animals that continued to groom contained very few number of surviving GABA-IR cells in the spinal cord **(E,F)**. Scale bars = 500 μm.

transplant area did not colocalize with NeuN indicating immaturity 3 weeks post-transplantation into host spinal cord. Similar numbers were obtained when sections were processed for GAD65/67 (data not shown).

DISCUSSION

Results from the present study show that intraspinal transplantation of predifferentiated embryonic GABAergic NPCs, but not bovine fibroblast cells, significantly decreases self-injurious overgrooming lesions observed in QUIS injured rats. The decrease in overgrooming may be mediated by the GABA released from the transplanted precursor cells since the reduction in grooming was observed primarily GABA-transplated QUIS animals. Manifestation of overgrooming behavior is thought to have a relation with dysesthetic and nociceptive sensations since subdural transplantation of analgesic compound-releasing chromaffin cells can reverse mechanical and thermal nociceptive thresholds in QUIS rats, in addition to significant reduction on the size of overgrooming area by 2 weeks post-transplantation compared to muscle transplanted control rats (Brewer and Yezierski, 1998).

In our experiment, reversal of overgrooming lesion was observed in 55.6% ($n = 15/27$) of GABA-transplanted rats from which seven rats recovered completely from self-inflicted dermal injuries 3 weeks post-transplantation. The main contributing factor for these recoveries may be the secretion of GABA in the lesioned dorsal horn by the embryonic GABA-IR NPCs transplanted into the host spinal cord, since fibroblast-transplanted control rats ($n = 7$) or QUIS animals with only a small number of surviving transplanted GABA cells did not show a decrease in their overgrooming lesion. In fact, the latter group of animals showed increasing overgrooming lesion size overtime closely resembling the pattern observed in fibroblast-transplanted control rats. The results from our experiment show intraspinal transplantation of GABA secreting embryonic precursor cells are effective in reducing centrally- and peripherally mediated nociceptive behaviors without causing sensory and motor deficits, similar to observations reported by others (Hendricks et al., 2006; Mukhida et al., 2007; Wolfe et al., 2007).

Changes in the number and expression of endogenous GABA leading to abnormal inhibitory signaling following spinal or peripheral nerve injuries have been well documented in several rodents pain models, yet their role in expression of nociceptive behaviors remain divided (Polgar et al., 2003; Lee et al., 2008; Polgar and Todd, 2008; Meisner et al., 2010). The postulated loss of GABAergic neurons is controversial, however, as a reduction in GABAergic immunoreactivity in parallel with the development of neuropathic pain symptoms is not always observed (Polgar et al., 2003, 2004; Polgar and Todd, 2008). Nevertheless, alternative explanations for disinhibition after nerve injury include reduced GABA synthesis in existing intact, but dysfunctional neurons or reduced excitatory drive to GABAergic dorsal horn neurons following loss of primary afferent input to these cells. Alterations in GABA receptors, e.g., loss of receptors or reversion to a neonatal excitatory and pronociceptive GABA phenotype may also occur. Diminished GABAergic inhibitory effects via reduced expression of the potassium co-transporter KCC-2 and disruption of the chloride gradient has been suggested as an underlying mechanism of activated microglia in neuropathic pain induction (Coull et al., 2003, 2005). However, pharmacologic findings argue against a complete excitatory reversion since GABA and GABAergic agonists can reduce neuropathic pain behaviors. In addition, electrophysiological studies suggest that diminished GABA release, rather than changes in receptor density, account for the loss in GABAergic inhibition in peripheral nerve injury models (Moore et al., 2002). Regardless whether or not overt GABAergic cellular death occurs, it is clear that loss of spinal inhibitory tone and consequent abnormal hyperexcitability contribute to the maintenance of neuropathic pain, and is a promising target for intervention.

Previously, our group reported selective loss of endogenous GABA-IR cells following excitotoxic QUIS lesion in rats (Lee et al., 2008), similar to the previously mentioned animal pain/nociceptive models that reported loss of GABAergic cells. It is not clear whether the decrease of GABA-labeled cells is due to apoptosis, down-regulation of GAD genes, or another mechanism. Spinal contusion injury-induced secretion of excitatory amino acids and subsequent activation of metabotropic glutamate receptors (mGluRs) has been shown to increase the GABA concentration in the spinal cord by 160% (4.8 μM) within half an hour of injury (Mills et al., 2001). Blocking mGluR1/5 with antagonist agents (10 nM AIDA, LY 367385 + MPEP) can prevent GABA release. It is possible that excessive mGluR1/5 activity after SCI

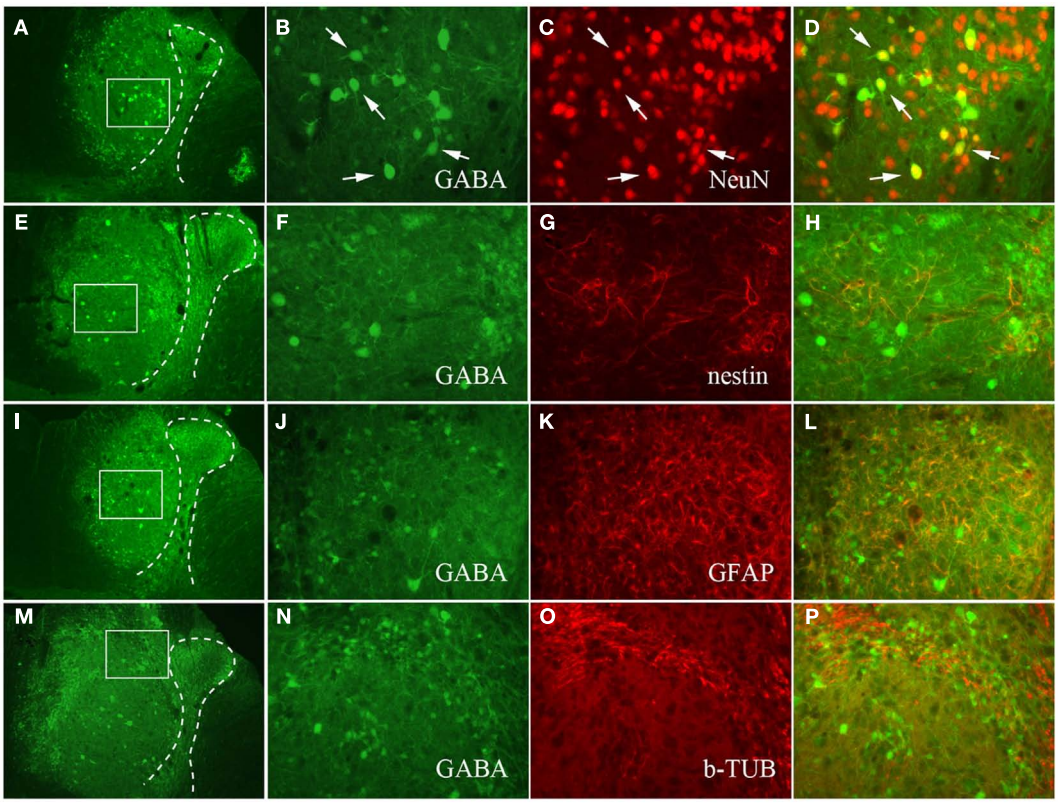

FIGURE 8 | Photomicrographs of QUIS spinal cord transplanted with GABA-IR NPCs. The NPCs were double labeled with NeuN **(A–D)**, nestin **(E–H)**, GFAP **(I–L)**, and β-III-tubulin **(M–P)**. Medium **(A,E,I,M)** and high magnification **(B,F,J,N)** of QUIS spinal cord stained for GABA-IR NPCs. The dotted lines represent the spinal gray matter, and white boxes represent the ROI in panels **(B,F,J,N)**. Majority of GABA-IR NPCs double labeled with NeuN-IR **(B–D)**. However, the GABA-IR NPCs did not colocalize with nestin **(F–H)**, GFAP **(J–L)**, or with β-III-tubulin **(N–P)**.

Table 1 | Stereological quantitation of predifferentiated GABAergic precursor cells following intraspinal transplantation of QUIS animals.

ID	GABA only (single)	GABA + NeuN (double)	GABA total	% of GABA only	% of GABA + NeuN	Total counting area (μm^2)	Total volume (mm^3)
Q12	606	401	1007	60	40	4350	0.74
Q16	952	422	1374	69	31	3600	0.61
Q24	314	137	451	70	30	3600	0.61
Q26	536	204	740	72	28	4350	0.74

could contribute to depletion of cellular GABA stores, indicated by decreased GABA immunoreactivity. We also provide evidence that glycine is reduced similar to GABA in the injured dorsal horn. Since GABA and glycine are frequently co-released at synapses, in part due to the shared vesicular transporter VIAAT (Wojcik et al., 2006; Juge et al., 2009), excessive mGluR1/5 activity could contribute to depletion of glycine stores as well.

The VIAAT loads GABA and glycine into presynaptic vesicles at inhibitory synapses (Dumoulin et al., 1999). In the current experiments, we found that VIAAT-IR was decreased in the QUIS-lesioned dorsal horn. Furthermore, colocalization of GABA, VIAAT, and the synaptic protein synaptobrevin was substantially decreased in the injured dorsal horn. An injury-induced decrease in VIAAT expression could lead to an inability to load available GABA into presynaptic vesicles, which could be reflected by increased free GABA-IR in the injured dorsal horn. Another possible mechanism is that QUIS-induced cell death in the dorsal horn deprives inhibitory neurons of their targets, causing axonal retraction, and decreased axonal trafficking of inhibitory vesicles. Alone or in combination, these potential causes of decreased inhibitory synaptic communication could operate in concert with previously described mechanisms of spinal cord hyperexcitability that are associated with injury-induced chronic pain.

Embryonic stem and precursor cells can be grown in culture and, depending on growth factors, can be induced to differentiate into many different cell types. In this study, we explored ways to promote and increase the number of cultured cortical precursor cells to differentiate *in vitro* into GABAergic phenotype by varying

Table 2 | Non-stimulation-induced released GABA concentration in the N2 growth media over time for P0 and P1 embryonic precursor cells (μM/20 μl).

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Passage 0	0.3955	0.3187	0.3844	0.5609	N/A	N/A	N/A
Passage 1	0.0941	0.0993	0.1540	0.2088	0.6500	0.9475	1.0673

the concentration level of FGF-2 present in the growth media. Cells grown under standard concentration of FGF-2 then switched to low concentration (0.1 ng/ml) for several hours facilitated transformation to GABAergic phenotype. The molecular biochemistry underlying such transformation remains unclear. It is important to note that not all phenotypic GABA cells colocalized with the neuronal marker NeuN. The vast majority of GABAergic cells colocalized with β-III-tubulin and MAP-2 suggesting incomplete neuronal differentiation 24–36 h after FGF-2 removal.

In addition to GABA, the vast majority of NPCs in these cultures were nestin-IR, indicating they were undifferentiated progenitor cells. All GABA-positive cells were double labeled with nestin. But no GABA cells colocalized with GFAP-IR cells indicating that most of our GABA cells were still in immature state and GABA was not present in the astrocytes. The majority of GABA cells were found as a cluster inside the neurospheres, suggesting they are clonally derived. Functional biochemical properties of our GABAergic neurospheres were demonstrated by high-pressure liquid chromatography (HPLC) where the secreted/released concentration of GABA in the culture media increased 10-fold to 1 μM/20 μl culture media over a 7-day period *in vitro* (**Table 2**). We were not able to obtain *in vitro* GABA HPLC data for P0 cells beyond 4 days due to decreased representation of GABA neurons in the culture with time (data not shown). Only the P0 precursor cells proliferate in the N2 culture media while the P0 GABA cells do not. Therefore in the non-proliferating P0 GABA cells that are already differentiated cells become a negligible fraction by day 4. In contrast the large numbers of P1 precursors differentiate into GABAergic cells persist longer in the culture in the absence of any cell proliferation. These data demonstrate that brief exposure of embryonic cortical precursor cells to low concentration of FGF-2 stimulates GABAergic cell differentiation, but that the differentiation status remains largely incomplete.

Overgrooming behavior observed in QUIS rats is thought to have a nociceptive component in its expression. Previously, subdural transplantation of chromaffin cells in the spinal cord of QUIS animals significantly decreased their overgrooming area directly demonstrating analgesic compound secreted from chromaffin cells, mainly norepinephrine, can reduce the overgrooming behavior (Brewer and Yezierski, 1998). In the present experiment, intraspinal transplantation of predifferentiated embryonic GABAergic cells also significantly decreased the overgrooming area at all-time points studied compared to fibroblast-transplanted control QUIS rats. The main factor for such an effect was most likely the absence of GABA cells since QUIS animals with very few surviving GABA cells also showed increased overgrooming. We know from past reports that only about 5–10% of transplants survive following incorporation to the host CNS (Chow et al., 2000; Cao et al., 2002; Hendricks et al., 2006; Lepore et al., 2006) but this number is sufficient for producing a significant effect on host behavior. Our recent paper (Jergova et al., 2012) shows that intraspinal injection of GABAergic progenitors partially restore inhibitory tone in the spinal cord in CCI model. Behavioral and electrophysiological experiments showed that the analgesic effect of grafted cell is positively or negatively modulated by GABA reuptake inhibitor or GABA receptor antagonist respectively.

In present study, quantitation of surviving GABAergic cells from select tissues showed that over 67% of transplanted cells remained as incompletely differentiated GABAergic cells. However, about 40% of predifferentiated cells displayed neuronal phenotypes positive for NeuN immunoreactivity.

Several reports exist that describe transplantations of predifferentiated GABAergic and non-GABAergic embryonic precursor cells to alleviate peripheral and central neuropathic pain (Eaton et al., 1999b; Stubley et al., 2001; Hendricks et al., 2006; Mukhida et al., 2007; Jergova et al., 2012). However, the present study is first to describe the effect of predifferentiated embryonic GABAergic cells transplanted in SCI animals to reverse dysesthetic/nociceptive behaviors.

In summary, the current study suggests transplantation of predifferentiated embryonic GABAergic cells in QUIS-lesioned rats can greatly reduce the overgrooming lesion size. Such reduction may be mediated with the release of GABA from the transplants. GABA replacement therapy through cell transplantation could be used to offset losses of inhibitory signaling molecules in injured spinal cord gray matter. The reversal of overgrooming behavior was associated with the density of surviving GABA-IR transplants in the injured host spinal cord.

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Accommodation of the spinal cat to a tripping perturbation

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Adult cats with a complete spinal cord transection at T12–T13 can relearn over a period of days-to-weeks how to generate full weight-bearing stepping on a treadmill or standing ability if trained specifically for that task. In the present study, we assessed short-term (milliseconds to minutes) adaptations by repetitively imposing a mechanical perturbation on the hindlimb of chronic spinal cats by placing a rod in the path of the leg during the swing phase to trigger a tripping response. The kinematics and EMG were recorded during control (10 steps), trip (1–60 steps with various patterns), and then release (without any tripping stimulus, 10–20 steps) sequences. Our data show that the muscle activation patterns and kinematics of the hindlimb in the step cycle immediately following the initial trip (mechanosensory stimulation of the dorsal surface of the paw) was modified in a way that increased the probability of avoiding the obstacle in the subsequent step. This indicates that the spinal sensorimotor circuitry reprogrammed the trajectory of the swing following a perturbation prior to the initiation of the swing phase of the subsequent step, in effect "attempting" to avoid the re-occurrence of the perturbation. The average height of the release steps was elevated compared to control regardless of the pattern and the length of the trip sequences. In addition, the average impact force on the tripping rod tended to be lower with repeated exposure to the tripping stimulus. EMG recordings suggest that the semitendinosus, a primary knee flexor, was a major contributor to the adaptive tripping response. These results demonstrate that the lumbosacral locomotor circuitry can modulate the activation patterns of the hindlimb motor pools within the time frame of single step in a manner that tends to minimize repeated perturbations. Furthermore, these adaptations remained evident for a number of steps after removal of the mechanosensory stimulation.

Keywords: EMG, spinal locomotor circuits, mechanosensory stimulation, tripping response, spinal cord transection

INTRODUCTION

While experience-dependent plasticity clearly occurs in supraspinal circuits controlling motor tasks, we now know that experience-dependent plasticity also occurs in spinal circuits that control posture and locomotion. For example, Di Giorgio and Menzio (1946) reported that the asymmetric positions of the lower limbs that are acquired after being exposed to an asymmetric vestibular input for about 30 min persists after a complete spinal cord transection. Chopin and Buerger (1976) demonstrated that mid-thoracic, complete spinal rats learned to avoid shock by keeping the paw elevated above a threshold level within 5–20 min of being exposed to a conditioning–learning paradigm. Complete spinal rats also can adapt the hindlimb kinematics to a perturbing force field so that a more normal stepping pattern is achieved within a time frame of seconds to minutes (de Leon et al., 1999; Timoszyk et al., 2002; Heng and de Leon, 2007). Motor learning that occurs over a period of days and weeks has been demonstrated in numerous experiments in which complete spinal animals regain and improve stepping or standing performance with daily practice of these tasks (Barbeau and Rossignol, 1987; Lovely et al., 1990; de Leon et al., 1998a,b). Given that learning-related phenomena can occur within a short time frame (within seconds) presumably within supraspinal circuits (Choi and Bastian, 2007), we hypothesized that a similar learning phenomenon could occur in the spinal cord within a similar time frame. In the present experiments, we demonstrate learning-related responses that occur within seconds of a tripping stimulus applied during the swing phase of a step cycle in the cat after a complete, low-thoracic spinal cord transection. We also found that the spinal circuitry is capable of perceiving a mechanical perturbation of the step cycle in a way that enhances the probability of sustaining successful locomotion, further demonstrating the wide repertoire of sensorimotor processing within the spinal locomotor circuitry.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Four adult female cats (2–3 years of age) were used for these studies, with most of the data shown derived from three cats. Prior to any surgery, the cats were acclimated to the treadmill and the testing environment for several sessions, i.e., the cats were made

to walk bipedally on a treadmill at speeds ranging from 0.2 to 1.0 m/s while in a harness supporting the upper body (de Leon et al., 1998a). Intramuscular EMG electrodes were implanted in selected hindlimb muscles and the spinal cord was completely transected at a low-thoracic level. Starting 4 days after surgery, the cats were step trained for 30 min/day, 5 days/week as described by de Leon et al. (1998a). After 6 months of training, the cats were subjected to tripping experiments periodically as described below. All procedures were performed in accordance with the American Physiological Society Animal Care Guidelines and were approved by the Animal Use Committee at the University of California, Los Angeles.

INTRAMUSCULAR EMG IMPLANTS

All surgical procedures were performed under aseptic conditions. The cats were administered sodium pentobarbital (35 mg/kg, i.p.) following pre-treatment with atropine sulfate (0.05 mg/kg) and acepromazine maleate (0.2 mg/kg) administered intramuscularly. Supplemental doses of anesthesia were administered as needed to maintain a surgical level. All incisions were closed in layers, i.e., 4.0 chromic gut and 4-0 Ethilon suture were used to suture the fascia and skin, respectively.

Prior to spinal cord transection, intramuscular recording electrodes were implanted in selected hindlimb muscles using procedures described in detail previously (Pierotti et al., 1989; de Leon et al., 1994). Briefly, one amphenol connector having nine Teflon-insulated stainless steel wires (AS 632, Cooner Wire, Chatsworth, CA, USA) was secured to the skull with screws and dental cement. The wires were passed subcutaneously from the connector to the hindlimb. One wire with ∼2 cm of the Teflon removed at the distal end was embedded in the back region and served as a common ground. Two wires were implanted in the following muscles unilaterally: deep mid-region of the distal compartment of the semitendinosus (St), mid-belly of the soleus (Sol), deep portion of the mid-belly of the tibialis anterior (TA), and medial deep portion of the mid-belly of the medial gastrocnemius (MG). The wires were passed through the muscle using a 23-gage hypodermic needle and ∼0.5–1.0 mm of insulation was removed from each wire to form the recording electrodes. After stimulation of the muscle through the connector to ensure the proper placement of the electrodes, each lead was secured with a suture at its entry and exit from the muscle. The proper placement of all electrodes also was verified post-mortem.

SPINAL CORD TRANSECTION

The spinal cord of each cat was transected completely at ∼T12 as described in detail previously (Roy et al., 1992). Briefly, a skin incision was made on the back to expose the vertebral processes between ∼T10 and L1. A partial laminectomy was performed at the T12–T13 vertebral level to expose the spinal cord. Fine scissors and forceps were used to cut the dura longitudinally and to perform a complete transection beginning on the dorsal surface of the cord while preserving the lateral and ventral dura. After the transection, the ends of the cord retracted leaving a clear space. The cut ends of the cord then were lifted gently using fine forceps to assure that no residual spinal cord matter remained between the two cut ends of the cord. Gel foam was packed between the rostral and caudal segments of the cord as an anticoagulant. This procedure allowed for the preservation of the large ventral artery of the spinal cord.

ANIMAL CARE PROCEDURES

Post-spinal cord transection management of the spinal cats has been detailed elsewhere (Roy et al., 1992). The cats were housed together in spacious cage with the cage floors covered with shredded newspaper. The bladders and colons of the cats were expressed twice daily for the duration of the experiment. Dry kibble and water were given *ad libitum* and wet food was given once daily.

ANIMAL TESTING

Prior to the surgeries, the animals were trained to step on the treadmill bipedally. Beginning 7 days post-surgery the animals were step trained for 30 min/day, 5 days/week for 6 months. All tripping experiments were performed over a period of ∼3 months following the 6-month training period. During this period stepping performance remained stable. During step training, the cats were placed in a thoracic vest that wrapped around the chest and shoulder girdle to provide stability, but allowed the hindlimbs to bear weight and to move freely on the treadmill belt. During the testing while the cats were stepping on the treadmill, an in-house designed tripping stainless steel rod mounted with a strain gage was placed using a sliding carriage in the normal trajectory of the limb such that it made contact with the dorsal surface of the paw during the swing phase of the step. The tripping rod was mounted onto a sliding chassis that utilized ball bearings to minimize friction and vibration while at the same time maintaining an exact position once in position. This design allowed for a smooth entrance and exit of the rod from the plane of motion of the hindlimb. There was no noise associated with the rod sliding in and out. The chassis was locked into a housing unit that had five different vertical slots allowing the chassis to be moved and locked into five different vertical positions. The results reported below are from the tests when the rod was placed at 1.1 and 3.7 cm above the treadmill. The base of the housing unit contained slots that allowed horizontal movement of the housing unit itself before it was locked down into position on the treadmill. Thus there was freedom to move the rod horizontally and vertically in the plane of motion before it was locked into place to adjust the system to induce the perturbation at a consistent point during the swing phase of the step cycle. Once the tripping device was set, the rod entered the plane in the same coordinates every time. The strain gage on the rod was calibrated using weights between 10 and 200 g so that the magnitude of the impact force of the paw onto the rod could be quantified. Each sequence consisted of a series of steps that were either obstructed (trip sequences) or not obstructed (control or release sequences) by the tripping rod during the swing phase of the step cycle. Control steps were either at the beginning of a sequence or at least 50 steps after a series of obstructed steps. Release steps consisted of steps not obstructed that followed immediately after a series of obstructed steps. A typical sequence consisted of a series of control steps (10 steps) followed by an alternating series of trip (range from 1 to 60) and release steps (range from 10 to 20 steps).

DATA RECORDING

A flexible insulated cable was used to connect the amphenol connector to a recorder. Raw EMG signals were conditioned through a wide band AC differential amplifier at a gain of 1000 and recorded on an analog tape recorder (TEAC Model XR-510, TEAC Corporation, Montebello, CA, USA) with a system bandwidth of D.C. to 2.5 kHz. EMG signals were calibrated at the beginning of each recording session using a 1 or 2 mV sine wave (500 Hz). To record kinematics data, a camera (Panasonic System Camera, WV D5100, Panasonic, Cypress, CA, USA) was oriented perpendicular to the plane of motion. The video was recorded at 30 frames (60 fields) per seconds on VHS tape simultaneously with a signal from a time code generator. The time code generator was used to synchronize the EMG and force recordings. Strain gage force and muscle EMG signals were calibrated, amplified, recorded, and then stored on FM tape using a TEAC recorder. The hindlimbs were shaved and small, round pieces of light-reflecting tape were placed on bony landmarks, i.e., the iliac crest, greater trochanter, head of the tibia, lateral malleolus, base of the calcaneus, head of the fifth metatarsal (MTP), and the first phalanx of the fifth digit, to be used to determine limb segment movement (**Figure 1A**).

DATA ANALYSIS

The x and y coordinates of all the bony landmarks were digitized using SIMI Motion and tracked using 2-D tracking within the program. The location of the force bar and the level of the treadmill (zero line) were digitized. The digitized points were exported into text files and then graphed for trajectories and stick diagrams in Excel. Step height was determined by measuring the largest distance between the paw (MTP) and the treadmill belt during the swing phase of the step cycle. Three markers were used to determine changes at each joint angle. Hip angle was based on the iliac crest, greater trochanter, and head of the tibia. Knee angle was based on the greater trochanter, head of the tibia, and lateral malleolus. Ankle angle was based on the head of the tibia, lateral malleolus, and fifth metatarsal.

Kinematics analyses were performed on the same stepping sequences from which EMG activity was recorded and analyzed. Force data were calibrated and expressed in grams. EMG data were sampled at 2 kHz, filtered with a 20–500 Hz band-pass filter, and rectified. Briefly, the onset and offset of each EMG burst were marked to calculate burst duration. Mean EMG per burst was calculated by averaging the EMG amplitude within a burst. Integrated EMG (IEMG) was calculated as the product of the mean burst EMG amplitude and duration. To examine the modulation of EMG amplitudes between two muscles, joint probability distribution plots (**Figure 10**) were generated. These amplitudes were derived from a running five-point average of the mean amplitude of multiple normalized step cycles.

STATISTICAL ANALYSES

Statistically significant differences were determined using a one-way analysis of variance (ANOVA) using Monte Carlo simulations. The mean data from all the groups were pooled into a single data set before randomly sampling the data with replacement. Means from each group were used to estimate the F value. This process was repeated 10,000 times. The original F value was compared to the simulated F value to determine any overlap in the confidence bands. When the original F value was outside the 95% confidence interval, the null hypothesis was rejected and there was a significant difference across groups at the $P < 0.05$ level (Efron and Tibshirani, 1991).

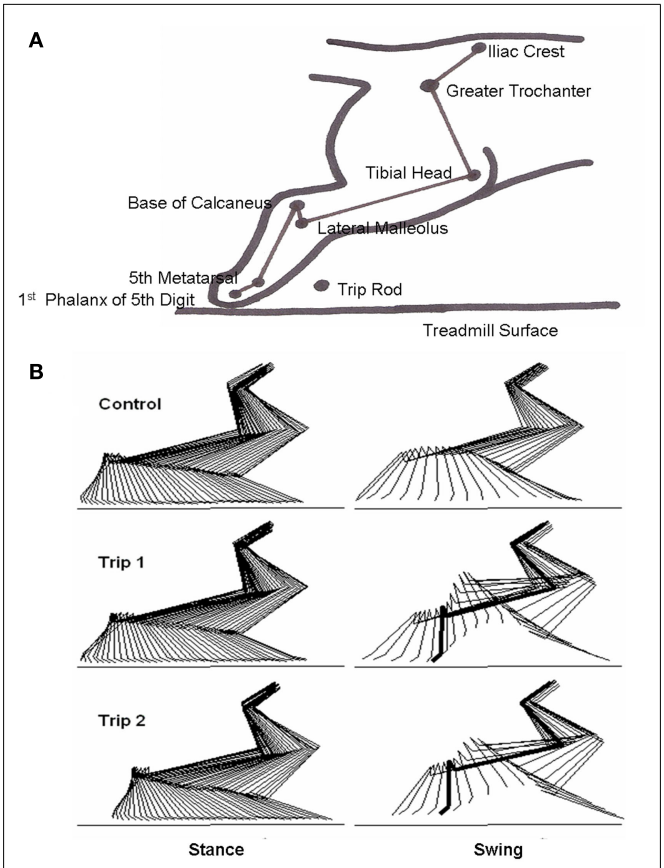

FIGURE 1 | (A) Schematic showing the bony landmarks in the cat hindlimb used for kinematics analyses, the position of the trip rod, and the treadmill surface. **(B)** Representative stick diagrams (17 ms between sticks) showing the stance and swing phases for a pre-trip (Control) step and the first (Trip 1) and second (Trip 2) trip steps. The horizontal lines denote the surface of the treadmill. The thick lines in the stick diagrams during Trip 1 and Trip 2 indicate the frame when the foot touches the trip rod during the swing phase.

RESULTS

ADAPTATION IN JOINT KINEMATICS DURING THE TRIP RESPONSE

Stick diagrams of the stance and swing phases for a control, first trip step (Trip 1), and second trip step (Trip 2) of a five-trip step sequence, are shown in **Figure 1B**. For the control step, the hindlimb was allowed to proceed with an unperturbed swing phase. For Trip 1, the paw hit the trip rod, lifted over the rod, and extended forward further than the previous control step. For Trip 2 that immediately followed Trip 1, the trajectory of the paw changed prior to contact with the tripping rod, i.e., the paw changed its trajectory before it hit the trip rod. These observations were observed consistently during a number of sequences for the three cats analyzed.

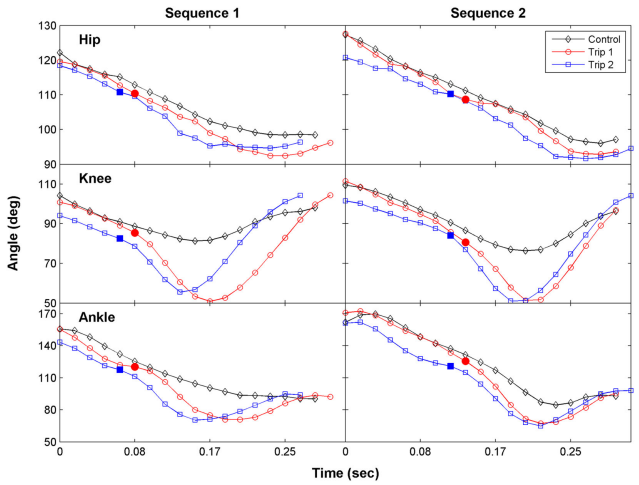

FIGURE 2 | The time course of the changes in the hip, knee, and ankle joint angles during the swing phase for a control, Trip 1, and Trip 2 step are shown for two tripping sequences in a representative cat. The solid symbols indicate the time when the foot touches the trip rod. The kinematics of Trip 2 reflect an adaptive response relative to Trip 1 at all three joints in both sequences. The stick figures in **Figure 1B** were generated from the data shown for the first sequence.

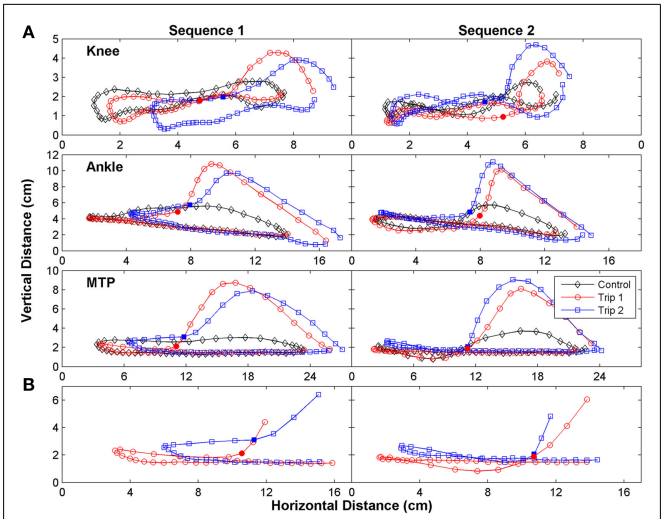

FIGURE 3 | The trajectory of the limb was changed immediately after the initial trip. (A) The trajectory of the knee (tibial head), ankle (lateral malleolus), and foot (metatarsophalangeal, MTP) for the same steps in **Figure 2** are shown. **(B)** An expanded plot of the trajectory of the MTP during late stance and early swing of the Trip 1 and Trip 2 steps are depicted. The solid symbols in **(A,B)** indicate when the foot touches the trip rod. Note the earlier initiation of the swing phase in Trip 2 than Trip 1 for both sequences.

The hip, knee, and ankle joint angles for Trip 1 during swing were similar to the control step before contacting the trip rod, but each of these angles were smaller after contact (**Figure 2**, sequence 1). For Trip 2, the joint angles were smaller prior to the paw contacting the rod, indicating that the cat began to lift its hindlimb before contacting the rod. The magnitude of the change during the trip steps was greatest at the knee joint. Thus the increase in step height during the trip compared to the control steps was primarily attributable to an increase in knee flexion. Similar observations were made in a second sequence of the tripping experiment within that same session (**Figure 2**, sequence 2).

The trajectories of the knee, ankle, and foot (MTP joint) throughout the step cycle for the same steps shown in **Figure 2** are shown in **Figure 3**. There was an earlier initiation of the swing phase in Trip 2 than Trip 1 in both sequences. These kinematics changes are shown for three tripping sequences in all three cats studied (**Figure 4**). The mean time when the foot started to lift prior to contact with the trip rod (Pre-contact time) was 150–350 ms shorter for Trip 2 than Trip 1 in all three cats (**Figure 4A**). In addition, the distance from toe off to contact was shorter for Trip 2 than Trip 1 in all sequences in all three cats (**Figure 4B**). These data demonstrate a clear modification in the kinematics of the swing phase to allow the paw to be at a higher position when it reaches the point of the previous perturbation, thus increasing the likelihood for a successful completion of the swing phase.

INCREASES IN MEAN STEP HEIGHT DURING THE RELEASE STEPS AFTER A SINGLE, SHORT, OR LONG BOUT OF TRIP STEPS

Sequences with a varying number of trip steps resulted in an increase in the mean step height during the release steps compared to the control steps. For example, the mean step heights for the control (10 steps), long trip (60 steps), and release (10 steps) steps during a single sequence were 2.8, 5.5, and 4.8 cm, respectively (**Figure 5A**). In another cat, the mean step heights for the control (9 steps), shorter trip series (10 steps), and release (9 steps) steps during a single sequence were 2.1, 4.9, and 2.8 cm, respectively (**Figure 5B**). In fact, the mean step height was significantly higher during the release steps compared to the control steps after a single trip sequence (ranging from 10 to 60 steps) in all four cats studied. In another example, a trip sequence of only two steps resulted in an increase in step height from 1.5 cm for the control steps (10 steps) to 2.5 cm during the release steps (9 steps; **Figure 5C**). This elevated step height for the release steps occurred in spite of the fact that the hindlimb collapsed in the first release step (this step was excluded when calculating step height). The subsequent series of 10 trip steps had no further effect on mean step height during the ensuing release steps, but the mean step height still was elevated compared to the control steps.

To further determine the temporal features of the tripping stimulus, a perturbation was imposed on every third step (**Figure 5D**). The sequence involved 9 control steps, a total of 10 trip steps with 2 release steps in between each perturbation, and a final 9 release steps. In every case the height of the two release steps between each trip step was elevated above control. In addition, the mean step height for the final nine release steps was higher than for the control steps, i.e., 3.2 vs. 2.5 cm. Occasionally the cats adapted the height of the swing phase sufficiently to step over the rod without touching it. For example, as shown in **Figure 6** the cat's paw cleared the bar at steps 14, 16, 30, and 35 in a series of 52 tripped steps. All of these data demonstrate that the spinal cats adjusted the step trajectory as early as in the second trip step to lift the leg higher.

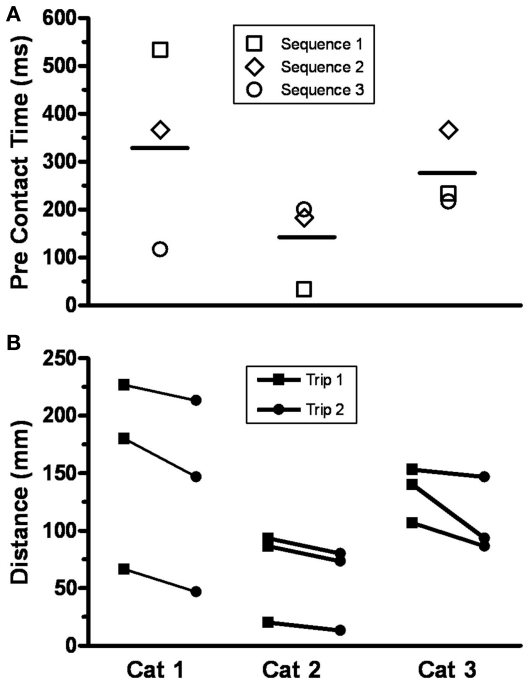

FIGURE 4 | (A) The pre-contact time, defined as the time when the foot started to lift prior to contact with the trip rod, for Trip 2 compared to Trip 1 for three sequences is shown for three cats. The horizontal bar is the mean for three sequences for each cat (range, 150–350 ms). A shorter pre-contact time for Trip 2 than Trip 1 was present in all nine cases. **(B)** The distance from toe off to contact with the trip rod for Trip 1 and Trip 2 for three sequences is shown for the same three cats. All distances were shorter for Trip 2 than Trip 1.

IMPACT FORCE ON THE TRIP ROD DECREASES WITH CONSECUTIVE SEQUENCES

In general, the impact force decreased with repeated sequences. For example, during the performance of three sequences the mean impact force progressively decreased from 554 g (range, 282–644 g) during the first sequence, to 319 g (range, 202–512 g) for the second sequence, to 247 g (range, 173–302 g) for the third sequence (**Figure 7A**). Note that the average step height during the release steps after each series of trip steps was higher than during the control steps. The ratio of impact force:step height (**Figure 7B**) showed the same pattern as for impact force (**Figure 7A**). This decrease in the ratio across trip sequences was, at least in part, due to a lower horizontal velocity during the swing prior to contact, i.e., 0.75, 0.63, and 0.57 m/s for in first, second, and third tripping sequence. A similar pattern was observed in three cats. Combined, these data indicate that the spinal sensorimotor circuitry made kinematics adjustments to minimize the impact force as early as the second trip step.

MODULATION IN THE EMG PATTERNS DURING TRIP-RELEASE SEQUENCES

EMG analysis was performed on four hindlimb muscles (St, TA, Sol, and MG) of the tripped hindlimb in two cats and on two muscles (St and TA) in a third cat. The mean EMG burst durations,

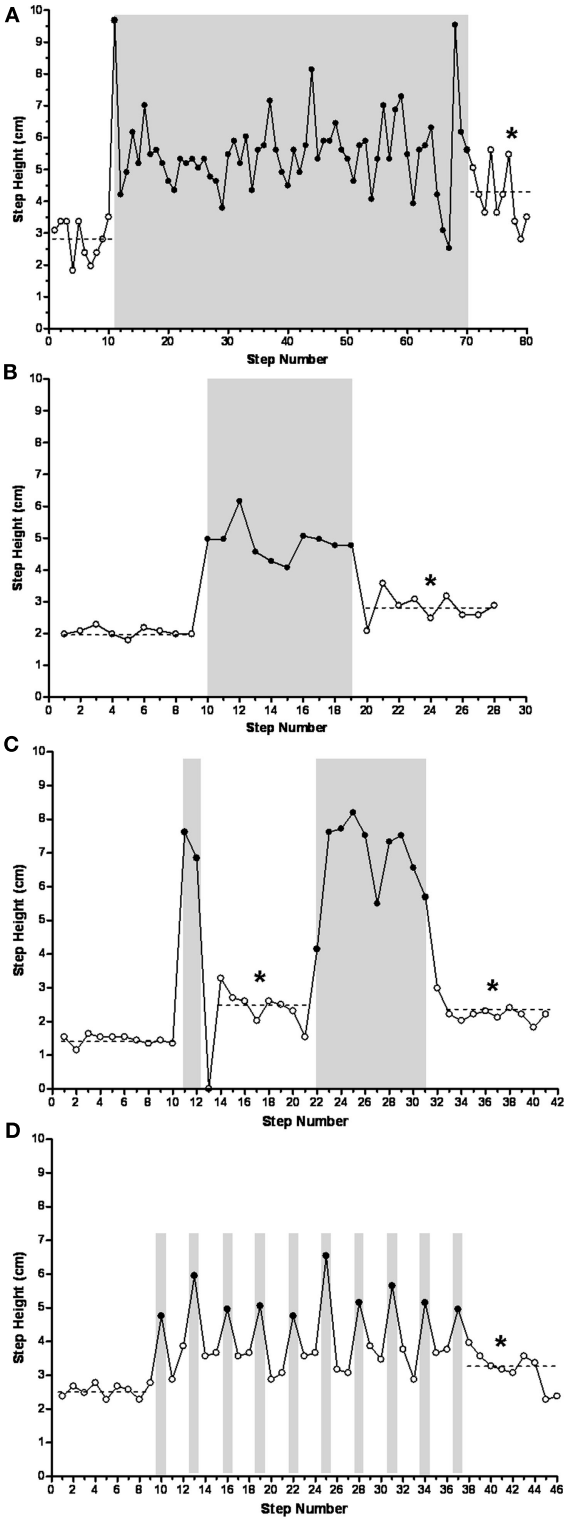

FIGURE 5 | Mean step heights during the release steps after long [(A) 60 trip steps], short [(B) 10 trip steps and (C) 2 and 10 trip steps], and single [(D) trip every third step] trip series were higher than during the control steps ($P \leq 0.005$), reflecting a learning-related phenomenon. Solid symbols, trip steps; open symbols, control steps; shaded areas, trip series. *Significantly different from control.

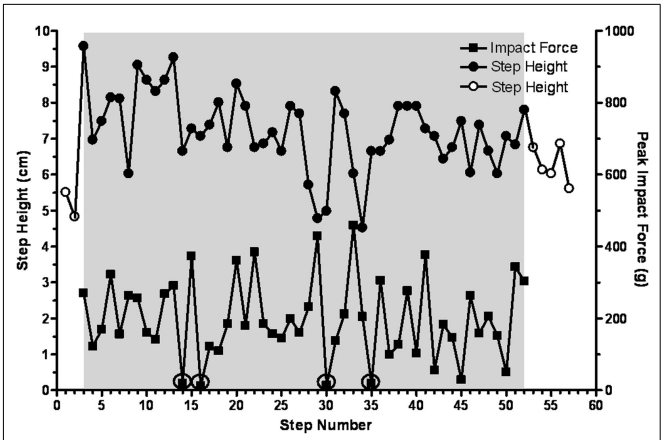

FIGURE 6 | Step height and peak impact force on the trip rod are shown for 52 consecutive trip steps for a representative cat. Four steps during which the limb avoided and cleared the trip rod are circled. Solid symbols, trip steps; open symbols, control steps; shaded area, trip series.

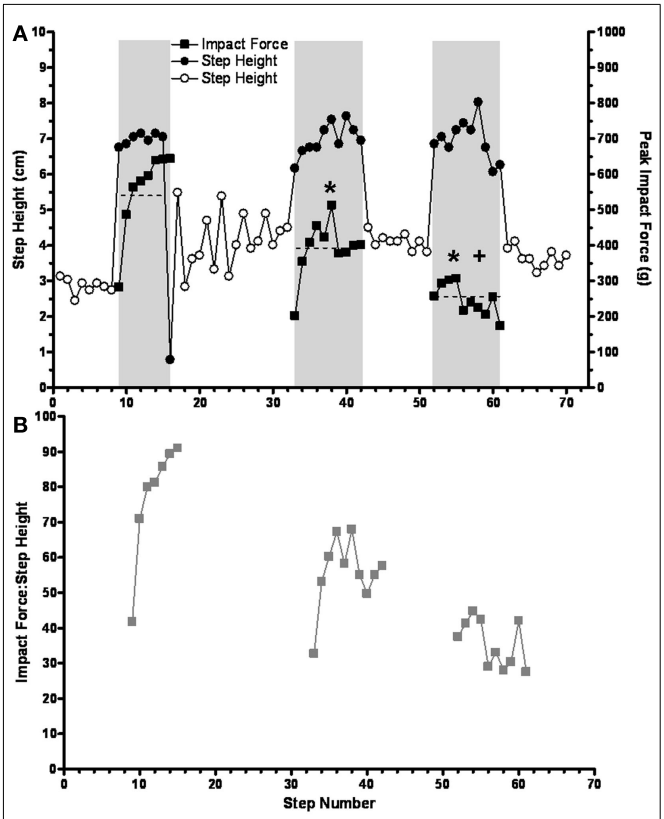

FIGURE 7 | (A) The mean impact force on the trip rod (horizontal dashed lines) is shown to progressively decrease during three successive short trip-release sequences. Solid symbols, trip steps; open symbols, control steps; shaded areas, trip series. **(B)** The ratios of peak impact force and step height are shown for the same trip sequences in **(A)**. Note that the results in both **(A)** and **(B)** reflect a learning-related phenomenon that occurs over a time frame of seconds. * and $^{+}$Significantly different from the first and second trip sequence, respectively.

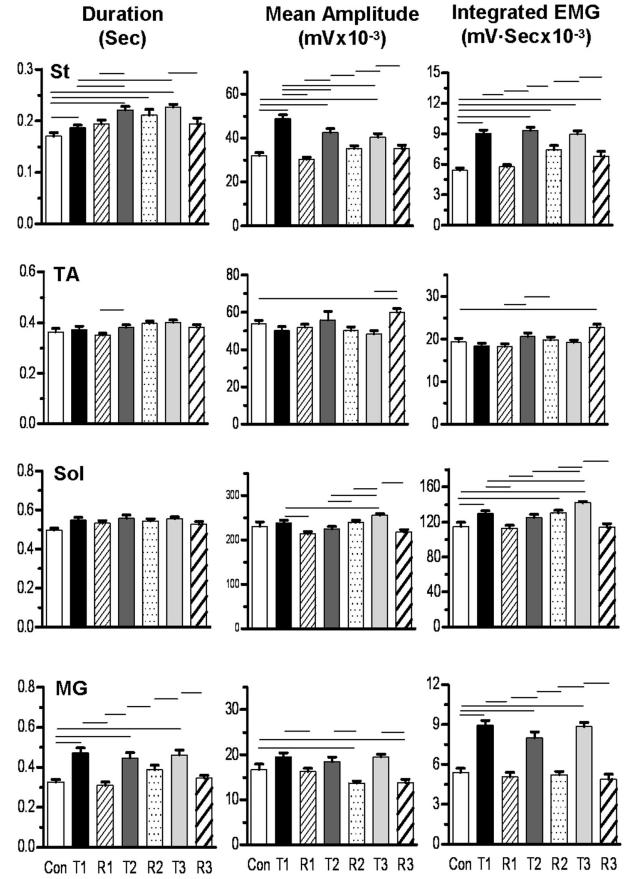

FIGURE 8 | Bar graphs showing the mean (SEM) burst duration, amplitude, and integrated EMG for the semitendinosus (St), tibialis anterior (TA), soleus (Sol), and medial gastrocnemius (MG) muscles for three trip-release sequences for a representative cat. Con, control sequence, 10 steps; T1, first trip sequence, 20 steps; R1, first release sequence, 20 steps; T2, second trip sequence, 20 steps; R2, second release sequence, 20 steps; T3, third trip sequence, 20 steps; R3, third release sequence, 20 steps. The horizontal lines above the bars denote significant differences between conditions at $P < 0.05$. Note that the St showed the highest activation levels compare to control during the trip steps and that the MG showed a qualitatively similar pattern as the St.

EMG burst amplitudes, and IEMGs for a control (10 steps), three trip (20 steps per series), and three release (20 steps per series) sequences for one cat are shown in **Figure 8**. In general, the mean burst durations for the St and MG, but not the Sol and TA, were longer for the trip compared to control steps. The mean EMG burst durations for the release steps generally were shorter than for the trip steps within a sequence for the MG, with no apparent trend for all other muscles. Mean EMG amplitudes and IEMG in the St, MG, and Sol were generally lower for the release steps compared to the trip steps. The other two cats in which EMG was determined showed similar activation patterns.

To look at the changes in the timing of the EMG, rectified raw EMG for the control, trip 1, release 1, and trip 2 sequences shown in **Figure 8** are plotted in **Figure 9**. For the St the amplitude at the end of the EMG burst was markedly higher ($P < 0.05$) for the

first trip series than the control series (**Figure 9A**). For the second trip sequence (Trip 2), the pattern of the St EMG was similar to control (**Figure 9B**). For the first trip sequence, the TA appears to be activated earlier and to have an EMG burst amplitude similar to control. The EMG amplitudes of the extensor muscles (Sol and MG) were higher ($P < 0.05$) in the beginning of the stance phase for the trip compared to the control and release sequences (see * in **Figure 9A**). The levels of activation of the TA, Sol, and MG vs. the St (the muscle showing the most consistent changes in response to the tripping stimulus) for a sequence of 10 control, 20 trip, and 20 release steps are shown in **Figure 10**. Note that the plots for the control and release steps are similar (**Figures 10A,C**), whereas the plots for the trip steps show an elevated level of activation for the St and a higher level of co-activation for the St with the TA (**Figure 10B**). The temporal sequence of the modulation of the St EMG amplitude immediately after impact on the rod is shown by the line connecting consecutive data points (green symbols) in **Figure 10B**. Note that the elevated St activity and the co-activation between the TA and St during the tripping steps had no impact on the clear reciprocal relationship between the St with either the Sol or MG. Similar observations were made in two other cats.

DISCUSSION

The "stumbling response" of the hindlimbs in complete, low-thoracic spinal cats was characterized more than three decades ago (Forssberg, 1979). These experiments demonstrated that a single instantaneous perturbation of a complex motor task, i.e., stepping, would result in a successful and instantaneously newly adopted neural control strategy, at least for the duration of the ipsilateral swing and contralateral stance phase of the step cycle for the whole hindquarters to sustain continuous stepping. If the same mechanical or electrical stimulus was applied during the stance phase of the step cycle of the spinal cat, there was an immediate hyperextension of the ipsilateral limb, indicating a "state dependent" feature of spinal "decision making." There, however, has been no systematic examination as to whether there are residual effects manifested in subsequent step cycles in complete spinal animals.

McVea and Pearson (2007) reported an elevated height of the swing phase after 20 or more consecutive perturbations of the swing phase in normal cats. This change in swing trajectory persisted up to 24 h and the magnitude of this effect generally reached a plateau within 120 repetitions of the perturbation. This effect was not clearly evident after decerebration, and the authors concluded that this phenomenon was mediated by supraspinal descending systems. There were, however, some short-term residual effects of repetitive stumbling in the decerebrated cats, but the elevated height of the swing phase was sustained for only about five to seven steps after removal of the perturbation. Given the multiple observations that have shown learning-related phenomena in the performance of a motor task within the lumbosacral spinal circuitry with time courses of the learned tasks ranging from minutes to weeks (Edgerton et al., 2001b; de Leon et al., 2002; Timoszyk et al., 2002; Liu et al., 2005), we examined the persistence of the tripping response in adult, chronic spinal cats that had been trained to step. In these experiments we examined the time frame of seconds over which experience-dependent modulation of the control of stepping can occur in the spinal circuitry without any supraspinal influence.

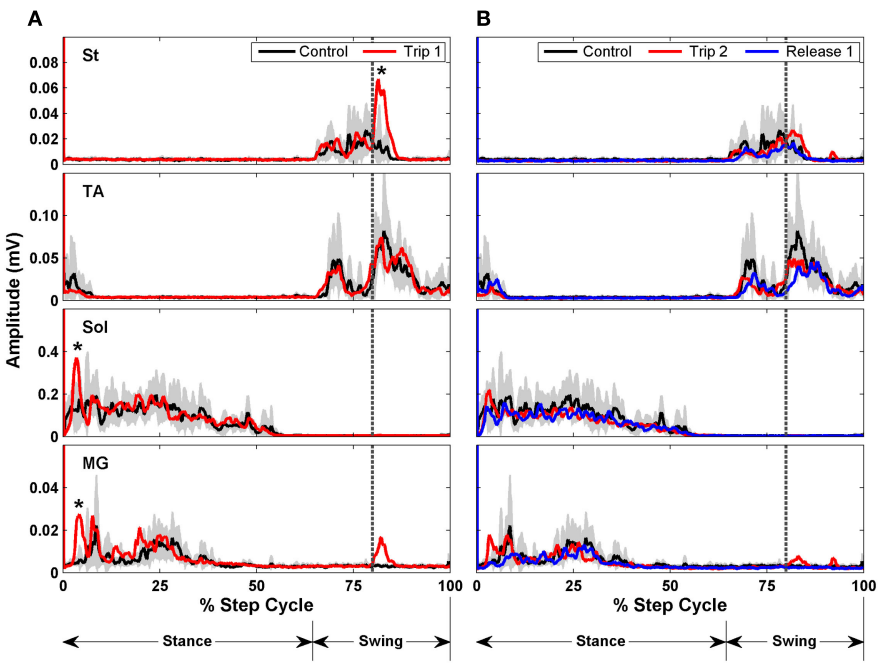

FIGURE 9 | Rectified EMG bursts for the St, TA, Sol, and MG of control (black traces, 10 steps preceding Trip 1), Trip 1 [red traces in (A) 20 steps], Trip 2 [red traces in (B) 20 steps], and release 1 [blue traces in (B), 20 steps] sequences. The gray shading is the SD for the control sequence. Muscle abbreviations, same as in **Figure 8**. *Significantly higher than in the control and release sequences.

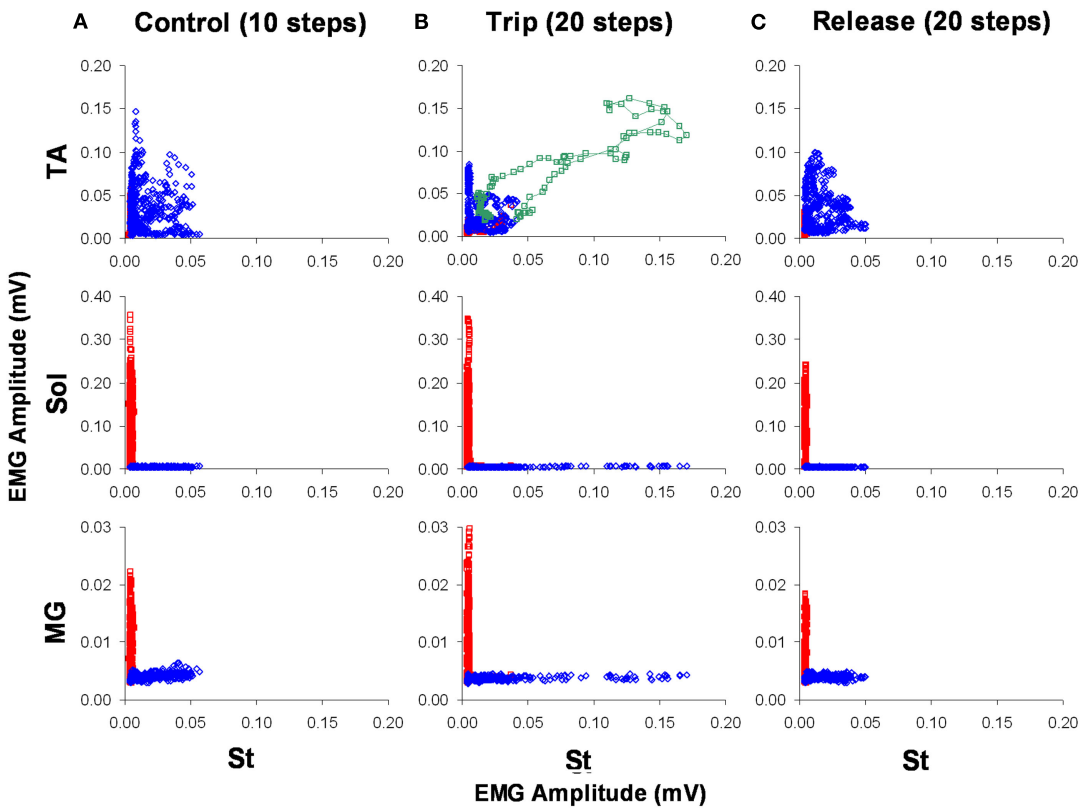

FIGURE 10 | The level of activation of the TA, Sol, and MG vs. the St for a sequence of 10 control (A), 20 trip (B), and 20 release steps (C) is shown. Blue and red represent the swing and stance phases of the step cycle, respectively. The green symbols in **(B)** represent the co-activation between the TA and St immediately after the trip. The data points are a five-point running average of the mean normalized EMG. Note the higher level of activation of the St during the trip compared to the control and release steps.

The present data demonstrate adaptive responses to a perturbation of the kinematics of the hindlimb in complete spinal cats occurring within a single step cycle as well as over as many as 60 continuous step cycles. These adaptive responses enhance the probability of sustaining successful stepping while being challenged with a specific mechanical perturbation by elevating the hindlimbs in a manner that tends to avoid the perturbation. From a more highly integrative and conceptual perspective, these responses reflect "recognition" of a specific complex afferent pattern induced by the stimulus that results in an adjustment to a subsequent encounter within the time frame of a single step cycle, i.e., within the same step cycle the trajectory of the paw is changed in a way that tends to avoid the previous perturbation (**Figures 1–4**). The neuromotor strategy that mediates this ipsilateral hyperflexion during the swing phase after the tripping stimulus seems to be rather consistent for different experimental preparations ranging from intact locomotion to fictive locomotion (Quevedo et al., 2005; McVea and Pearson, 2007). A common feature of this response is an increase in step height and earlier initiation of activation, particularly of motor pools associated with knee flexion. In the present study these were the most predominant and consistent changes observed in response to the tripping stimulus.

In essence the immediate response (within the same step cycle as the initial perturbation) demonstrates a feed-forward control mechanism within the spinal circuitry, i.e., a single instantaneous sensory event that interrupts a step can be recognized as such, and a response is initiated which modifies the limb trajectory not only to complete that swing phase, but also to adjust the neural control needed to avoid or minimize the perturbation in the succeeding step cycle. The initial response in modifying the limb trajectory within a single step cycle would seem to reflect "evolutionary learning" (Edgerton et al., 2001a) in that a similar ability to adjust the kinematics of a limb to a continuously changing environment apparently has been acquired in a wide range of invertebrates and vertebrates (Grillner, 1981). The more sustained responses that occur as a result of repetitive presentations of the perturbations, however, could be attributed to more classical learning phenomena occurring over a range of time frames, undoubtedly engaging different mechanisms.

Much of the emphasis on the response to stumbling during locomotion has been on the initial "reflex" response with less attention given to the responses that are more delayed and sustained over multiple step cycles. These latter responses are highly coordinated responses requiring larger scale circuitries to control those motor pools that generate locomotion. Early and late responses to

stumbling perturbations that can sustain successful stepping after a stumble during locomotion have been observed in uninjured human adults (Schillings et al., 2000) and infants (Pang et al., 2003), decerebrated ferrets (Lou and Bloedel, 1988), intact cats (McVea and Pearson, 2007), and chronic spinal cats (Forssberg et al., 1975). Other more complex perturbations, such as applying force fields at specific phases of the step cycle in spinal rats, also demonstrate that there is a range of highly coordinated and immediate as well as delayed motor responses that have memory-related properties (Timoszyk et al., 2002; Heng and de Leon, 2007). The combination of the studies noted above demonstrate that the locomotor spinal circuitry can recognize, predict, and adapt to brief, instantaneous as well as more prolonged perturbations of complex motor tasks such as stepping.

Other examples demonstrate the detailed control that can be exhibited by the proprioceptive input to the spinal cord in complete spinal animals. Spinal rats and cats have the ability to step in different directions when stepping on a treadmill (Grillner, 1981; Heng and de Leon, 2007; Courtine et al., 2009). Decerebrate (Musienko et al., 2012) and spinal (unpublished observations) cats can make corrective responses to proprioceptive input (lateral displacement of the hindquarters) to maintain balance during stepping. Similar responses in balance control during standing have been reported in a human subject after complete paralysis (Harkema et al., 2011). In addition, Wernig and Muller (1991) reported that a completely paralyzed individual could initiate stepping by shifting his body weight, stretching the hip, and leaning forward. Each of these observations, in addition to the present data, demonstrate a significant level of sensory control that goes well beyond the control of the more stereotypical unperturbed stepping that has been demonstrated in chronic spinal cats. These observations reflect more than simple reflex-driven responses as they encompass a more sophisticated processing of sensory input, including feedforward as well as feedback mechanisms.

The present results may be interpreted as a reflection of central pattern generation. Central pattern generation, however, is cyclic activity that occurs without any phasic sensory input. The spinal model used herein, in contrast, encompasses the ability of the spinal central pattern generators to dynamically process complex sensory ensembles, make detailed decisions, in real time, and execute the appropriate motor output (Courtine et al., 2009). In fact, the spinal cord circuitry recognizes and habituates (learns) to the sensory input, facilitating the avoidance of an obstacle during stepping. Thus the present results should be interpreted within a context that exceeds what is routinely considered to be central pattern generation.

In summary, the spinal locomotor circuitry can recognize and translate a simple, instantaneous stimulus (perturbation) into a "useful" adaptation of a complex motor task (e.g., stepping). This ability to sustain such an adaptive strategy demonstrates the degree to which the spinal locomotor circuitry accommodates in a rather routine way to continuing changes in environmental events, providing a neural substrate for making these behaviors essentially "automatic," i.e., requiring little or no supraspinal intervention. This capability is clearly evident in normal human adults and infants and decerebrate cats and some features of these immediate and later responses have been observed during fictive locomotion in the cat (Quevedo et al., 2005). The present data, combined with other studies addressing longer time frames (Liu et al., 2005), demonstrate that a wide range of accommodating responses covering a time frame ranging from milliseconds to minutes can be generated and that these responses also can be "remembered" over this time frame in a manner that facilitates sustained locomotion within the spinal locomotor circuitry.

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Maladaptive spinal plasticity opposes spinal learning and recovery in spinal cord injury

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Synaptic plasticity within the spinal cord has great potential to facilitate recovery of function after spinal cord injury (SCI). Spinal plasticity can be induced in an activity-dependent manner even without input from the brain after complete SCI. A mechanistic basis for these effects is provided by research demonstrating that spinal synapses have many of the same plasticity mechanisms that are known to underlie learning and memory in the brain. In addition, the lumbar spinal cord can sustain several forms of learning and memory, including limb-position training. However, not all spinal plasticity promotes recovery of function. Central sensitization of nociceptive (pain) pathways in the spinal cord may emerge in response to various noxious inputs, demonstrating that plasticity within the spinal cord may contribute to maladaptive pain states. In this review we discuss interactions between adaptive and maladaptive forms of activity-dependent plasticity in the spinal cord below the level of SCI. The literature demonstrates that activity-dependent plasticity within the spinal cord must be carefully tuned to promote adaptive spinal training. Prior work from our group has shown that stimulation that is delivered in a limb position-dependent manner or on a fixed interval can induce adaptive plasticity that promotes future spinal cord learning and reduces nociceptive hyper-reactivity. On the other hand, stimulation that is delivered in an unsynchronized fashion, such as randomized electrical stimulation or peripheral skin injuries, can generate maladaptive spinal plasticity that undermines future spinal cord learning, reduces recovery of locomotor function, and promotes nociceptive hyper-reactivity after SCI. We review these basic phenomena, how these findings relate to the broader spinal plasticity literature, discuss the cellular and molecular mechanisms, and finally discuss implications of these and other findings for improved rehabilitative therapies after SCI.

Keywords: pain, nociception, plasticity, spinal cord injury, spinal cord learning, recovery of function

INTRODUCTION

Research into spinal plasticity over the past 50 years has shown that neurons within the spinal cord gray matter have a remarkable degree of plasticity, and in recent years we have seen a surge of interest in this field (Raineteau and Schwab, 2001; Edgerton et al., 2004; Cai et al., 2006; Grau et al., 2006). Spinal synapses can be strengthened or weakened in response to external stimulation, demonstrating the basic properties required for use-dependent learning and memory. This capacity is of great importance after spinal cord injury (SCI). Prior work has shown that peripheral injury or inflammation can induce chronic neuropathic pain states and that this outcome is due, in part, to sensitization of nociceptive pathways within the spinal cord (for review see Woolf and Salter, 2000; Ji et al., 2003). Aspects of this process have been shown to be mechanistically analogous to brain-dependent learning and memory, and thus represents a lasting form of maladaptive spinal plasticity. Work from our team over the past 15 years has built upon this foundation, focusing on the ways in which central sensitization impacts and informs both adaptive and maladaptive forms of spinal learning and memory (Ferguson et al., 2006; Grau et al., 2006; Hook et al., 2008; Huie et al., 2012a,b). Our work suggests that exposure to uncontrollable/unpredictable peripheral stimulation induces a central sensitization-like state that inhibits adaptive spinal learning and undermines recovery of locomotor function after spinal contusion injury.

The present review discusses the features of spinal cord plasticity with a specific emphasis on protecting against maladaptive plasticity in nociceptive systems and promoting adaptive forms of spinal plasticity for rehabilitation after SCI. We will first review the cellular and physiological evidence for use-dependent spinal cord plasticity and draw parallels to brain-dependent plasticity.

We then review behavioral evidence for learning and memory within the spinal cord and discuss how specific changes in stimulation parameters can tip the balance between adaptive and maladaptive outcomes. Finally, we discuss how noxious input below the level of SCI may induce a similar nociceptive plasticity that undermines recovery, and the potential application of appropriate spinal cord training to overcome these maladaptive effects to restore function after SCI.

CELLULAR AND ELECTROPHYSIOLOGICAL EVIDENCE FOR SYNAPTIC PLASTICITY IN THE SPINAL CORD

Research of spinal plasticity over the past 50 years has eroded the perception of the spinal cord as a simple conduit of neural information. We now know that that the spinal cord is capable of supporting a number of forms of plasticity, yet spinal plasticity remains understudied in comparison to the field of brain plasticity. We will begin this review by highlighting some of the major findings that reshaped our view of the spinal cord, in order to provide the reader with a contextual background for our later discussions of the ways in which spinal plasticity is modulated.

Synaptic changes in the spinal cord have been often studied in the context of spinal nociceptive plasticity. In the early 1980s Clifford Woolf demonstrated that tissue injury to the lateral hind-paw produces cutaneous hypersensitivity to light tactile (von Frey) stimulation both on the ipsilateral and contralateral hind-paw (Woolf, 1983). This suggested that post-injury pain hypersensitivity was due, in part, to an increase in excitability of spinal cord neurons. This increase in spinal activity, known as "central sensitization," reflects a form of neuroplasticity in the spinal cord (Woolf and Salter, 2000).

In many ways central sensitization involves changes in the spinal cord gray matter that mimic hippocampal-mediated activity-dependent plasticity. In the hippocampus, tetanic stimulation of afferent pathways can increase responsiveness of subsequent post-synaptic potentials, a phenomenon known as long-term potentiation (LTP) (Bliss and Lomo, 1973). LTP is widely believed to be a synaptic mechanism for learning and memory in the CNS. Early pharmacological evidence suggested that both hippocampal LTP and hippocampal-dependent learning tasks are blocked by antagonists to the glutamate NMDA receptor (Collingridge and Bliss, 1987). Other lines of evidence suggested that electrophysiologically overdriving (saturating) LTP prevented both later LTP and spatial learning (McNaughton et al., 1986; Moser et al., 1998). Recent data have shown that spatial learning experience produces LTP in the hippocampus that is detectable both electrophysiologically (as increases post-synaptic currents) and biochemically as an increase in phosphorylation of the glutamate AMPA receptor and trafficking of AMPA receptors to synapses (Whitlock et al., 2006). These AMPA receptor changes are thought to be fundamental to LTP and other forms of plasticity at excitatory synapses in the CNS (Malinow and Malenka, 2002).

Many of the characteristics of hippocampal LTP have been identified in the spinal cord, providing a potential cellular mechanism for central sensitization. For example, tetanic stimulation of primary nociceptive afferents (C-fibers) has the capacity to increase post-synaptic responses in the superficial spinal lamina, a phenomenon known as "wind-up" (Mendell and Wall, 1965; Mendell, 1966). In addition, like hippocampal LTP, central sensitization can be blocked with NMDA receptor antagonists (Woolf, 1983; Woolf and Thompson, 1991; Dougherty et al., 1992), providing a strong pharmacological link between nociceptive sensitization and LTP. This apparent common mechanism between changes in nociception and LTP (which has been suggested as a substrate for learning and memory) has led led to the idea that nociceptive sensitization may act as a form of 'pain memory' (Woolf and Costigan, 1999; Ji et al., 2003). Work by Sandkuhler and colleagues have provided direct evidence that nociceptive stimuli can produce spinal LTP (Liu and Sandkuhler, 1995; Sandkuhler and Liu, 1998). In addition, this team has recently found that high doses of short-acting opioids can reverse spinal pain memory, as measured by losses of hyper-reactivity, reduced spinal LTP, and reduced phosphorylation of glutamate AMPA receptors (Drdla-Schutting et al., 2012). Together, these data provide strong evidence that activity-dependent plasticity in pain pathways reflects a form of learning and memory within the spinal cord.

Spinal glia have also been implicated in spinal LTP. Once believed to simply provide structural support, the capacity for glial cells to affect glutamatergic signaling through the release of a host of neuromodulators has led researchers to assess the importance of glia in CNS plasticity (Muller, 1992; Allen and Barres, 2005). Using high-frequency stimulation of the sciatic nerve to induce LTP of C-fiber-evoked field potentials in the dorsal horn, Ma and Zhao demonstrated that glial activity is required for this effect, as spinal treatment with the glial metabolic inhibitor fluorocitrate blocked the induction of spinal LTP (Ma and Zhao, 2002). As spinal pain memory is believed to be encoded by a LTP-like effect in the dorsal horn, this finding illustrates the essential role of glia in modulating spinal plasticity. A similar effect has been demonstrated behaviorally as well. Watkins et al. induced pain hyper-reactivity in rats using a peripheral injection of the common irritant formalin. They found that if glial activity was inhibited using fluorocitrate prior to formalin administration, the induction of this hyper-reactivity was blocked (Watkins et al., 1997). Others have investigated the key glial products that mediate neuromodulation of nociceptive plasticity in the spinal cord, and have shown a number of these products to be involved, including nitric oxide, prostaglandins, and the cytokines IL-1b, IL-6, and tumor necrosis factor alpha (TNFα). TNFα in particular has been shown to enhance spinal LTP in rats with neuropathic pain (Liu et al., 2007). In both hippocampal culture and *in vivo* spinal cord, TNFα has been shown to increase trafficking of AMPA receptors to synaptic sites, providing a potential mechanism for TNFα-induced increases in spinal LTP (Beattie et al., 2002; Ferguson et al., 2008b; Choi et al., 2010). Recent work aimed at elucidating the role of spinal glia and TNFα in maladaptive forms of spinal nociceptive plasticity is discussed later in this review.

Similarly, metabotropic glutamate receptors (mGluRs) modulate spinal plasticity within pain pathways by altering the plasticity of the ionotropic NMDA and AMPA receptors (Mills et al., 2002). In particular, the group I mGluRs (mGluR1 and mGluR5) have been shown to enhance ionotropic receptor-dependent central

nociceptive plasticity in the spinal cord (Fisher and Coderre, 1996a,b). These systems have also been implicated in brain-dependent plasticity as well as multiple forms of spinal plasticity. We will return to a discussion of mGluRs in the "cellular and molecular mechanisms" section of this review.

In summary, the spinal cord is capable of supporting memory for prior noxious experience that manifests behaviorally, pharmacologically, and electrophysiologically. This spinal memory depends on mechanisms similar to learning and memory in the higher CNS, including induction and expression of LTP at spinal synapses. Spinal LTP is mediated by at least some of the same receptor pathways as in the brain, providing further evidence of a common mechanism of plasticity. Notably, the expression of LTP in spinal pain pathways has been shown to contribute to central sensitization in nociceptive systems, providing a mechanism for some maladaptive neuropathic pain states.

SPINAL CORD LEARNING AND MEMORY

Plasticity within the spinal cord is not limited to maladaptive plasticity within nociceptive pathways. The spinal cord also demonstrates several forms of adaptive motor plasticity. In the following section, we will move beyond spinal nociceptive pathways to investigate how spinal plasticity in motor pathways can induce robust behavioral changes, and how these changes can be used as outcome measures in a simple model of learning in the spinal cord.

Inducing adaptive plasticity in spinal motor systems can have profound effects on locomotor behavior. For example, following complete thoracic transection, the lumbar spinal cord can regain the capacity to sustain weight-supported stepping with extensive step training (Lovely et al., 1986; Barbeau and Rossignol, 1987; de Leon et al., 1998; Harkema et al., 2011). The capacity for locomotor re-training after SCI is thought to be possible because the lumbar spinal cord contains central neural networks that control reciprocal activity of extensor and flexor efferents during locomotion (Grillner, 1975; Grillner and Zangger, 1979). These "central pattern generators" in the lumbar cord can be tuned by generating a specific pattern of afferent input during physical rehabilitation training, thereby promoting recovery of function (Dietz and Harkema, 2004; Prochazka and Yakovenko, 2007; Edgerton et al., 2008).

However the specific learning capacities of the spinal cord that underlie this recovery of function remain a topic of intensive study. Work from the field of neurobiology of learning and memory has revealed that the isolated spinal cord can support simple forms of motor learning. There is well-documented evidence that spinal neurons can sustain single stimulus learning (habituation/sensitization), stimulus association (Pavlovian conditioning), and response-outcome (instrumental) learning (Sherrington, 1906; Thompson and Spencer, 1966; Fitzgerald and Thompson, 1967; Grau et al., 1998).

Early demonstrations of habituation and sensitization in the spinal cord provided fundamental evidence that the spinal cord could learn from repeated activity, and demonstrated a form of spinal memory that manifested behaviorally. Repeated exposure to a stimulus was found to decrease (habituate) a spinally mediated flexion response. This habituation was not due to adaptation at sensory receptors in the periphery or to a change at the neuromuscular junction, suggesting that the memory for stimulus training history resided in spinal interneuronal synapses (Sherrington, 1906; Prosser and Hunter, 1936; Thompson and Spencer, 1966). In contrast to habituation, which occurred with moderate stimuli, exposure to a single strong stimulus had the capacity to increase subsequent responsiveness in a process known as "sensitization". Groves and Thompson (1970) went on to characterize different interneuronal pools that were responsible for habituation and sensitization, providing one of the early neurobiological theories of activity-dependent plasticity in the spinal cord.

Another line of work revealed that spinal neurons were capable of encoding relationships between different stimuli, a hallmark of Pavlovian (classical) conditioning. For example, the isolated spinal cord was shown to be capable of associating weak thigh stimulation (conditioned stimulus; CS) with strong plantar stimulation of the foot (unconditioned stimulus, US). With repeated CS–US pairings the thigh stimulation came to modulate the flexion withdrawal reflex, a type of Pavlovian conditioning known as "pairing-specific enhanced sensitization" (Fitzgerald and Thompson, 1967; Groves et al., 1969). A similar form of Pavlovian conditioning had been demonstrated in the aquatic mollusk, Aplysia, in classic work in the Kandel laboratory (Carew et al., 1981). Together these data suggest that not all forms of learning and memory require the brain.

Much of our recent work has focused on response-outcome (instrumental) training of the spinal cord, with the goal of uncovering the basic principles that dictate spinal cord learning. The translational goal of this work is to provide basic knowledge that can help improve training-based rehabilitative therapies after SCI. Our spinal training model is based on the Horridge paradigm originally developed in headless insects and then later adapted to spinalized mammals to study learning and memory within the spinal cord (Horridge, 1962; Buerger and Fennessy, 1970; Grau et al., 1998). Horridge found that after experimentally instituting a relationship between leg position and electrical stimulation of an ankle flexor, insects could learn to hold the hind limb in a flexed position. This acquired leg flexion emerged after repeated trials, demonstrating an acquisition curve that resembles brain-dependent escape learning. To rule out the possibility that the change in flexion response reflected a peripheral mechanism such as muscle tetanus, Horridge used a clever design that involved testing insects in "master/yoked" pairs. One insect, the master, received leg stimulation only when the leg was extended. The other rat served as a yoked control that received passive leg stimulation whenever the master received stimulation. Although both rats received equal stimulation, only the master insect learned to hold the leg in a flexed position, suggesting that the acquired flexion response was not due to a simple unconditioned effect of stimulation, but rather depended on the response-outcome contingency between leg position (response) and stimulation (outcome).

Master/yoked training of the hind limb after spinal transection in rats revealed that the mammalian spinal cord is also sensitive to response-outcome relationships (**Figure 1A**; Buerger and Fennessy, 1970). Building upon this foundation, our group

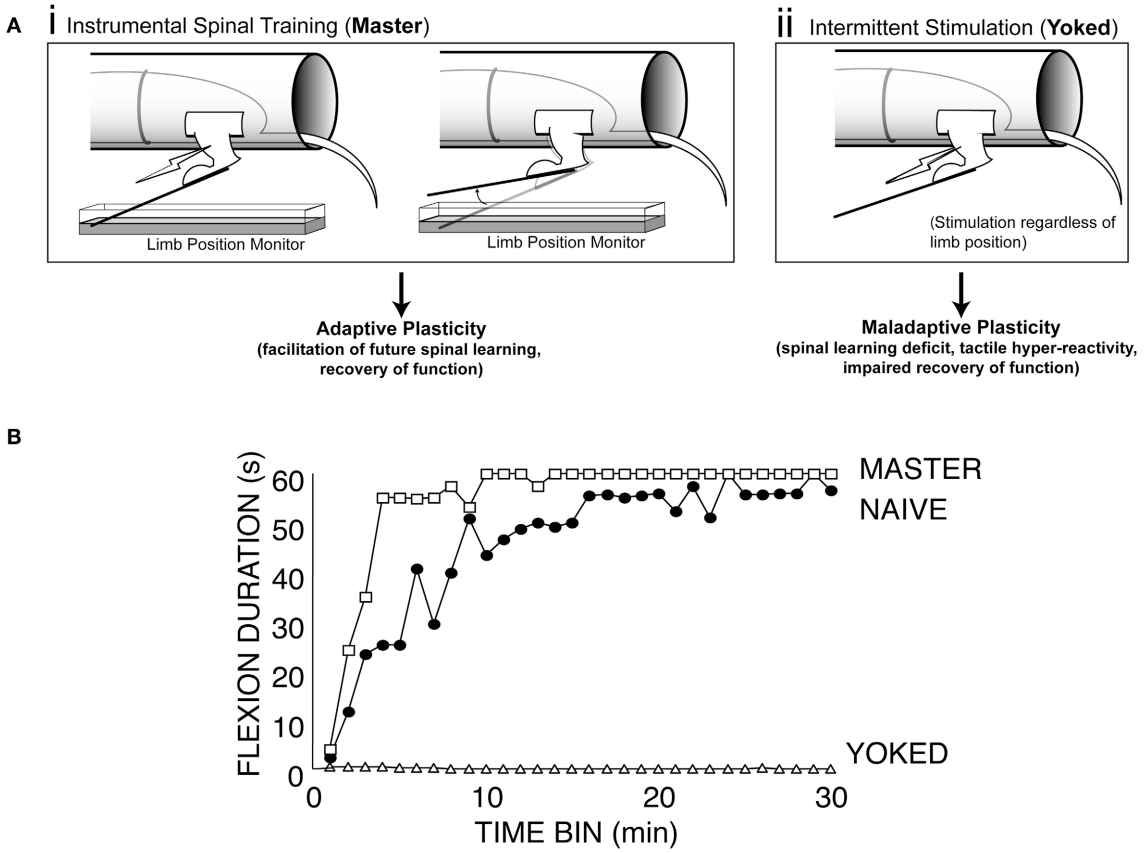

FIGURE 1 | Effects of instrumental vs. uncontrollable nociceptive stimulation on spinal function. (A) Modes of stimulation. (i) Instrumental spinal training. For Master rats, electrical stimulation is delivered to the tibialis anterior muscle when the hindlimb is unflexed, and terminated when the hindlimb is flexed. Over a 30 min training session, master rats learn to keep the hindlimb flexed to reduce stimulus exposure (spinal learning). This spinal training promotes future adaptive plasticity. (ii) Uncontrollable stimulation. The yoked rats receive stimulation whenever the master does, regardless of their hindlimb position. **(B)** Exposure to this uncontrollable (yoked) stimulation causes a learning deficit that is evident when these rats are later tested with the spinal learning assay. Spinal learning is assessed by monitoring the ability of spinally transected rats to maintain the hindlimb in a flexed position as manifested by increasing average response duration in each 1 min time bin for 30 min test during instrumental training. Modified from Grau et al. (1998).

has shown that this example of spinal cord plasticity meets the behavioral criteria (Grau et al., 2006) for instrumental learning and has a lasting effect on spinal function that impacts clinically relevant phenomena (e.g., Grau et al., 1998, 2004; Ferguson et al., 2006; Hook et al., 2008). Over the past 14 years we, and others (Jindrich et al., 2009), have shown that by imposing a relationship between leg position and stimulation of the tibialis anterior muscle (master condition/controllable stimulation) we can produce beneficial effects that improve future spinal cord training (**Figure 1B**, master; Grau et al., 1998). In this sense the spinal cord shows memory for training history and can re-learn the flexion response more rapidly after instrumental training (**Figure 1B**, master vs. naïve).

However, not all spinal memory for stimulus training is adaptive. Rats that are given stimulation that is independent of leg position (yoked condition/uncontrollable stimulation) show persistent deficits in future learning when tested with response-contingent stimulation. This spinal learning deficit appears to reflect a long-term form of maladaptive spinal plasticity that endures for at least 48 h in completely transected rats (**Figure 1B**, yoked; Grau et al., 1998; Crown et al., 2002b). Thus, using this master/yoked learning paradigm has allowed us to observe and manipulate the expression of both adaptive and maladaptive forms of plasticity in the spinal cord.

The training-dependent effects of stimulus controllability on future spinal learning can be interpreted as "plasticity of plasticity" or "metaplasticity," that regulates the capacity for future learning in a bidirectional manner (Abraham and Bear, 1996; Crown et al., 2002a,b; Grau et al., 2006; Ferguson et al., 2008b). On the one hand, controllable stimulation produces a positive and adaptive form of plasticity that promotes future spinal learning and limits the development of nociceptive plasticity. The benefits of controllable stimulation are discussed in detail in Grau et al. (2012) within this same issue. On the other hand, uncontrollable stimulation produces a lasting, maladaptive effect that undermines the capacity for future spinal learning. A large body of work from our group and others has focused on the stimulus parameters that are critical to this effect, the neurobiological mechanisms that mediate this form of maladaptive plasticity, and how it may relate to the development of central sensitization.

Perhaps most importantly, we have worked to uncover how such an effect impacts recovery of function following SCI.

The remaining sections of this review will delve into these critical issues. The general theme is that uncontrollable/yoked stimulation produces a maladaptive form of spinal metaplasticity that is associated with impaired spinal learning, reduced recovery of function after SCI, and nociceptive hyper-reactivity (Ferguson et al., 2006; Hook et al., 2008). The findings above suggest that the specific patterning of peripheral stimulation exerts exquisite control over the nature of activity-dependent plasticity that develops in the spinal cord. In sections that follow we will review the environmental conditions that determine whether stimulation impacts spinal function in an adaptive or maladaptive manner and the underlying neurobiological mechanisms.

STIMULATION PARAMETERS FOR MODULATING SPINAL LEARNING

The capacity for adaptive spinal cord learning is thought to contribute to functional recovery after SCI (Edgerton et al., 2001; Grau et al., 2006). However, the specific stimulus conditions that promote effective spinal cord learning are not fully understood. Similarly, those conditions that lead to a spinal learning deficit require elucidation. Given that SCI is likely to be accompanied by peripheral noxious input from other concomitant injuries (as well as noxious input as a result of secondary injury processes), a more complete understanding of the stimulus parameters that may undermine adaptive spinal plasticity is essential. This section will highlight findings that have shed light on the conditions under which maladaptive plasticity may be induced.

After careful study of yoked animals, Crown and Grau (2001) developed a computer program that emulated the shock schedule produced by master rats during the early phase of training. This enabled the experimental evaluation of the effects of uncontrollable stimulation in a single "virtually yoked" rat to explore the parameters that disable future learning ability. The program delivers 80 ms, 1.5 mA AC current (60 Hz) stimulation on a randomized variable interstimulus interval that ranges from 0.2 to 3.8 s (mean interval = 2 s). Through a series of parameteric studies Crown et al. (2002a) found that delivering as little as 6 min of uncontrollable stimulation to either the leg or the tail after complete spinal transection produces lasting (>48 h) impairments in spinal learning (Crown et al., 2002a; Joynes et al., 2003). Later work went on to show that the same stimulation procedure produced long-term (>6 weeks) impairments in recovery of function after contusive SCI (Grau et al., 2004). As these effects are seen in both transection and contusion injuries the findings suggest that intermittent, uncontrollable stimulation has a generalized negative effect on adaptive spinal plasticity.

The finding that intermittent uncontrollable stimulation to the tail produced the same disruption of adaptive motor learning as stimulation to the leg provided important support for the idea that these maladaptive changes are centrally mediated in the spinal cord. But the question remained, what is it about these particular stimulation parameters that drives such robust inhibition of spinal learning and recovery? Is it the uncontrollability, the number of shocks presented, the unpredictability, or some combination thereof that is essential to this phenomenon?

The lasting differences in learning capacity between master/yoked pairs suggested that controllability is an essential characteristic. The differential effects of controllable and uncontrollable stimulation on spinal plasticity can be invoked even when the total amount of stimulation is equalized using master/yoked training protocol. In master/yoked training, rats are run in pairs where one rat (master) is given response-contingent stimulation and the other rat (yoked) receives stimulation whenever the master does. Even though the two rats receive stimulation at the same time, only master rats show instrumental learning. But is it necessary that stimulation pulses be separated at all to induce the yoked learning failures? Is it the total amount of shock delivered that predicts the induction of a spinal learning deficit, or the discontinuous nature of shock administration? Delivering 80 ms stimulation at an average of 2 s intervals over 6 min produces a total of approximately 180 shocks, and a total stimulation time of approximately 15 s. Thus, to test whether these temporal gaps are necessary, we applied a continuous 1.5 mA AC (60 Hz) shock for 15 s to one group, while another group received a continuous AC shock for the entire 6-min session. A third group received the typical intermittent stimulation schedule, and a control group received no stimulation. All rats were then subsequently tested for instrumental learning. As expected, the rats that received no stimulation were able to learn, and those that received intermittent stimulation failed. Interestingly, both groups that received continuous stimulation were able to learn, suggesting that the long-term effect of shock stimulation depends upon whether the pulses are separated by a temporal gap. Moreover, presenting a continuous tailshock while rats received intermittent legshock negated the adverse effect of intermittent stimulation, suggesting that continuous stimulation induces an opponent process that counters the induction of the learning deficit (Crown et al., 2002a).

Further work revealed that continuous and intermittent stimulation have divergent effects on nociceptive reacitivity. Exposure to continuous shock reduces reactivity (antinociception) to a noxious thermal stimulus applied to the tail (tail-flick test; Crown et al., 2002a). In contrast, exposure to intermittent shock has no effect on thermal reactivity (Crown et al., 2002a), but enhances reactivity to tactile stimulation. Rats were given uncontrollable intermittent stimulation to the hindleg and then tested for tactile reactivity on the ipsilateral and contralateral leg using calibrated von Frey hairs. The results indicated that uncontrollable stimulation to the leg produces a bilateral tactile hyper-reactivity, whereas controllable (instrumental) stimulation has the opposite effect, mitigating central sensitization (**Figure 2**; Ferguson et al., 2006; Hook et al., 2008; Huie et al., 2012b). Thus, it appears that one key feature that may differentiate the effects of intermittent stimulation from other stimulation schedules is the induction of nociceptive sensitization.

The enhanced reactivity to tactile stimulation observed after intermittent shock resembled the mechanical allodynia observed after peripheral inflammation and injury (Ji et al., 2003; Hook et al., 2008). Given this, we hypothesized that the maladaptive effect of uncontrollable stimulation may be linked to the induction of a state akin to central sensitization. If so, it would be expected that the spinal learning deficit should be induced not only within the confines of specific electrical stimulation

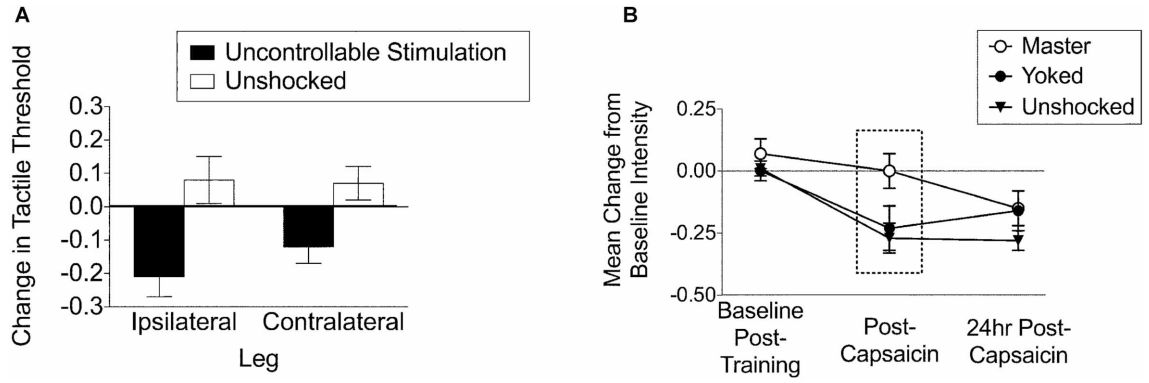

FIGURE 2 | Training history alters nociceptive hyper-reactivity. (A) Uncontrollable stimulation (6 min, 80 ms stimuli, variable inter-stimulus interval: 0.8–3.2 s, 1.5 mA AC) to the leg produces tactile hyper-reactivity on both the ipsilateral and contralateral leg. The same stimulation pattern undermines spinal cord learning on both leg for over 24 h and impairs recovery of locomotor function for over 6 weeks (adapted from Ferguson et al., 2006). **(B)** Master and yoked training have differential effects on central sensitization to intradermal capsaicin. Rats that have been trained with controllable stimulation (Master) have decreased tactile responsiveness immediately post-capsaicin injection [adapted from Hook et al. (2008)]. Similar effects are reported with central sensitization by intradermal formalin in Ferguson et al., 2012 within this same issue.

parameters, but by using more generalized nociceptive activators. We discovered that intradermal injection of the inflammatory agent carrageenan, an agent often reported in the pain literature to induce central sensitization, also produced a marked inhibition of spinal learning (**Figure 3**; Ferguson et al., 2006). This effect was time-dependent with the most dramatic loss of spinal learning at 3–6 h, and recovery of learning by 24 h after injection. This time-course precisely mirrors the established time-course for central sensitization by carrageenan (Hargreaves et al., 1988; Zhang et al., 2003). Similar effects have subsequently been observed with other peripheral nociceptive stimuli including intradermal capsaicin and formalin (Hook et al., 2008). These findings suggest that intermittent stimulation undermines adaptive spinal modifications by inducing nociceptive plasticity that is akin to central sensitization.

In all of the experiments reviewed above, intermittent shock was presented in a variable manner using a program that emulated the temporal distribution of shocks produced by a master rat. Does this temporal variable matter, or would intermittent shock undermine spinal plasticity independent of whether it is presented in variable or regular (fixed spaced) manner? Baumbauer et al. (2008) explored this issue and found that spacing does not matter when rats are given just 6 min of stimulation (approximately 180 shocks); both fixed and variably spaced shock impaired subsequent learning (**Figure 4**; Baumbauer et al., 2008). In contrast, when rats were exposed to an extended series of shocks (1800 given over 30 min), only variable shock induced a learning deficit. Introducing temporal regularity (predictability) not only eliminated the learning deficit, it induced a protective effect analogous to instrumental control that both prevented, and reversed, the learning deficit (Baumbauer et al., 2009). The protective effect of fixed spaced shock lasted 24 h was mediated by a protein synthesis-dependent process, and involved a form of NMDAR-mediated plasticity (Baumbauer et al., 2009). These observations suggest that both instrumental control and temporal predictability can negate the adverse consequences of intermittent stimulation. Variable shock may lead to a central sensitization-like state independent of shock number because this pattern of stimulation emulates the erratic pattern of neural activity observed in C-fibers (Sandkuhler, 2007). Further, intermittency may be an essential feature because repeated shock onsets are required to drive an electrophysiological response over extended periods; a continuous stream of shock pulses may lead to a form of physiological habituation that reduces C-fiber induced activity (Groves and Thompson, 1970).

In summary, several studies have demonstrated that the patterning of peripheral stimulation after SCI can have an enormous effect on whether adaptive or maladaptive plasticity emerges in the lumbar spinal cord. Response-contingent (master), predictable nociceptive stimulation promotes future spinal cord learning whereas unpredictable, intermittent nociceptive stimulation undermines future spinal learning and generates central sensitization. Therefore, discovering the biological mechanisms for these stimulus-induced forms of spinal plasticity has implications for both pain modulation and recovery of function after SCI.

CELLULAR AND MOLECULAR MECHANISMS DICTATING SPINAL LEARNING

The preceding sections have outlined the fundamental features of spinal cord learning, and highlighted the importance of stimulus patterning in tipping the balance between adaptive and maladaptive forms of spinal plasticity. In the present section we will discuss the cellular and molecular mechanisms dictating spinal learning potential. The purpose of this line of research has been to discover biological mechanisms that modulate spinal learning, with the goal of developing therapeutic approaches to promote rehabilitation and recovery of function after SCI (Grau et al., 2006). Because of its rapid-throughput nature (30 min), the instrumental training assay has provided a powerful tool to identify a number of neuropharmacological targets for improving adaptive spinal cord function. In general, this work has focused on either promoting adaptive plasticity to improve future spinal

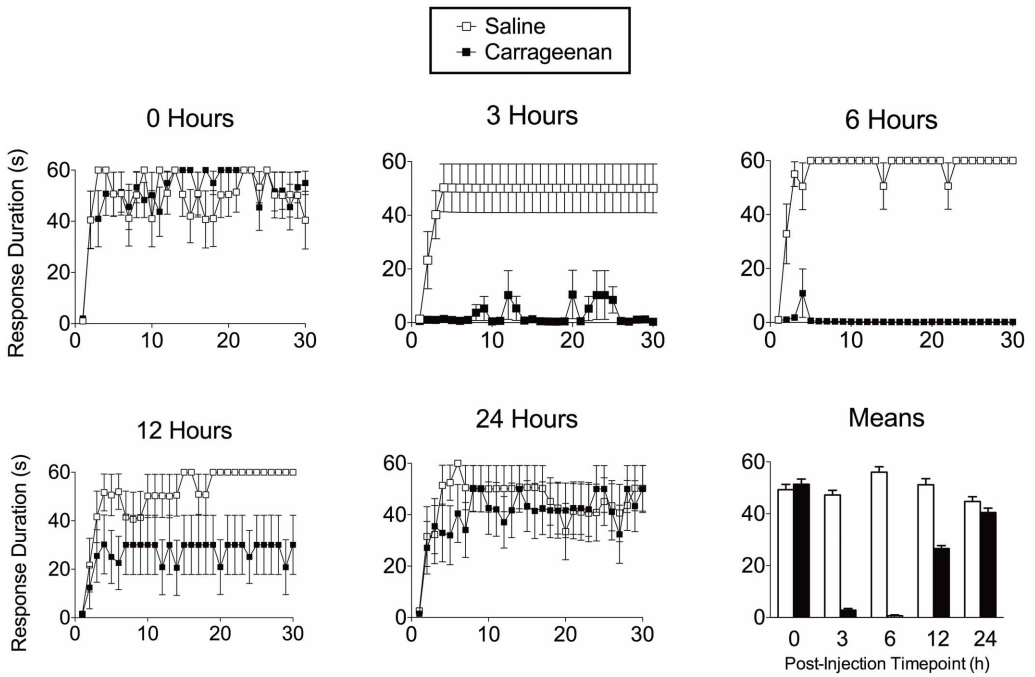

FIGURE 3 | Peripheral inflammation with intradermal carrageenan produces transient impairment in spinal cord learning on the contralateral leg. The timecourse of these learning deficits mimic the known timecourse for carrageenan-induced central sensitization. Adapted from Ferguson et al. (2006).

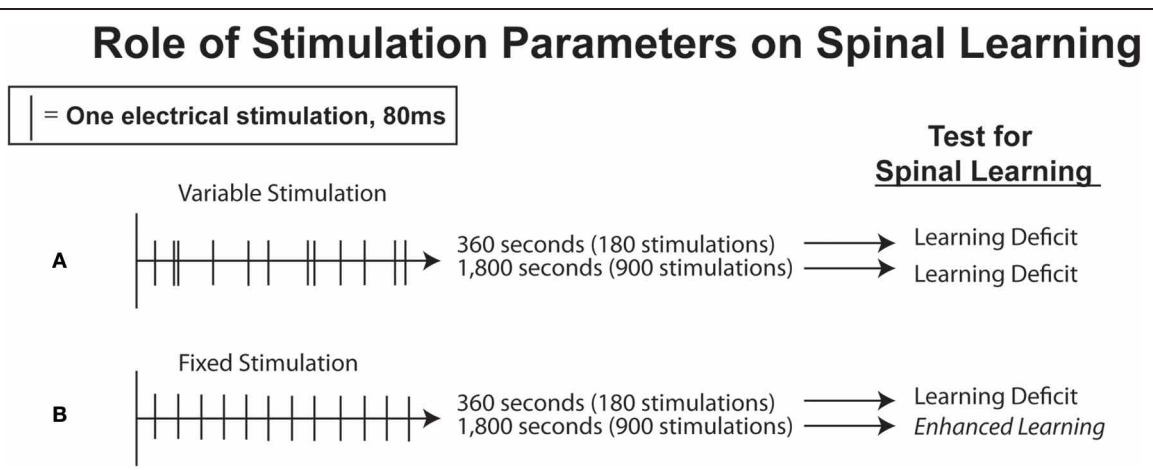

FIGURE 4 | Stimulation parameters affecting spinal learning. (A) If nociceptive electrical stimulation is delivered to the tail in a randomized, intermittent fashion, rats will fail to learn when later tested with controllable stimulation to one hindlimb. This deficit is exhibited after either short (360 s) or long (1,800 s) trains of intermittent, randomized stimulation. **(B)** If stimulation is administered to the tail with a fixed interval between stimulations over the course of 360 s, rats will also later fail to learn, but if the train of fixed stimulation is extended to 1800 s rats then exhibit enhanced learning when later tested. Adapted from Baumbauer et al. (2008).

learning, or preventing maladaptive plasticity to reverse learning deficits and limit nociceptive plasticity. This section will first briefly consider the mechanisms that mediate spinal learning, and will then focus on the neurobiological pathways that have been implicated in the maladaptive effect of uncontrollable, intermittent nociceptive stimulation.

Adaptive spinal cord learning in the Horridge paradigm is blocked by intrathecal lidocaine, indicating that spinal neuronal activity is required for the learned flexion response (Crown et al., 2002b). In addition, intrathecal antagonists to glutamate NMDA and AMPA receptors have each been independently shown to block acquisition of the flexion learning (Joynes et al., 2004; Hoy et al., 2012). These findings mimic what is observed in visuo-spatial learning paradigms, indicating that spinal cord learning in the Horridge paradigm depends on biological mechanisms similar to hippocampal-dependent learning in

the brain (Whitlock et al., 2006). This suggests that mechanisms that enhance hippocampal-dependent learning have potential to enhance spinal cord training as well. Gomez-Pinilla et al. (2007) tested the relationship between spinal training and several known molecular biomarkers of CNS plasticity. Quantitative RT-PCR revealed that master rats have an increase in spinal mRNA levels compared to naïve controls for several pro-plasticity biomarkers including brain-derived neurotrophic factor (BDNF), calcium/calmodulin activated kinase II (CaMKII), cAMP response element binding protein (CREB), and the pre-synaptic terminal protein synapsin I. Moreover, the degree of mRNA correlated highly with performance on the spinal learning task. In contrast, yoked animals demonstrated significant decreases in spinal levels of BDNF, CaMKII, and CREB relative to naïve animals. Together these data suggest that pro-plasticity markers may play a role in spinal cord learning.

BDNF appears to be particularly important for spinal learning. Intrathecal BDNF supplementation can promote spinal cord learning when task difficulty is increased beyond a level that naïve rats can normally perform (Gomez-Pinilla et al., 2007). In contrast the BDNF inhibitor TrkB-IgG can block the adaptive benefits of prior training reducing master animals to the performance level of untrained rats. This suggests that BDNF, a well-established modulator of synaptic plasticity in the brain, also has beneficial effects on adaptive spinal plasticity that promotes future spinal learning (reviewed in McAllister et al., 1999; Fritsch et al., 2010). In contrast, the learning deficit following yoked stimulation involves a down regulation of BDNF, suggesting that bidirectional modulation of synaptic plasticity mechanisms dictate spinal learning potential. A similar role for BDNF in promoting adaptive spinal plasticity has also recently been shown to translate to a model of locomotor recovery. Boyce et al. demonstrated that in rats with a complete transection at the T10 thoracic spinal cord segment, treatment with adeno-associated virus expressing BDNF was able to induce weight-supported hindlimb stepping without the assistance of step-training (Boyce et al., 2012).

Further work has indicated that uncontrollable/unpredictable intermittent nociceptive stimulation engages a maladaptive form of plasticity that involves its own signature of cellular molecular changes. Initial studies indicated that intrathecal lidocaine (Joynes et al., 2003) and protein synthesis inhibitors (Patton et al., 2004; Baumbauer et al., 2006) protect learning potential when delivered prior to intermittent nociceptive stimulation. This suggested that spinal cord circuitry actively encodes the maladaptive stimulation patterns through a form of activity-dependent CNS plasticity. Indeed, intrathecal delivery of an NMDA receptor antagonist (at doses that are known to block central sensitization of nociceptive systems) prior to intermittent nociceptive stimulation protected spinal learning potential (Ferguson et al., 2006). This observation led to the hypothesis that central sensitization undermines spinal cord learning potential by engaging a generalized hyper-excitability that prevents adaptive spinal learning (Ferguson et al., 2006). Several converging lines of molecular and cellular evidence have lent support for this concept. The key cellular and molecular mechanisms associated with both long-term spinal learning deficits and central sensitization are depicted in **Figure 5**. Intermittent nociceptive stimulation has been found to engage several glutamate receptor systems, as well as the proinflammatory cytokine TNFα and substance P (Baumbauer et al., 2007; Huie et al., 2012a). All of these systems have been implicated in central nociceptive processing as well (Sandkuhler and Gruber-Schoffnegger, 2012).

One of the conundrums outlined above is that both adaptive learning and maladaptive plasticity could be blocked by NMDA receptor antagonists (Joynes et al., 2004; Ferguson et al., 2006). This suggested that NMDA receptors serve as executors of spinal plasticity in the spinal learning preparation. The NMDA receptor in spinal motoneurons has been shown to be highly mobile, and can induce glutamatergic plasticity by rapidly trafficking from extrasynaptic to synaptic sites in response to changes in glutamatergic input (Shanthanelson et al., 2009). Interestingly, Shanthanelson et al. showed that recovery of NMDA-mediated excitatory tone was more pronounced at monosynaptic input sites coming from segmental dorsal roots, as opposed to central inputs coming from the ventrolateral funiculus. This finding suggests that NMDA-mediated plasticity is highly regulated by, and highly susceptible to, peripheral input. As such, differences in the timing and pattern of peripheral input (such as those seen in controllable vs. uncontrollable stimulation) may induce differential NMDA receptor trafficking and activation, leading to divergent forms of spinal plasticity.

Critical systems for switching between maladaptive and adaptive plasticity may also lie upstream of NMDA receptor changes. One of the well-established neuropharmacological modulators of NMDA receptor plasticity is group I mGluRs. The group I mGluRs consist of two receptor subtypes (mGluR1 and mGluR5) that are known to modulate plasticity in the CNS through downstream effects on iGluRs, including NMDA and AMPARs (Fisher et al., 2002; Fundytus et al., 2002). In addition activation of group I mGluRs in the spinal cord increases NMDA-dependent central sensitization after intradermal formalin (Coderre and Melzack, 1992), and central pain after SCI (Mills et al., 2002).

Therefore, mGluRs are a good candidate for a central role in dictating spinal metaplasticity in the spinal learning paradigm. We tested this hypothesis using a combination of pharmacological, biochemical, and behavioral methods. We found that mGluR1 and mGluR5 antagonists protect spinal learning potential in the face of uncontrollable stimulation. On the other hand the group I mGluR agonist DHPG can substitute for uncontrollable stimulation to produce long-term impairments in spinal cord learning (Ferguson et al., 2008b). Group I mGluRs were the first receptor system identified to be both necessary and sufficient for inducing persistent maladaptive alterations in spinal learning potential. Maladaptive mGluR effects depend on downstream activation of protein kinase C (PKC), a molecule that has been implicated in hippocampal plasticity (Akers et al., 1986; Malinow and Malenka, 2002; Ferguson et al., 2008b). Activation of mGluRs and PKC has previously been implicated in maladaptive plasticity within spinal pain pathways, once again suggesting an interaction between spinal learning deficits and nociceptive systems (Munro et al., 1994; Young et al., 1995; Ferguson et al., 2008b).

One of the major downstream effects of mGluR and PKC activation is post-translational modification of the ionotropic

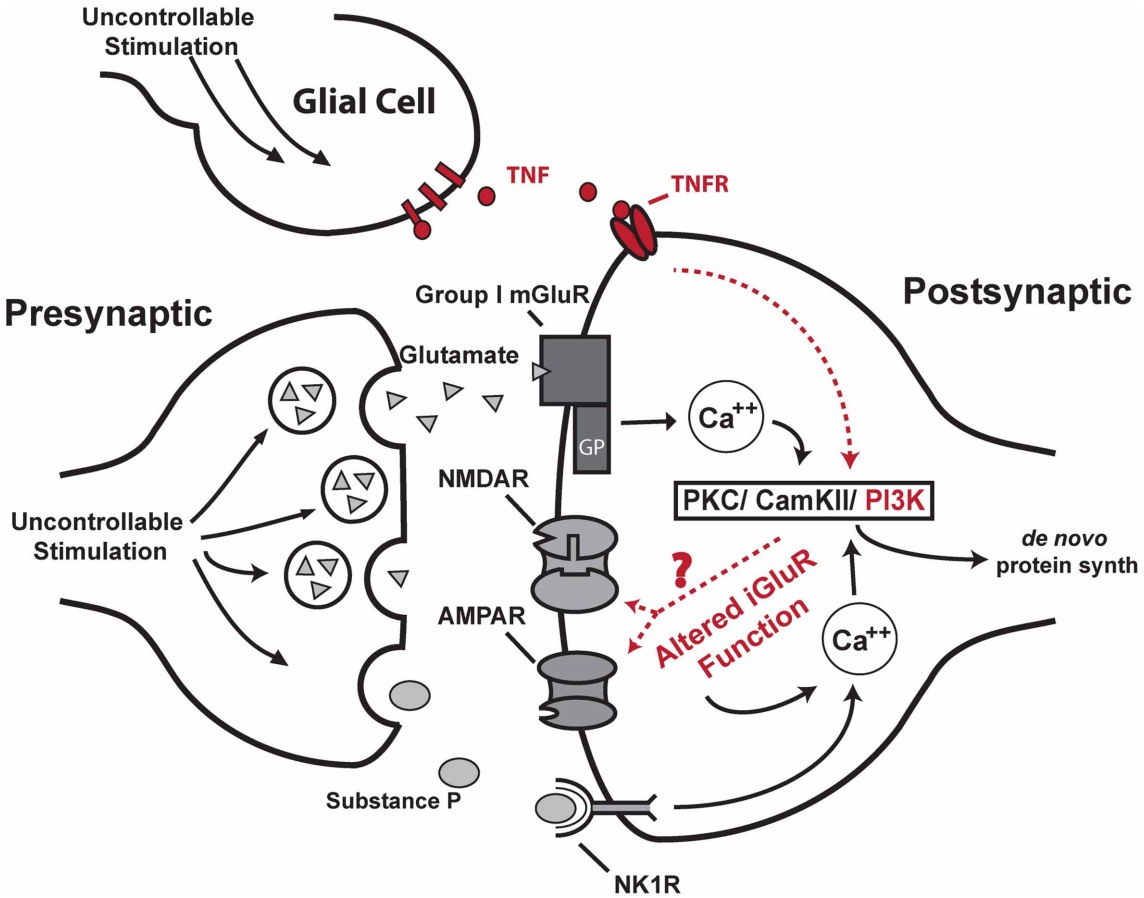

FIGURE 5 | Proposed neurobiological model for maladaptive spinal plasticity. The uncontrollable stimulation-induced spinal learning deficit requires the activation of group I metabotropic glutamate receptors and the substance P receptor NK1R which liberate intracellular calcium (Baumbauer et al., 2008; Ferguson et al., 2008a). This in turn activates downstream protein kinases PKC and CamKII, (Ferguson et al., 2006, 2008a; Baumbauer et al., 2007). These kinases are known to produce long-term alterations in ionotropic glutamate receptor (iGluR) function; however, the specific role of this signaling cascade in spinal learning remains an open question ("?"; dashed lines). Altered iGluR activation is known to further increase post-synaptic calcium levels through the NMDA receptor channel and calcium-permeable AMPA receptors, which may provide a mechanism for altered associative learning in the spinal cord. Increases in intracellular calcium can induce further protein kinase activity and *de novo* protein synthesis, all of which have all been shown necessary for the development of the stimulation-induced spinal learning deficit (Patton et al., 2004; Baumbauer et al., 2006; Huie et al., 2012a). Well-characterized features are shown in black. Areas of ongoing study are shown in red. Adapted from Ferguson et al. (2008a).

AMPA receptor (Ugolini et al., 1997; Xiao et al., 2001). AMPA receptors are thought to mediate the majority of rapid excitatory neurotransmission in the CNS, and their post-translational modification can have major effects on synaptic plasticity. For example, phosphorylation of AMPARs can increase their open channel time, resulting in enhanced post-synaptic currents and LTP (Derkach et al., 1999; Lee et al., 2000). In addition rapid trafficking of AMPA receptors to the plasma membrane can have a major effect on CNS plasticity (Malinow and Malenka, 2002). In the spinal cord, AMPAR phosphorylation and trafficking to synapses have been implicated in central sensitization and other forms of maladaptive plasticity (Galan et al., 2004; Ferguson et al., 2008a; Drdla-Schutting et al., 2012).

Pharmacological evidence has recently linked AMPAR over-activation to spinal learning deficits after intermittent nociceptive stimulation. Intrathecal delivery of a general AMPA receptor antagonist prevents induction of learning deficits by intermittent nociceptive stimulation (Hoy et al., 2012). However, this protective effect can only be observed at 24 h post-drug because general AMPAR antagonism reduces performance of the learned flexion response in the Horridge paradigm. A specific antagonist to AMPA receptors lacking the GluA2 subunit, however, selectively reverses the spinal learning deficit with no measurable side-effect on performance of the flexion response (Huie et al., 2012a). This is particularly relevant from a plasticity perspective because the GluA2 subunit renders AMPA receptors impermeable to Ca++ and GluA2-lacking receptors increase intracellular Ca++ levels which then, in turn, can activate a series of intracellular changes that can lead to a feedback loop of ever increasing excitatory plasticity and ultimately cellular dysfunction (**Figure 6**). Therefore, by engaging GluA2-lacking AMPARs intermittent nociceptive stimulation may produce lasting maladaptive plasticity that is characterized by spinal hyper-activity and reduced capacity for future spinal cord

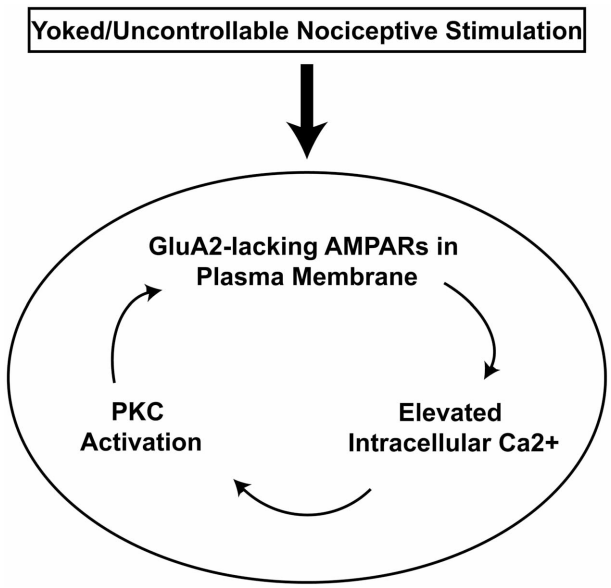

FIGURE 6 | AMPA receptor positive feedback-loop hypothesis for spinal metaplasticity. Spinal AMPARs are hypothesized to rapidly traffic to the plasma membrane in response to uncontrollable nociceptive stimulation in the periphery (box). Because GluA2-lacking AMPARs function as calcium ionophores, their localization to the membrane generates a cascade of increased intracellular calcium followed by activation of intracellular calcium detectors such as PKC that can then produce further increases in trafficking of AMPARs to the plasma membrane. This feedback loop (oval) of increasing AMPA receptor numbers may account for the observed pattern of saturation of spinal learning plasticity, spinal learning deficits, and nociceptive hyper-responsiveness that is observed after yoked/uncontrollable stimulation training.

learning. However, the specific mechanisms by which intermittent nociceptive stimulation engages AMPAR receptors remains a topic of intensive ongoing study.

One prevailing hypothesis is that proinflammatory cytokine TNFα contributes to maladaptive spinal plasticity mediated by AMPA receptors. Prior work has shown that the glial TNFα increases trafficking of GluA2-lacking AMPA receptors to the neuronal plasma membrane, thereby increasing synaptic receptor levels (Beattie et al., 2002; Stellwagen et al., 2005; Stellwagen and Malenka, 2006; Leonoudakis et al., 2008). A series of studies have linked TNFα modulation of AMPARs to altered synaptic plasticity in the cortex (Leonoudakis et al., 2004; Jia et al., 2007), hippocampus (Furukawa and Mattson, 1998; Beattie et al., 2002; Ogoshi et al., 2005), and spinal cord (Hermann et al., 2001; Ferguson et al., 2008a). In addition, TNFα induced AMPAR trafficking has recently been implicated in maladaptive plasticity in pain pathways (Choi et al., 2010). We have recently undertaken a series of experiments to test whether TNFα plays a role in maladaptive plasticity in the spinal learning paradigm (Huie et al., 2012a). Results suggest that glial TNFα does indeed play a critical role in undermining spinal learning potential (Huie et al., 2012a). Intrathecal delivery of TNFα was found to be sufficient to induce a spinal learning deficit for at least 24 h. In addition, uncontrollable stimulation produces TNF release in the spinal cord, providing a link to stimulation-induced maladaptive plasticity. The maladaptive effects of uncontrollable stimulation and TNFα are reversible with glial inhibitors and TNFα sequestering agents, providing strong evidence that glial TNF plays a central role in modulation of synaptic mechanisms of spinal cord learning. We also found that specifically blocking GluA2-lacking AMPARs protected against both the TNF- and stimulation-induced learning deficits (Vichaya et al., 2009; Huie et al., 2012a). The specific mechanisms of these effects remain a topic of intensive study (Garraway et al., 2012; Stuck et al., 2012). However, several converging lines of evidence suggest that TNFα may play an important role in tipping spinal plasticity toward a maladaptive form that undermines future spinal cord training and promotes nociception. This crucial role for TNFα in mediating maladaptive spinal plasticity caudal to a complete transection may also provide insight into the recovery of function after spinal contusion injury. TNF mRNA is significantly increased following spinal contusion, peaking within hours, but remaining elevated for days (Wang et al., 1996). Likewise, others have shown that TNF protein signaling at the site of injury may not peak until 2 days after injury and remain elevated up to one week (Gorio et al., 2005). Given our findings that increased TNFa expression can undermine adaptive plasticity, it is possible TNFa may also contribute to limiting behavioral recovery after contusion injury by altering plasticity.

Taken together, these findings provide strong evidence that the maladaptive effect of intermittent stimulation on spinal learning is a distinct form of plasticity that shares many mechanistic similarities with central sensitization. Ongoing research into these molecular mechanisms may have great potential for improving rehabilitation therapies. The final section explores how the findings from the spinal learning work has been extended to spinal contusion injury, and how our work on maladaptive spinal plasticity may provide insights toward improved rehabilitation after injury.

IMPLICATIONS FOR REHABILITATION AFTER SPINAL CORD INJURY

We have shown how uncontrollable nociceptive stimulation can undermine adaptive spinal modifications below a SCI. Although much of this work was done in an isolated spinal system, the implications of these findings on recovery of function after incomplete SCI are profound. In the present section we will first show how our instrumental spinal training paradigm can be applied to a more general spinal injury model, and then discuss the broader implications of spinal training as means to overcome maladaptive plasticity, and promote functional recovery.

Knowing that the spinal cord is capable of exhibiting varying types of spinal plasticity in response to subtle changes in stimulation, we reasoned that spinal instrumental training could provide a window into how these divergent forms of spinal plasticity may dictate functional recovery after SCI. To test the relationship between instrumental training and recovery of function after SCI we evaluated the long-term effects of acute master/yoked training in a T12 contusion model of SCI (Grau et al., 2004). We delivered two 30 min sessions of master/yoked training to rats 24 and 48 h post-contusion injury and then monitored recovery

of locomotor function over 42 days using the Basso, Beattie, Bresnahan (BBB; Basso et al., 1995) locomotor scale with behavioral raters who were blind to instrumental training condition. Yoked training produced persistent impairments in recovery of locomotor function (**Figure 7A**) as well as measures of sensory and autonomic recovery (Grau et al., 2004). Surprisingly, master rats did not show significant impairments. The yoked stimulation effect was further replicated, as just a single 6-min bout of uncontrollable stimulation to the tail, given 1 day post-injury, produced a deficit in locomotor recovery that was still apparent 6 weeks after injury (**Figure 7B**).

Such a finding illustrates just how vulnerable the spinal cord is to nociceptive input after injury, and how important the spinal cord is in processing nociceptive signals. This has clear translational relevance to human SCI, given that acute nociceptive input is a common feature of polytraumatic automobile accidents, the most common etiology of human SCI (Marino et al., 1999). Pain is a prevalent feature in SCI, affecting between 65–85% of the patient population (Siddall et al., 2003; Siddall, 2009). Although the mechanisms for neuropathic pain after SCI are not fully understood, the work reviewed in the present paper strongly suggests that co-morbid peripheral injuries may play a role in dictating both functional recovery and nociceptive sensitization.

One of the most clinically difficult forms of pain following SCI involves pain below the injury site. This phenomenon, known as dysesthetic pain syndrome, may represent an example of plasticity within spinal pain systems following SCI (Davidoff et al., 1987; Yezierski, 1996; Bennett et al., 2000; Hains et al., 2001; Bruce et al., 2002; Mills et al., 2002; Finnerup et al., 2003; Andersen et al., 2004; Carlton et al., 2009; Gwak et al., 2009; Hulsebosch et al., 2009; Leem et al., 2010). It has been argued that dysesthesia involves dysregulation of descending pain inhibitory mechanisms that affect spinal nociceptive circuitry. Two observations provide support for this. First, dysesthetic pain syndrome is most prevalent when there is damage specific to the spinal and mesencephalic trajectories of the ascending pain transmission pathways (Beric et al., 1988). These pathways are thought to be involved in activating segmental and descending pain inhibitory

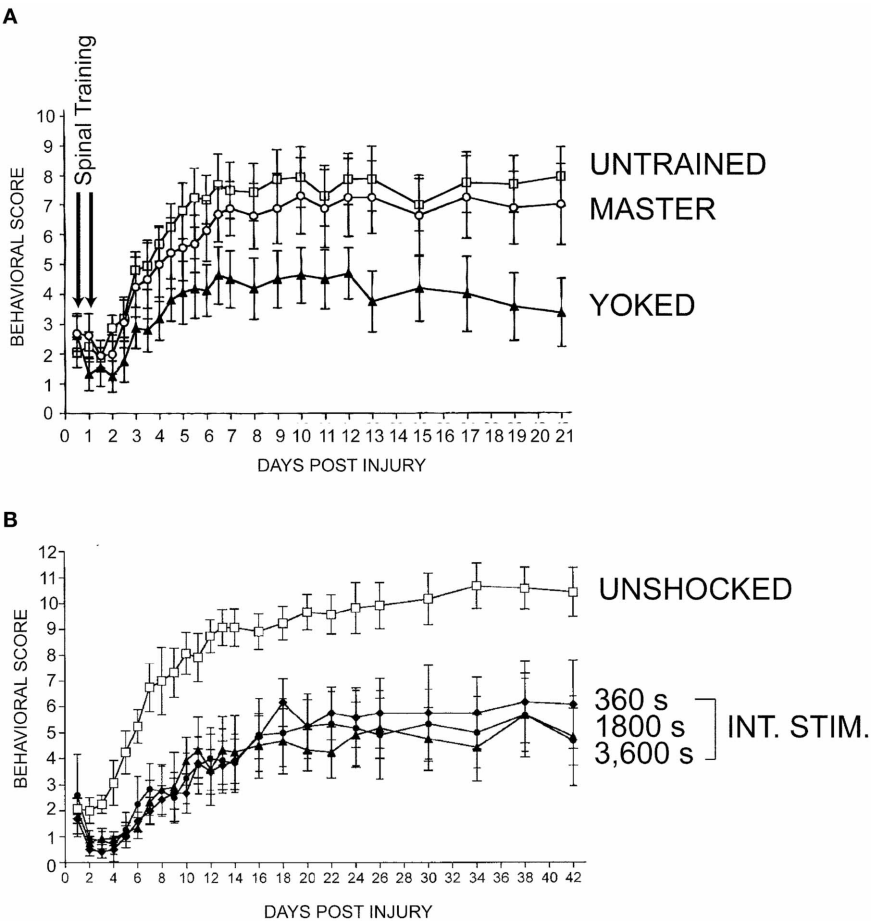

FIGURE 7 | The spinal learning deficit is associated with other forms of maladaptive plasticity such as impaired recovery of function after SCI. (A) Rats were given 2 days of spinal training in the acute phase after a thoracic contusive SCI delivered with the NYU/MASCIS impactor (Gruner, 1992). **(B)** Varying amounts (in seconds, s) of intermittent stimulation (INT. STIM.) were administered to the tail one day following contusive SCI. Data show as little as 6 min (360 s) of intermittent stimulation to the tail is sufficient to undermine recovery of function. Y-axes represent a 12-point modified version of the Basso, Beattie, Bresnahan (BBB) locomotor scale, over 3 weeks **(A)** or 6 weeks **(B)** (Basso et al., 1995; Ferguson et al., 2004). Modified from Grau et al. (2004).

mechanisms. In contrast, most studies have shown that complete injury to the anterolateral ascending pain transmission pathways does not produce dysesthetic pain (Beric, 1993; Yezierski, 1996; Finnerup et al., 2003; Wasner and Brock, 2008). Second, there is evidence that many of the descending fibers damaged by SCI have a net inhibitory effect in uninjured subjects. Under non-pathological conditions, nociceptive plasticity in the spinal cord is tightly controlled by descending modulation from the brain, preventing spontaneous emergence of maladaptive pain syndromes. Following SCI there is significant alteration of reflexes below the lesion (Hubscher and Johnson, 2000; Grau and Patterson (eds.), 2001). Nociceptive reflexes demonstrate increased excitability accompanied by a loss of GABAergic inhibition (Zhang et al., 1994; Yezierski, 1996), as well as loss of descending inhibition through noradrenergic and serotonergic fibers located in the dorsolateral funiculus (Watkins et al., 1984; Faden et al., 1988; Liu et al., 1990). This increased excitability can be reduced by administration of the $GABA_B$ agonist baclofen (Hao et al., 1992) and serotonergic/noradrenergic drugs (Barbeau and Norman, 2003; Hains et al., 2003).

Interestingly, the expression of spinal LTP is normally inhibited by descending pathways (Sandkuhler and Liu, 1998; Gjerstad et al., 2001). Using our spinal training paradigm, we showed that intermittent stimulation given *prior* to complete transection was not sufficient to produce a learning deficit, indicating a brain-mediated descending protection against the deleterious effects of intermittent stimulation. Further, we showed the necessity for the DLF in this effect. We lesioned the DLF at T2, then gave intermittent stimulation followed by a complete transection at T8, and found that by removing the protection of this descending system, rats exhibited a spinal learning deficit (Crown and Grau, 2005). Similarly, as SCI may disrupt these fibers, it is possible that the vulnerability to nociceptive plasticity after injury reflects a loss of descending inhibition (Hains et al., 2002). Thus, spinal injury may create a predisposition toward the lasting maladaptive effects of nociceptive input. This notion has even greater clinical impact, when one considers the potential blockade of descending inhibition conferred by surgical anesthesia. To test this, Washburn et al. (2007) assessed the effect of the anesthetic pentobarbital on the stimulus-induced spinal learning deficit. We first delivered 6 min of intermittent uncontrollable stimulation (AC 60 Hz, 1.5 mA) to rats that were under pentobarbital anesthesia, 24 h after complete T2 transection. The next day these rats were tested for spinal instrumental learning. These rats failed to learn, indicating that pentobarbital anesthesia does not protect against the spinal mechanisms responsible for the induction of the stimulation-induced learning deficit. We next assessed whether pentobarbital anesthesia could protect against the induction of the learning deficit if *intact* rats were given intermittent uncontrollable stimulation under anesthesia. Intact rats were given an injection of either pentobarbital or saline vehicle, followed by 6 min of uncontrollable intermittent stimulation to the tail and the next day all rats were given a complete transection, followed 24 h later with spinal instrumental testing. Rats that were not under anesthesia during the intermittent uncontrollable stimulation (saline-treated), did not exhibit a learning deficit, replicating the earlier finding from Crown and Grau (2005) that descending supraspinal input provides protection against the stimulation-induced deficit. Interestingly, those rats that were under pentobarbital anesthesia during the intermittent uncontrollable stimulation did exhibit a learning deficit when later tested, indicating that the descending protection was undermined by anesthesia, leaving the spinal cord susceptible to the induction of maladaptive nociceptive plasticity. These findings highlight how important it is to be cognizant of the capacity of the spinal cord for nociceptive plasticity, particularly when supraspinal controls are removed under anesthesia. The pain memory that can be formed during surgical procedures could contribute to the development of neuropathic pain, and in the instance of SCI, could undermine functional recovery.

From our studies detailing the varying stimulation parameters that produce divergent effects on adaptive and maladaptive forms of spinal plasticity, it is clear that the timing and controllability of stimulation are very important predictors of recovery of function. Harnessing the specific capacity for spinal cord learning may improve rehabilitative training of limb position after SCI. The neurobiological basis of training-induced plasticity within the spinal cord has been studied in some detail. For example, following chronic complete SCI there is an increase in glycinergic (de Leon et al., 1999; Cantoria et al., 2012) and GABAergic inhibitory tone (Edgerton et al., 2001; Tillakaratne et al., 2002) that has been shown to hinder locomotor performance. Systemic administration of the glycinergic antagonist strychnine improves locomotion in untrained animals, whereas animals with previous step training do not require strychnine (de Leon et al., 1999). Moreover, strychnine does not yield further improvement beyond step-training alone, suggesting that spinal training has the capacity to modulate inhibitory neurotransmitter levels in the spinal cord. However, the type of training used has a profound influence on glycinergic tone. If animals are re-trained to stand after treadmill step training, there is a reemergence of glycinergic inhibition, resulting in poor treadmill performance that was reversible by strychnine.

Indeed the locomotor physiology literature has produced substantial evidence that following complete transection the spinal cord can be trained to elicit stepping (for review see Edgerton et al., 1997). For example, Lovely et al. (1990) found that treadmill training can improve stepping in spinally transected cats. A similar improvement can be quantified through the use of kinematics and electromyography (EMG) in rats and mice, and consistent differences have been observed between untrained and trained animals in a variety of different contexts (Drew and Rossignol, 1987; Roy et al., 1998; Courtine et al., 2009; van den Brand et al., 2012). Importantly, motor training after SCI has been shown to be exquisitely task-specific; training on one task can interfere with training on other tasks (Garcia-Alias et al., 2009; de Leon et al., 2011). Collaborative work has revealed that rats that are trained to stand on a treadmill have impaired instrumental learning (Bigbee et al., 2007). Interestingly, stand training is also known to interfere with locomotor training after SCI (de Leon et al., 1999), suggesting that instrumental training in the Horridge paradigm may be able to predict locomotor recovery. These findings illustrate how critical it is to control the types of input that are received after SCI. A study from Petruska et al. (2007) highlighted this importance. They trained transected rats to walk on a treadmill, and found that this training regimen successfully improved locomotor performance in most of the

trained animals. Interestingly, a subset of rats was found to have lesions on their hindpaws and the beneficial effects of training were markedly diminished in these rats. This finding suggests that the noxious input that these animals were receiving may have been undermining their capacity for adaptive locomotor plasticity. Further, recent research indicates that even manipulations that are considered relatively passive or innocuous may be inducing unintended maladaptive plasticity, and doing untold damage to recovery of function. Studies in the human patient population of the efficacy of relatively passive rehabilitative efforts after SCI (including stretching to increase range of motion) have revealed that these interventions may have few beneficial effects on locomotor recovery (Harvey et al., 2002, 2009). It has been shown in rodents that peripheral input such as leg immobilization in a wheelchair model, muscle stretching, or repeated tactile stimulation produce lasting changes in spinal circuitry that can impair recovery (Hutchinson et al., 2004; Hoschouer et al., 2010; Caudle et al., 2011).

SUMMARY

The existing literature indicates many similarities between central sensitization within spinal pain pathways and stimulus-induced deficits in spinal learning after SCI. Taken together the findings indicate that central nociceptive sensitization and adaptive spinal learning are opposing forms of spinal plasticity (**Figure 8**). Adaptive training of the spinal cord undermines the development of central sensitization, and conversely central nociceptive stimulation undermines the capacity for spinal learning.

While studies of spinal cord plasticity after SCI have uncovered a remarkable degree of plasticity, it must also be remembered that this plasticity is a two-edged sword; adaptive processes can foster recovery and reduce neuropathic pain while maladaptive mechanisms have the opposite effect. This review has aimed to bring to light the ongoing struggle between nociceptive and adaptive forms of spinal plasticity after injury. We have shown that certain peripheral stimuli limit adaptive spinal learning and may actually promote maladaptive forms of spinal plasticity. This maladaptive plasticity may include hyperexcitability of spinal nociceptive systems, leading to pain and spasticity. Maladaptive spinal plasticity may ultimately lead to intractable pain in SCI, a common problem for SCI patients (for review see Yezierski, 1996, 2000, 2009; Bruce et al., 2002; Weaver et al., 2002; Finnerup and Jensen, 2004; Crown and Grau, 2005; Crown et al., 2006, 2008; Finnerup et al., 2007).

The present paper has focused on the negative side of spinal plasticity: the mechanisms by which inappropriate spinal training (uncontrollable electrical stimulation) and persistent nociceptive stimuli can impair spinal cord learning. In the companion paper in this issue Grau et al. (2012) discuss the positive side to spinal training: the mechanisms by which specific types of spinal cord training can protect against the development of nociceptive plasticity and may promote spinal adaptation and recovery of function after SCI. An effective rehabilitation strategy will integrate the messages from both papers to maximize positive elements of spinal cord training while limiting the negative consequences of inappropriate stimulus timing and nociceptive input below the injury. Ongoing work is focused on determining the specific

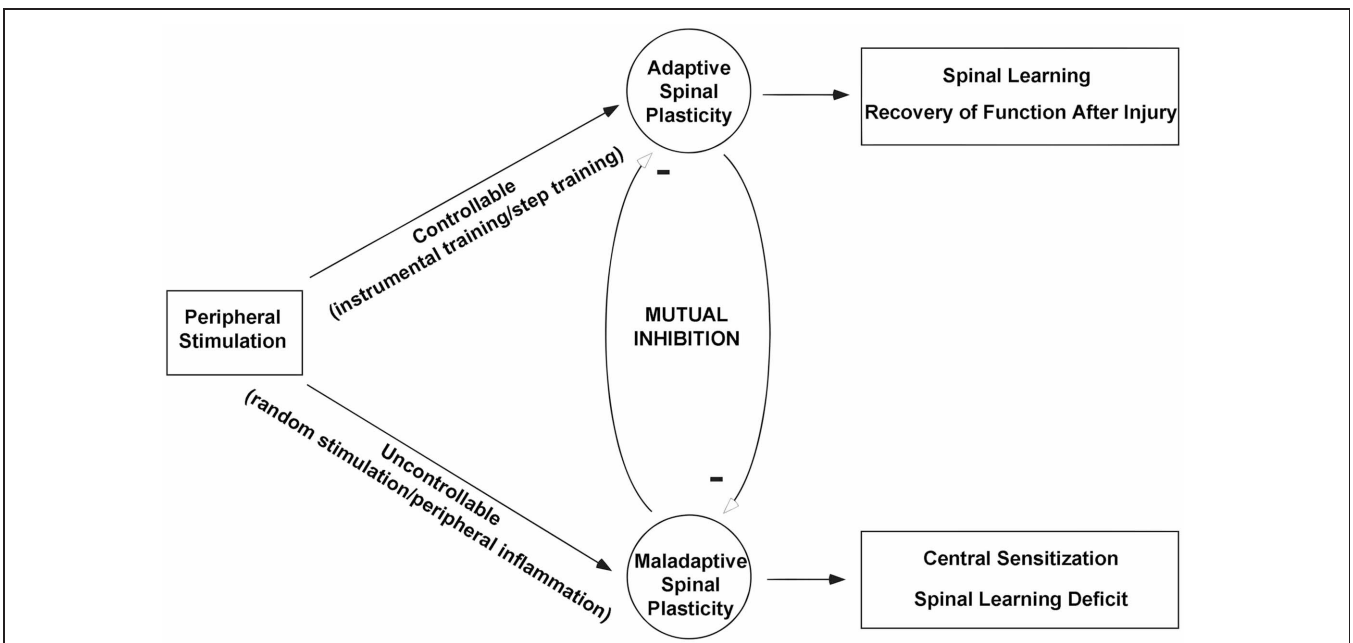

FIGURE 8 | Theoretical framework for understanding the relationship between adaptive and maladaptive spinal cord plasticity. After spinal cord injury peripheral stimulation can take a controllable form such as instrumental training or step training that promotes adaptive plasticity and leads to further improvements in spinal learning and recovery of function. On the other hand peripheral stimulation delivered in an uncontrollable form such as random (position-independent) stimulation or peripheral inflammation produces a maladaptive spinal plasticity that promotes central sensitization and deficits in future spinal cord learning. Modified from Ferguson et al. (2006).

conditions that limit maladaptive plasticity, while promoting adaptive spinal plasticity. This work will be critical in order to tailor rehabilitative and restorative therapies toward maximizing recovery of function after SCI.

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Central nociceptive sensitization vs. spinal cord training: opposing forms of plasticity that dictate function after complete spinal cord injury

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The spinal cord demonstrates several forms of plasticity that resemble brain-dependent learning and memory. Among the most studied form of spinal plasticity is spinal memory for noxious (nociceptive) stimulation. Numerous papers have described central pain as a spinally-stored memory that enhances future responses to cutaneous stimulation. This phenomenon, known as central sensitization, has broad relevance to a range of pathological conditions. Work from the spinal cord injury (SCI) field indicates that the lumbar spinal cord demonstrates several other forms of plasticity, including formal learning and memory. After complete thoracic SCI, the lumbar spinal cord can be trained by delivering stimulation to the hindleg when the leg is extended. In the presence of this response-contingent stimulation the spinal cord rapidly learns to hold the leg in a flexed position, a centrally mediated effect that meets the formal criteria for instrumental (response-outcome) learning. Instrumental flexion training produces a central change in spinal plasticity that enables future spinal learning on both the ipsilateral and contralateral leg. However, if stimulation is given in a response-independent manner, the spinal cord develops central maladaptive plasticity that undermines future spinal learning on both legs. The present paper tests for interactions between spinal cord training and central nociceptive sensitization after complete spinal cord transection. We found that spinal training alters future central sensitization by intradermal formalin (24 h post-training). Conversely intradermal formalin impaired future spinal learning (24 h post-injection). Because formalin-induced central sensitization has been shown to involve NMDA receptor activation, we tested whether pre-treatment with NMDA would also affect spinal learning in manner similar to formalin. We found intrathecal NMDA impaired learning in a dose-dependent fashion, and that this effect endures for at least 24 h. These data provide strong evidence for an opposing relationship between nociceptive plasticity and use-dependent learning in the spinal cord. The present work has clinical implications given recent findings that adaptive spinal training improves recovery in humans with SCI. Nociception below the SCI may undermine this rehabilitation potential.

Keywords: pain, nociception, plasticity, spinal cord injury, spinal cord learning, recovery of function

INTRODUCTION

Research over the past 50 years has revealed the spinal cord to be surprisingly plastic. The spinal cord has been shown to support a number of simple forms of learning, including habituation and sensitization, as well as Pavlovian and instrumental conditioning (Thompson and Spencer, 1966; Fitzgerald and Thompson, 1967; Grau et al., 1998). This remarkable capacity for adaptability in response to stimuli has led researchers to investigate how spinal plasticity might be utilized to promote functional recovery after spinal cord injury (SCI). To this end, researchers have designed behavioral training programs to engage the inherent plasticity in spinal motor systems. Training on a treadmill has been shown to evoke locomotor activity, and induce weight-supported stepping in completely transected cats (Lovely et al., 1986; Barbeau and Rossignol, 1987; De Leon et al., 1998). Similar effects have been observed in a variety of species, and under varying conditions, demonstrating that multiple forms of locomotor training can induce adaptive alterations in spinal plasticity that improves recovery after SCI (Edgerton et al., 1992; Bregman et al., 1997; Raineteau and Schwab, 2001; Edgerton and Roy, 2009). In order for functional recovery to be successful, appropriate sensory feedback, including proprioceptive and cutaneous afferent input, is necessary (Bouyer et al., 2001; Bouyer and Rossignol, 2003). Unilateral deafferentation of spinally transected

cats results in impaired locomotor recovery (Giuliani and Smith, 1987). Likewise, the disruption of even a small number of peripheral nerves prior to SCI can greatly limit adaptive locomotor recovery (Bouyer and Rossignol, 2003; Frigon and Rossignol, 2009). These findings highlight the essential role of afferent input in inducing plasticity within locomotor circuits. If the delicate interaction between sensory input and motor output is disturbed, recovery of spinal function can be severely impaired (Frigon and Rossignol, 2009).

These findings illustrate the plastic nature of the spinal cord, and highlight the importance of promoting adaptive spinal modifications in order to combat the deleterious effects of SCI.

The capacity for plasticity after SCI also creates an environment in which the spinal cord is vulnerable to maladaptive changes. Potentiation of the response to nociceptive afferent input in the superficial dorsal horn can produce lasting changes in pain reactivity. This phenomenon, known as central sensitization, may be a mechanism by which intractable neuropathic pain is induced (Woolf, 1983; Woolf and Salter, 2000). Interestingly, this effect bears a striking resemblance to long-term potentiation (LTP), an NMDA-mediated process that has been long-believed to be the neurobiological basis for learning and memory (Bliss and Lomo, 1973; Ikeda et al., 2003; Ji et al., 2003; Woolf, 2007). The similarity between LTP in the brain and central sensitization in the spinal cord has raised the possibility that nociceptive plasticity is akin to a form of learning, and lasting pain states are in essence a nociceptive memory (Sandkuhler et al., 2000; Ji et al., 2003; Crown et al., 2005, 2006, 2008).

From the evidence presented above, it is apparent that the spinal cord is capable of supporting a wide range of plastic processes that, depending on the type of stimulus, can either promote adaptive changes or exacerbate nociceptive activity. Despite our knowledge of these opposing processes, the relationships between spinal nociceptive memory and spinal memory for adaptive training are not well-understood. Yet, uncovering the interactions between these different forms of spinal neuroplasticity has clinical relevance for developing rehabilitative therapies that maximize beneficial recovery and minimize harmful side effects such as pain. To study spinal plasticity mechanistically we have used a simple model of instrumental learning in the isolated spinal cord (Buerger and Fennessy, 1970; Grau et al., 1998). Rats with complete spinal transections receive electrical stimulation to the tibialis anterior muscle of one hindlimb whenever that limb is extended (*controllable stimulation*). Rats learn over time to keep the limb flexed in order to reduce stimulation exposure, thus exhibiting a simple form of instrumental (response-outcome) learning. This training paradigm has been shown to produce a number of beneficial effects on spinal function (see Ferguson et al., 2012 and Grau et al., 2012, in this issue).

Conversely, if rats receive electrical stimulation of the tibialis anterior muscle that is independent of leg position (*uncontrollable stimulation*), they are unable to learn the instrumental response. Further, when these rats are later tested with controllable stimulation, they continue to exhibit a learning deficit, even if rats are tested on the contralateral limb (Joynes et al., 2003). This finding, along with a series of other pharmacological and physiological studies, indicates that prior exposure to uncontrollable stimulation does not simply produce a peripheral, motoric effect, but instead induces a lasting maladaptive alteration in spinal plasticity (Crown et al., 2002a; Baumbauer et al., 2009; for review see Grau et al., 2006). Interestingly, prior work indicates that uncontrollable stimulation also induces nociceptive plasticity (Ferguson et al., 2006; Huie et al., 2012a). Tests of tactile reactivity with von Frey filaments have shown that uncontrollable (yoked) stimulation can induce mechanical allodynia (Ferguson et al., 2006; Huie et al., 2012a). Interestingly, both the induction of the spinal learning response and the induction of the learning deficit have been shown to require NMDA activation, providing further mechanistic similarities between adaptive and nociceptive plasticity and learning in the spinal cord. To further elucidate whether uncontrollable stimulation induces spinal nociceptive activity, we have previously used electrophysiological and pharmacological methods to investigate the fiber types that are engaged by this stimulation regimen. Baumbauer et al. (2007), found that intrathecal blockade of the NK1 (substance P) receptor blocked the induction of a learning deficit normally produced by uncontrollable stimulation. Likewise, electrical stimulation of the sciatic nerve did not induce a learning impairment until shock intensity was increased to a level that engaged C-fibers (Baumbauer et al., 2008). While instrumental learning is not blocked by pretreatment with a NK1 antagonist, the shock intensity needed to elicit an intermediate flexion force is well within the range that Baumbauer et al. (2008) found to elicit some C-fiber activity and a robust A-delta response. On the basis of these observations, we have suggested that the induction of the learning deficit requires C-fiber activity.

Prior work has also shown that spinal learning deficits can be induced by the peripheral administration of inflammatory agents known to induce central sensitization, such as carrageenan, and capsaicin (Ferguson et al., 2006; Hook et al., 2008). As with uncontrollable stimulation, peripheral inflammation induced a learning deficit that was observed when testing was administered on the contralateral limb, yielding further evidence for central nociceptive plasticity. Thus, using this simple behavioral model of spinal plasticity provides a mechanism to study both maladaptive nociceptive plasticity and adaptive alterations in spinal learning. The present study is designed to gain further insight into these opposing processes, by testing the interaction between spinal training and nociceptive plasticity. We first ask how spinal training history affects nociceptive plasticity, and then conversely, how nociceptive activity may alter the capacity for spinal learning.

We first assessed the capacity for an inflammatory agent known to induce central sensitization (intradermal formalin) to produce tactile hyper-reactivity in the spinally transected rat. We then tested what effect spinal training (with either controllable or uncontrollable stimulation) prior to formalin administration may have on nociceptive responding. To further assess the interaction between nociceptive plasticity and spinal learning, we then tested whether formalin administration is sufficient to undermine spinal learning, and whether this effect is centrally-mediated. Finally, given the findings that central sensitization is an NMDA receptor-mediated process (Dickenson and Sullivan, 1991), we tested

whether direct central activation of NMDA receptors induces a spinal learning deficit.

MATERIALS AND METHODS

We performed three independent experiments to investigate the relationship between spinal learning and nociceptive sensitization. In the first experiment we delivered three different spinal cord training procedures and then 24 h later delivered intradermal formalin or vehicle (3 training groups × 2 formalin conditions; $n = 7$/group; $N = 42$ total). We then evaluated tactile responsiveness (**Figures 2–4**). In a separate group of rats we delivered intradermal formalin and then evaluated spinal learning potential immediately as a function of formalin dose-response (**Figure 5**; $n = 2$–6/dose group; $N = 17$ total). In an independent replication of the most effective dose, we tested the effects of formalin on spinal learning 24 h later ($n = 6$/group, $N = 12$ total). Finally, in a third set of rats we delivered intrathecal NMDA at doses that are known to produce spontaneous nociception and tested spinal training potential 24 h later ($n = 12$/group; $N = 48$ total). The experimental designs for each study are depicted in **Figures 2**, **5A**,**C**, and **6A**. The specific procedures are described below.

ANIMALS

Subjects were adult (100–120 day old, $N = 119$) male Sprague-Dawley rats (Harlan, Houston, TX, USA). Rats were individually housed in an AAALAC-approved, temperature-controlled environment with *ad libitum* access to food and water. Rats were maintained on a 12 h light/dark cycle with experiments performed during the last 6 h of the light cycle. All experiments adhered to the NIH Guide and were approved by the Animal Care Committee at Texas A&M University.

SPINAL CORD TRANSECTION AND INTRATHECAL CANNULA INSERTION

Surgery was performed under pentobarbital anesthesia (50 mg/kg, i.p.). Rats were placed in a stereotaxic instrument and a small gauze "pillow" was placed under the chest to raise and support the area around the second thoracic vertebra (T2). After localizing T2 through the skin, a rostro-caudal incision was made and the muscles were blunt dissected to reveal the intervertebral space rostral to T2. Rongeurs were used to clear ligaments and expose the spinal cord in between T1 and T2 and the spinal cord was transected by cauterization. Gel foam was placed in the transection site. For intrathecal drugs delivery experiments, a cannula was implanted after spinal transection. The cannula consisted of 25 cm of PE-10 tubing fitted with a 0.23 mm (diameter) stainless steel guide wire (Small Parts, USA) that was threaded 9 cm caudally from T2 into the subarachnoid space between dura and white matter to lie on the dorsal cord. The exposed end of the tubing was secured with cyanoacrylate and the guide wire was gently pulled from the tubing. Spinal transections were confirmed by (1) inspecting the cord during the operation, (2) observing the behavior of the rats after recovery to ensure that they exhibited paralysis below the level of the forepaws and did not vocalize to the leg shock, and (3) examining the spinal cord post-mortem in a randomly selected subset of the animals.

BEHAVIORAL APPARATUS

We assessed spinal learning capacity as well as tactile responsiveness using a behavioral apparatus previously described (Grau et al., 1998; Ferguson et al., 2006; **Figure 1**). Briefly, spinally transected rats were placed in plexiglas tubes containing slots to allow the hindlimbs to hang freely. Rats were secured with an insulated wire belt that was gently wrapped around the rat and passed through holes on the side of the tube.

SPINAL LEARNING PARADIGM

For spinal training studies, stainless steel leads from a BRS/LVE AC stimulator (Model SG-903; Laurel, MD, USA) were implanted into the tibialis anterior muscle, and the skin 1.5 cm above the ankle. Stimulation (60 Hz, constant current biphasic AC) intensity was initially set to 0.1 mA and then adjusted so that a single 0.3 s stimulus yielded a standardized flexion force of 0.4 N. Force was measured using strain gauge (Fort-1000; World Precision Instruments) attached to the foot with a monofilament plastic line (4-lb test; Stren, DuPont). The strain gauge output was fed through a multimeter calibrated to allow conversion between voltage and force in N. Instrumental (response-outcome) learning in the spinal cord was evaluated by arranging a relationship between leg position (response) and shock to the tibialis anterior muscle (outcome). Prior work has shown that in the presence of this controllable stimulation the spinal cord rapidly learns to hold the leg in a flexed position, minimizing shock exposure (Buerger and Fennessy, 1970; Grau et al., 1998; Jindrich et al., 2009). We assessed leg position by attaching a stainless steel contact electrode (7 cm × 0.46 mm) to the plantar surface of one hind leg and submerging the tip of the electrode 4 mm below the surface of an underlying saline solution. By placing a ground wire in the solution and attaching a fine wire to the contact electrode, we can monitor whether the leg is in an extended position, completing the circuit, or in a flexed position. The state of the circuit was monitored using an analog to digital converter (sample rate = 30 Hz) with digital outputs sent to a Macintosh computer. Stimulation was delivered each time the contact electrode touched the underlying solution. Stimulus onset occurred upon contact with the solution and stimulus offset occurred when the leg was lifted (minimum stimulus duration = 80 ms). Stimulation occurred for the duration of contact. Using this simple dichotomous measure of leg position (up vs. down) allows us to measure the time in the down position and the number of flexion responses. From these two measures we derive the mean response duration for each animal within 60 s time bins over the 30 min instrumental training period (Grau et al., 1998). Response duration for each animal was determined using the following formula:

$$\text{Response duration} = (60 - \text{seconds in solution}_i)/(\text{flexion number}_i + 1),$$

where i is the current training bin.

MASTER/YOKED TRAINING PROCEDURES

The spinal cord was trained using a well-established three-group design consisting of master, yoked, and unshocked rats

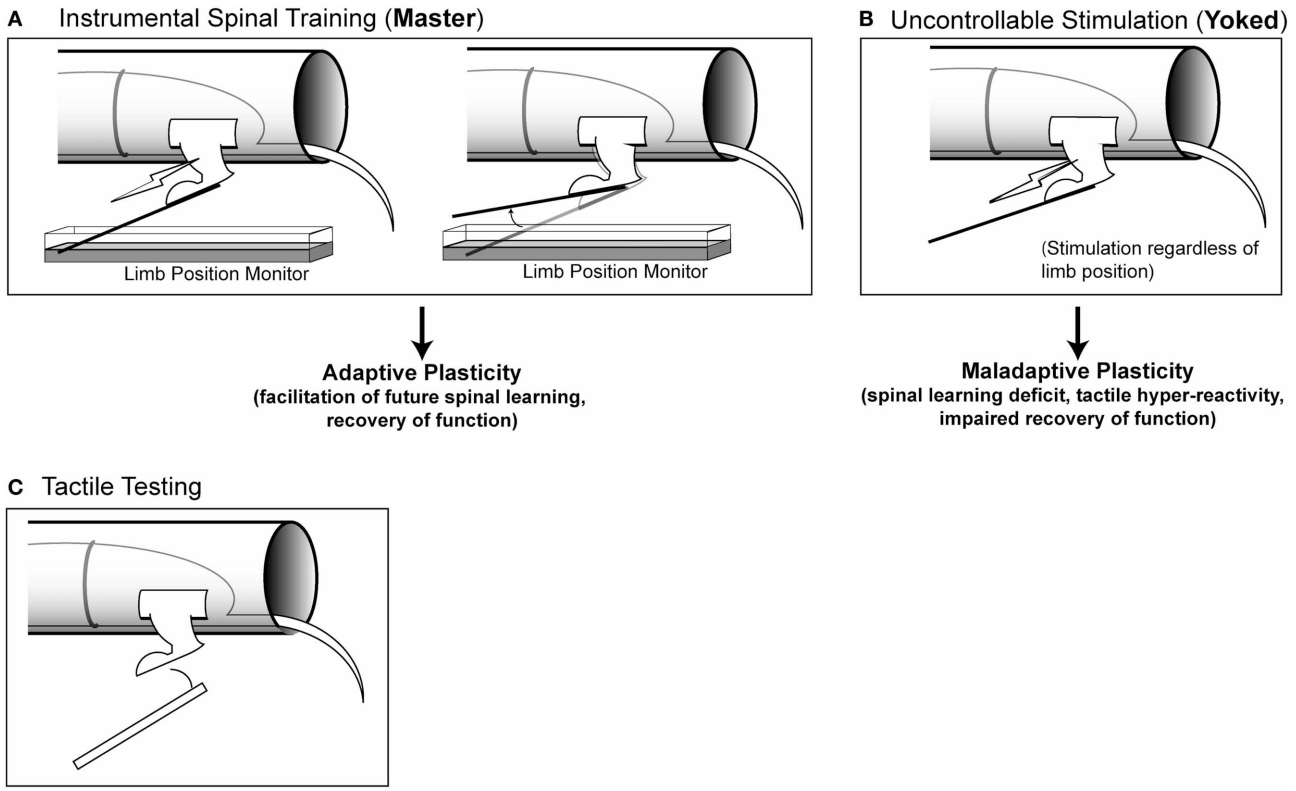

FIGURE 1 | Modes of stimulation and testing. (A) Instrumental spinal training. Master rats receive stimulation to the tibialis anterior muscle of one hindlimb that is contingent upon leg position. When the limb is extended, stimulation is delivered, when the limb is flexed, stimulation is terminated. Master rats learn over time to keep the limb flexed to reduce stimulation exposure (spinal learning). **(B)** Uncontrollable stimulation. Yoked rats receive stimulation at the same time as their Master counterparts, regardless of limb position. These rats do not learn a response-outcome association and continue to fail when later tested with controllable stimulation. **(C)** Tactile testing. To assess hyper-reactivity, calibrated von Frey filaments are used to probe the ventral surface of the hindlimb. Filaments of increasing stiffness are used, until a response is made, and the force necessary to elicit a response is recorded.

run simultaneously in sets of three rats (**Figures 1A** and **1B**; Horridge, 1962; Buerger and Fennessy, 1970; Grau et al., 1998). All rats were prepared for spinal training as described above and then randomized to the three different conditions. Master rats received response-contingent stimulation: stimulation was delivered to the leg when it was in an extended position. Yoked rats received uncontrollable leg stimulation: the tibialis anterior was stimulated whenever their paired master extended the leg. Unshocked rats received no leg stimulation. Prior work has shown that under these conditions the master rats will acquire the flexion response, and yoked rats will fail (Horridge, 1962; Buerger and Fennessy, 1970; Grau et al., 1998; Jindrich et al., 2009). When all three groups are later re-tested with response-contingent stimulation, the master animals re-acquire the response at a faster rate than unshocked controls and the yoked animals fail to learn (Grau et al., 1998, 2004; Crown and Grau, 2001; Huie et al., 2012a). The learning deficit produced by yoked training represents a lasting form of maladaptive spinal plasticity that endures for >24 h in complete transection injuries, and produces long-term (>6 week) impairments in locomotor recovery after contusive SCI (Crown et al., 2002a; Grau et al., 2004).

INTRADERMAL FORMALIN

To test for interactions between central sensitization and spinal training, we used the formalin test, a well-characterized central sensitization model from the pain literature (reviewed in Le Bars et al., 2001). Spinalized rats were given a single 50 μl subcutaneous injection of formalin in 1 of 4 concentrations (0, 5, 10, or 15%) in 0.9% saline into the dorsal surface of one hindpaw (in contrast to the plantar surface where tactile testing was performed). This manipulation produces a well-documented sensitization of spinal neurons (Coderre, 2001) that can be blocked by N-methyl-D-aspartate receptor (NMDAR) antagonists (Coderre and Melzack, 1992; Yamamoto and Yaksh, 1992).

TACTILE TESTING

Hindpaw tactile testing was performed on spinalized rats placed in loose restraint tubes and secured as described the "Behavioral Apparatus" section (**Figure 1C**). After a 5 min acclimation period baseline tactile reactivity was established using von Frey stimuli (Semmes-Weinstein). Von Frey stimuli consisted of standardized polymer monofilaments of differing diameters that were delivered serially with increasing von Frey filament forces until the stimulation elicited a flexion response. When flexed against the skin,

each filament delivers a standard force of a known intensity. All tactile reactivity testing was performed on the plantar surface of the hindpaw (in contrast to the dorsal surface where formalin was delivered). All tactile testing was performed by raters who were blind to experimental conditions. Baselines were established prior to spinal training (**Figure 2**). Baseline was established twice on both the ipsilateral (shocked) and contralateral leg in a counterbalanced ABBA order and then averages were produced for each leg. Rats were then given master/yoked/unshocked training to one leg. Experimenters who were blind experimental condition performed threshold testing using calibrated von Frey filaments on the ipsilateral and contralateral limb in a counterbalanced ABBA sequence every 5 min for 60 min post-injection. Re-testing of the same paw was spaced 2 min apart. Further details can be found in prior studies (Ferguson et al., 2006).

INTRATHECAL NMDA DELIVERY

Spinalized rats received intrathecal injections of NMDA (Tocris, Ellisville, MO) in 1 of 3 doses (1.0, 10.0, or 100 nmol) with a control of 15 μl 0.9% saline. The drug was delivered over the course of 3 min followed by a 10 μl saline flush over 2 min. Immediately after drug delivery rats were placed in Plexiglas tubes. Twenty-four hours later rats were prepared as described above under "Spinal Learning Paradigm" and tested with 30 min of response-contingent leg stimulation.

STATISTICS

Results were analyzed using balanced experimental designs and mixed, factorial analyses of variance (ANOVA) by the GLM protocol in SPSS v.19 (IBM). The general statistical workflow adhered to highly-cited analytical standards (Keppel and Wickens, 2004). The statistical plan consisted of testing higher-order interactions that were intrinsic to the *a priori* experimental design, and then significant effects were distilled in waves of lower order interaction testing, and ultimately testing of main effects followed by Tukey's *post-hocs* on group means where appropriate. Significance was assessed at $p < 0.05$. All graphs reflect group means and error bars reflect standard error of the mean (SEM).

RESULTS

The experiments were designed to test for cross-talk between spinal training and nociceptive plasticity in complete SCI. The experiments are complementary to those reported elsewhere and interested readers are encouraged to examine complementary studies in Ferguson et al. (2006, 2008a) and Huie et al. (2012a). The present paper is also linked to companion reviews by Grau et al. (2012) and Ferguson et al. (2012) in the present issue of Frontiers in Integrative Physiology which provide further theoretical background for the present studies.

IRRITANT-INDUCED SENSITIZATION OF TACTILE SENSITIVITY BELOW COMPLETE SCI

To test whether a nociceptive barrage could undermine normal spinal function below a complete SCI we used a well-established chemical irritant that directly drives nociceptors: intradermal formalin. Numerous papers have shown that a dilute formalin solution injected into the dorsal surface of the hindpaw strongly activates primary nociceptive afferents resulting in both peripheral and central sensitization (Hunskaar and Hole, 1987; Coderre and Melzack, 1992; Yamamoto and Yaksh, 1992; Le Bars et al., 2001; Yashpal et al., 2001). Behaviorally, the formalin test results in tactile hyper-reactivity as well as spontaneous nocifensive behaviors such as licking the paw, or hind limb guarding (Dubuisson and Dennis, 1977). In intact animals these nocifensive behaviors occur in two phases: an early phase (5 min), followed by a quiescent period and then re-emergence of a late phase (25–60 min). It has been argued that the different phases of formalin pain involve different mechanisms that may be organized at different anatomical levels. The early phase reflects hyperactivity in spinal nociceptive system that is then inhibited by segmental and descending brain stem pathways. The second phase is thought to reflect a brain-mediated change that alters descending control over spinal nociception, resulting in a secondary phase of hyper-reactivity (Abbadie et al., 1997; Xu et al., 2010). Accordingly, prior work has shown that complete spinal transection abolishes the late phase response whereas the early phase hyper-reflexia remains largely intact (Wheeler-Aceto and Cowan, 1991).

To confirm that formalin alters spinal nociceptive function in our complete transection model of SCI, (Grau et al., 1998), we performed a complete T2 transection by cautery and performed hindpaw formalin testing 48 h post-injury (**Figure 2**, unshocked group). Because complete transection abolishes supraspinal

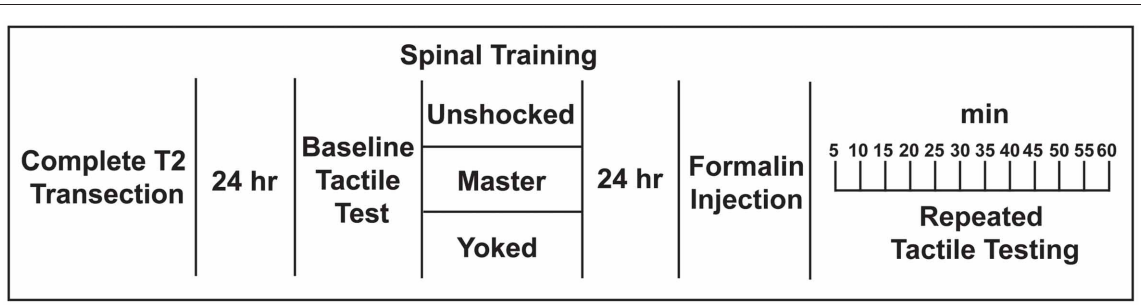

FIGURE 2 | Experimental design used to test whether spinal training history alters future nociceptive responsiveness in the formalin test.

responses to hind-paw formalin, we limited our behavioral testing to tactile responses of the plantar hindpaw.

Formalin produced persistent tactile hyper-sensitivity on the ipsilateral hind paw relative to vehicle injection (**Figure 3**). This hyper-reactivity to formalin was significant on the ipsilateral leg (**Figure 3A**) but not the contralateral leg (**Figure A1**). Because the early phase (first 5 min) and the late phase (25–60 min) of the formalin response involve distinct mechanisms (Coderre and Melzack, 1992; Yamamoto and Yaksh, 1992), we next performed separate analyses of the different phases on the ipsilateral leg (**Figures 3B,C**). Analysis of the early phase confirmed a pronounced hyper-sensitivity (**Figure 3B**), however, the late phase response was diminished (**Figure 3C**). Together the data confirm that formalin produces a behaviorally discernible nociceptive response in our complete transection SCI model. It is noteworthy that the most pronounced sensitivity was observed on the ipsilateral leg and in the early phase of the formalin test, replicating previous observations in complete transection injuries (Wheeler-Aceto and Cowan, 1991).

SPINAL TRAINING HISTORY AFFECTS IRRITANT RESPONSE IN COMPLETE SCI

To test whether spinal cord training produces central changes that alter formalin nociceptive reactivity, we randomized a new group of rats into master/yoked training pairs at 24 h post-injury (24 h pre-formalin; for design see **Figure 2**). For each training pair, master rats received leg position-dependent stimulation and yoked animals received stimulation along with the master, irrespective of leg position. This experimental design ensures that master rats and yoked rats receive the same amount of leg stimulation, yet the master experiences stimulation that is dependent upon their leg position while the yoked experiences leg stimulation that is uncontrollable (Horridge, 1962; Buerger and Fennessy, 1970; Grau et al., 1998). We have previously found that uncontrollable stimulation of one leg produces a bilateral tactile hyper-sensitivity in complete SCI (Ferguson et al., 2006), and the same response is observed with direct activation of nociceptors using intradermal capsaicin (Hook et al., 2008). It has been argued that ipsilateral hyper-reactivity reflects both peripheral and central changes, whereas only contralateral changes provide a

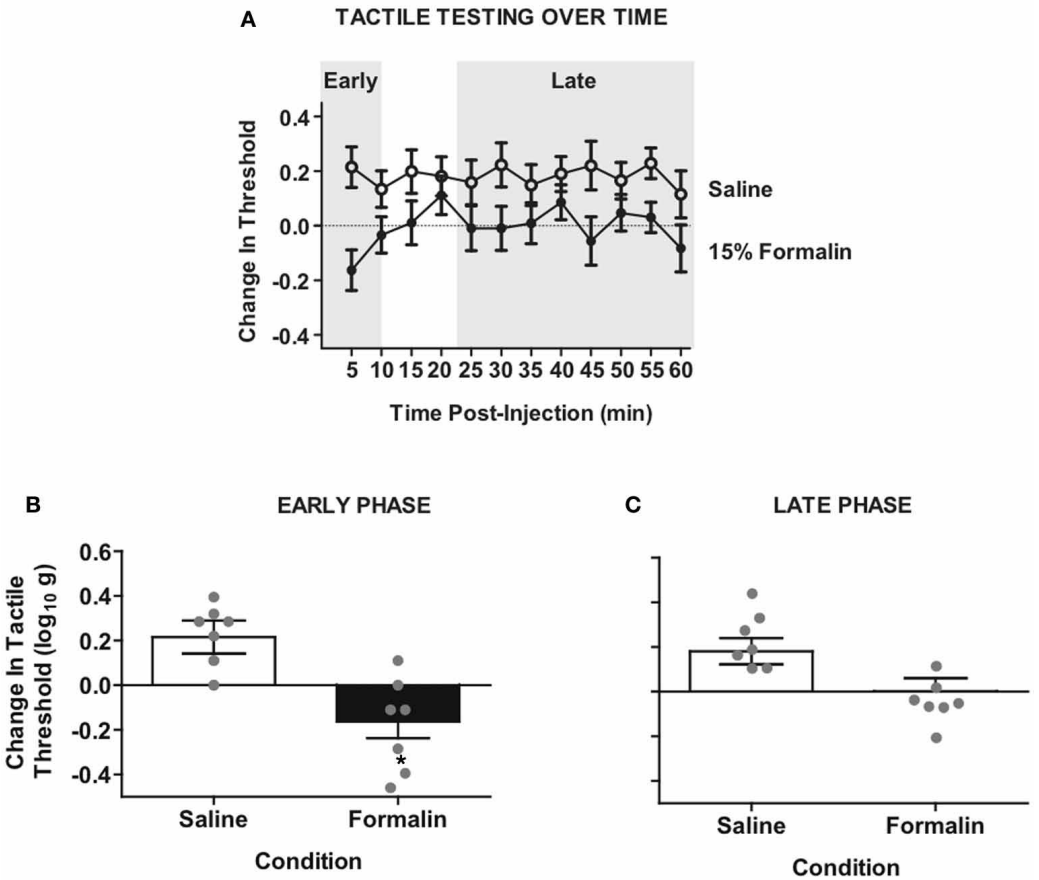

FIGURE 3 | Intradermal formalin produces ipsilateral hyper-reactivity in rats with complete SCI. (A) Time-course of hyper-reactivity with repeated von Frey testing on the plantar surface of ipsilateral hindpaw after intradermal formalin injection. Mixed factorial Three-Way ANOVA revealed significant interaction of testing side × condition, $F_{(1,\ 12)} = 5.59$, $p < 0.05$. **(B)** Significant hyper-reactivity on the early phase formalin response ipsilateral to injection $F_{(1,\ 12)} = 13.11$, $^*p < 0.01$, $n = 7$ rats/formalin condition. **(C)** Non-significant trend of hyper-reactivity in the late phase response, $p = 0.059$. Points and bars represent group means (± SEM), gray points reflect the individual animals.

pure measure of central sensitization (Woolf, 1983; Milligan et al., 2003). For this reason we contrasted the effects of master/yoked training on formalin reactivity ipsilateral and contralateral to the injection. Laterality of the training leg was counterbalanced with respect to the injection, thereby controlling for peripheral effects of training history. As shown in **Figure 4A,** early phase formalin responses on the ipsilateral limb did not differ across master/yoked training groups, and both groups showed similar hyper-sensitivity as unshocked rats (compare with **Figure 3B**). The findings suggest that spinal training history does not alter peripheral sensitization in the formalin test.

To assess central sensitization we used the classic approach of testing tactile responsiveness on the contralateral leg (Woolf, 1983). This analysis revealed that yoked (uncontrollable stimulation) training enhanced future hyper-reactivity contralateral to formalin injection (**Figure 4B**). Specifically, yoked animals demonstrated a significant tactile hyper-reactivity on the contralateral limb that was not observed in either master or unshocked rats. In addition the modulatory effect of training history on formalin hyper-reactivity was not significantly different between ipsilateral and contralateral sides, confirming prior findings that training on one leg induces a central change that alters bilateral responsiveness. This is in contrast to the unilateral effects observed in the untrained (unshocked) rats shown in **Figure 2**. The bilateral nature of the training effects underscores that nociceptive hyper-reactivity produced by yoked training history is indeed a form of central sensitization (Grau et al., 1998; Crown et al., 2002b; Joynes et al., 2003; Ferguson et al., 2006; Baumbauer et al., 2008; Hook et al., 2008; Young et al., 2008; Huie et al., 2012a).

The findings strongly suggest that prior exposure to uncontrollable (yoked) stimulation in SCI rats primes them for later central sensitization by formalin injection. Notably the equivalent amount of response-specific instrumental (master) training did not sensitize the central nociceptive system.

FORMALIN NOCICEPTION PRODUCES IMPAIRMENT IN SPINAL LEARNING ON THE CONTRALATERAL LEG

The preceding experiments indicate that spinal training history influences the degree of central sensitization produced by formalin. We next tested the converse: whether formalin produces a central change that influences spinal cord learning in complete SCI. We performed a set of two independent experiments (**Figures 5A,B**). We first delivered formalin to the hind-paw at a range of concentrations after complete SCI and then assayed spinal learning on the contralateral leg. As shown in **Figure 5C** formalin produced a dose-dependent impairment of spinal learning on the contralateral limb.

To test whether formalin induced a lasting central change, we performed an independent study where we delivered 15% formalin or vehicle and then assayed spinal cord learning on the contralateral leg 24 h later. As shown in **Figure 5D** formalin produced a long-term deficit in spinal learning. To contrast the long-term and short-term effects of formalin we performed an additional analysis comparing all doses and post-injection time points. The findings revealed equivalent deficits in spinal learning in immediate and 24 h post-formalin conditions (**Figure 5E**).

The findings indicate that peripheral nociceptive activation with formalin induces a lasting central change in spinal cord learning in rats with complete SCI. Together with the prior findings the data suggest that the specific pattern of peripheral stimulation dictates the form of central plasticity that is invoked after injury, leading to either central sensitization or adaptive spinal cord learning, depending on stimulus type.

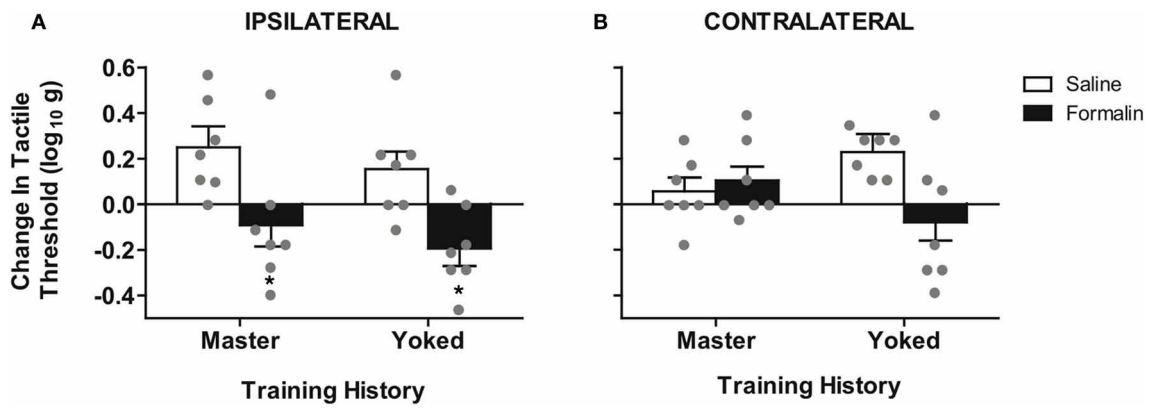

FIGURE 4 | Spinal training history alters formalin hyper-reactivity contralateral to the injection. (A) Significant hyper-reactivity response to formalin but no differential effect of master/yoked training history on the ipsilateral leg, $^*p < 0.05$ from saline, $n = 7$ rats/group. There was also no significant difference between master/yoked/unshocked on ipsilateral hyper-reactivity, all $p > 0.05$, (compare to **Figure 3B**). **(B)** Significant enhancement of formalin hyper-reactivity in yoked group contralateral to injection, $^*p < 0.05$ from yoked saline, $n = 7$ rats/group. Bars represent group means (± SEM) gray points reflect the individual animals. Four-Way mixed, double repeated measures ANOVA was used for an integrative test of full experimental design including: training history (between subjects), formalin condition (between subjects), leg laterality (within subjects), time (within subjects). Modulatory effects of training history on formalin reactivity were re-affirmed by significant interaction of training history × formalin × time, ($p < 0.05$). The Four-Way interaction of leg laterality × training history × formalin × time did not reach significance, $p > 0.05$, reinforcing the bilateral (central) nature of training-enhanced nociception.

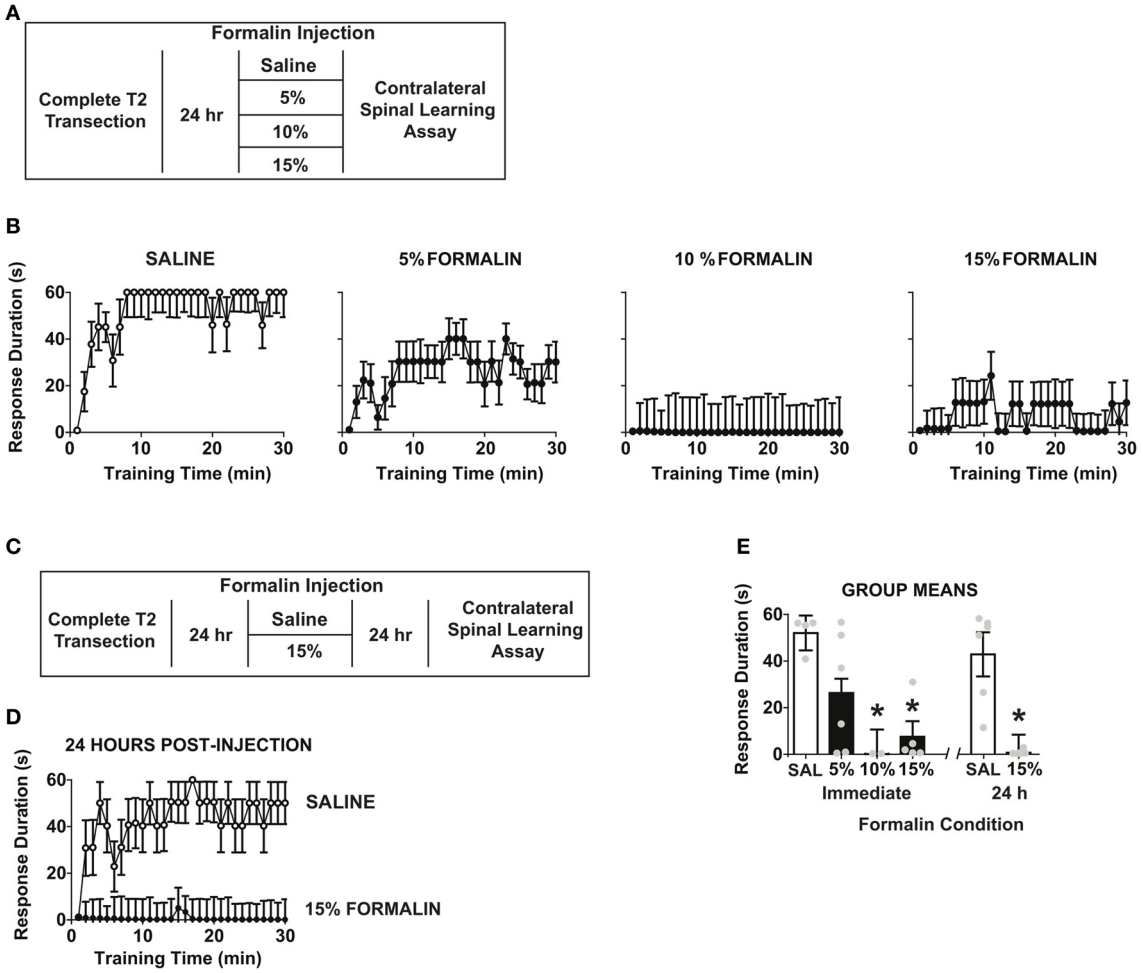

FIGURE 5 | Intradermal formalin produces contralateral impairments in spinal learning. (A) Experimental design used to test immediate dose-response characteristics of formalin concentration on spinal learning potential. **(B)** Concentration-dependent impairment in spinal learning contralateral to formalin injection (0%, $n = 4$; 5%, $n = 6$; 10%, $n = 2$; 15%, $n = 5$; the numbers of subjects is supported by statistical power analysis, partial eta squared = 0.58, power = 0.89). Mixed repeated measures ANOVA revealed a significant effect of time, $F_{(29, 377)} = 2.46$, $p < 0.05$, and formalin concentration, $F_{(3, 13)} = 6.15$, $p < 0.05$. Tukey's *post-hoc* test revealed that 10% and 15% formalin significantly impaired spinal cord learning relative to 5% formalin and vehicle, $p < 0.05$. **(C)** Experimental design used to test effects of formalin on spinal learning potential. **(D)** Spinal learning impairment on the contralateral leg 24 h after formalin injection ($n = 6$ rats/group). Mixed repeated measures ANOVA revealed significant main effects of time, formalin, and time × formalin, all $p < 0.05$. **(E)** Group means for all formalin conditions, One-Way ANOVA confirmed effect of formalin condition $F_{(5, 23)} = 8.64$, $p < 0.0001$. *Post-hoc* Tukey's revealed saline groups did not differ, whereas 10, 15, and 15% 24 h formalin groups had significant learning impairments,* $p < 0.05$ from saline groups. Points and bars represent group means (± SEM), gray points reflect the individual animals.

CENTRAL ACTIVATION OF SPINAL NMDA RECEPTORS PRODUCES ENDURING SPINAL LEARNING IMPAIRMENTS IN SCI ANIMALS

The preceding experiments demonstrated that chemical nociception with hindpaw formalin injection impairs spinal cord learning in complete SCI. The fact that this effect is observed contralateral to the irritant strongly suggests that a form of central sensitization is involved. Central sensitization by formalin is known to require activation of spinal NMDA receptors (Haley et al., 1990; Dickenson and Sullivan, 1991). In addition, direct intrathecal delivery of NMDA ligand (5–50 mM) sensitizes dorsal horn neurons and produces spontaneous nociceptive behaviors in intact rats (Raigorodsky and Urca, 1987; Dougherty et al., 1992; Bjorkman et al., 1994; Menendez et al., 1997; Alvarez-Vega et al., 2000; Sato et al., 2003; Kim et al., 2008; Roh et al., 2009).

To test whether NMDA-induced central sensitization impacts spinal cord learning, we delivered intrathecal NMDA at three doses (0.06, 0.6, or 6.0 mM) and assayed spinal learning 24 h later (**Figure 6A**). If NMDA produces a central sensitization that mimics the enduring effects of uncontrollable stimulation and formalin, then NMDA should alter spinal learning at this delayed time point. As shown in **Figure 6B**, NMDA produced a dose-dependent impairment in spinal learning when tested at 24 h post-injection. The most robust deficit was observed with 6 mM. Together the data indicate that central sensitization by NMDAR activation generates an impairment in

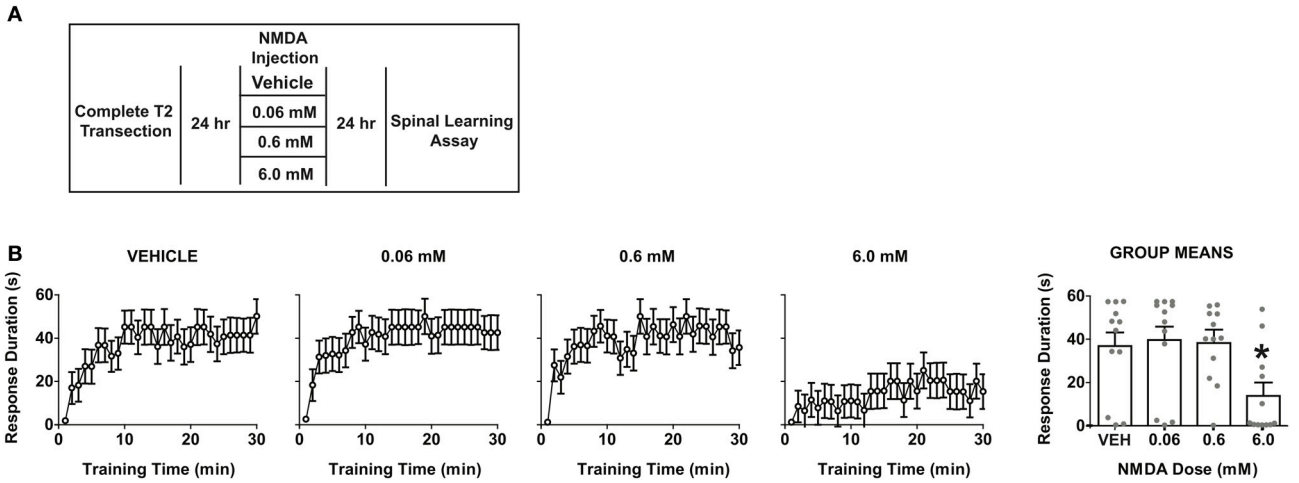

FIGURE 6 | Direct overactivation of spinal NMDA receptors impairs spinal learning 24 h later. (A) Experimental design used to test the effect of intrathecal NMDA on spinal learning potential 24 h after delivery. **(B)** Dose-response function for NMDA-induced impairment of spinal learning. Mixed-repeated measures ANOVA revealed a significant effect of time $F_{(29,\ 1276)} = 10.69$, $p < 0.0001$ and NMDA dose $F_{(3,\ 44)} = 4.08$, $p < 0.05$. Tukey's *post-hoc* revealed that the 6 mM dose produced a significant impairment relative to the other doses, $^*p < 0.05$ from other doses ($n = 12$ subjects/dose) gray points reflect the individual animals.

spinal learning. The findings strongly suggest that direct central sensitization undermines spinal cord learning in the injured spinal cord.

It is important to note that the dose necessary to impair spinal learning was relatively high when compared to prior studies of spontaneous nociceptive behaviors in intact animals, which have typically used doses ranging from 0.1 to 1 mM. This raises the possibility that the current effects could be due to an NMDA-induced excitotoxicity, which has been observed in primary cell culture at doses as low as 1 mM (Koh and Choi, 1988; Dawson et al., 1993). However, several lines of data suggest that our 6 mM intrathecal dose still falls within a normal physiological range *in vivo*. Studies of the locomotor central pattern generator (Grillner et al., 1981) have used intrathecal doses five times higher than our highest dose to elicit fictive locomotion in *in vivo* spinal rat preparations (Giroux et al., 2001). In addition studies in primate models have shown central sensitization of nociceptive responses with intrathecal NMDA doses of up to 50 mM. Together, this suggests our doses fall within a reasonable range for central sensitization and the learning deficits observed are more consistent with maladaptive plasticity than a wholesale excitotoxic lesion (Villanueva et al., 2002; Yoon et al., 2010). In combination with the other experimental findings and prior literature the NMDA findings reinforce the concept that central sensitization impairs adaptive spinal cord learning.

DISCUSSION

Prior literature and the new experimental findings presented here reinforce the concept that nociceptive plasticity opposes spinal learning adaptations after complete SCI. The results indicate that spinal training history can influence future nociceptive responsiveness in the formalin test. In particular pre-training with inappropriate uncontrollable (yoked) stimulation, but not instrumental (master) training, enhances later tactile hyperreactivity produced by intradermal formalin into the hind paw. This training-enhanced reactivity was evident on the contralateral paw, strongly suggesting a central mechanism. Follow up experiments revealed that intradermal formalin, delivered at doses that enhance tactile reactivity, disrupts future spinal learning on the contralateral paw for at least 24 h. Finally, directly driving central NMDA receptors, which produces central nociceptive sensitization, generates a lasting (>24 h) impairment in spinal learning. Taken together the findings indicate that central sensitization and adaptive spinal learning are opposing forms of spinal plasticity.

The existing literature indicates many similarities between central sensitization within spinal pain pathways and stimulus-induced maladaptive spinal plasticity that prevents future spinal learning after SCI. As with many other forms of plasticity, both central sensitization and the spinal learning deficit are mediated by changes in glutamatergic activity. The NMDA receptor antagonist MK-801 has been used to block central sensitization, as well as to inhibit the induction of the spinal learning deficit induced by uncontrollable stimulation (Woolf and Thompson, 1991; Ferguson et al., 2006). Further, group I metabotropic glutamate receptors (mGluR1 and mGluR5) have been shown to be necessary for formalin-induced nociception, as well playing a key role in the development of chronic pain following spinal contusion injury (Fisher and Coderre, 1996; Mills et al., 2002). We have previously shown that these same mGluR subtypes are also necessary in order for uncontrollable stimulation to produce a spinal learning deficit. Taken together with the current experiments demonstrating formalin-induced undermining of adaptive spinal learning, these findings provide further behavioral evidence that central sensitization and the maladaptive spinal plasticity that inhibits spinal learning engage common central mechanisms.

It should be noted that the main focus of the work with this spinal learning model has been behavioral and pharmacological, rather than strictly physiological. Historically, those studying spinal plasticity with this model have focused on the central neurochemical factors that mediate the behavioral effects, while the physiological circuitry that is engaged by stimulation of the tibialis anterior muscle has been investigated less extensively. Our goal is to understand the training potential of the spinal cord and all of our experiments are designed to rule out peripheral effects by testing all effects on the contralateral limb. The current study evaluated the potential for established nociceptive stimuli (formalin and TA stimulation) to alter the future capacity for spinal cord training of a flexion response on the contralateral limb. Similarly, our evidence for central nociceptive plasticity in the current study has been provided by behavioral outcomes on the contralateral limb, rather than direct testing of fiber activation. Although we have previously found that these behavioral effects depend on stimulation of c-fibers, direct electrophysiological confirmation for the recruitment of fiber types and muscle group activation following stimulation of the tibialis anterior represents an important area for further research.

An improved understanding of the fiber types could also potentially help explain some of the formalin dose-response features observed in the present study. We found that the most profound learning deficits were observed at a relatively high dosing range (5–15% formalin). Recent work suggests that lower doses (0.5%) of formalin selectively engage primary nociceptors that express transient receptor potential (TRP) channel subtype TRPA1, whereas increasing doses (2–5%) engage a wider range that includes non-TRPA1 nociceptors (Braiz and Bausbaum, 2010). Based on the dose-response function in the present study, one might hypothesize that spinal cord learning is more impaired when a broad range of different nociceptive fiber populations are engaged. However, the relative role of different nociceptive populations remains an open question for further research.

Much of the work to understand the neurobiology of our observed behavioral effects has come from pharmacological manipulation of known mediators of plasticity and nociception. Several nociception-inducing substances have been implicated in spinal learning deficits in our model including substance P, capsaicin, carrageenan, intrathecal glutamate agonists, and intrathecal delivery of the cytokine tumor necrosis factor alpha (TNFα) (Ferguson et al., 2006, 2008a,b; Baumbauer et al., 2007; Hook et al., 2008; Huie et al., 2012b) Our recent interest in TNFα as a modulator of spinal learning provides additional links between spinal cord learning impairments and nociception. Findings from the pain literature indicate that TNFα is a potent mediator of nociceptive plasticity (Czeschik et al., 2008; Choi et al., 2010; Park et al., 2011; Zhang et al., 2011). TNFα is known to modulate synaptic strength by acting to increase glutamatergic signaling, and we have demonstrated this effect to play a role in excitotoxicity following SCI (Beattie et al., 2002; Ferguson et al., 2008b). We have recently shown that blocking TNFα activity prior to uncontrollable stimulation protects against maladaptive spinal plasticity, and that administration of exogenous TNFα is sufficient to undermine adaptive spinal learning (Huie et al., 2012b). Beyond a protective effect, inhibition of TNFα activity can also restore adaptive plasticity after stimulation-induced maladaptive plasticity has been induced (Huie et al., 2012b). The use of TNFα inhibitors after SCI to aid in recovery is showing promise, and these recent findings suggest that TNFα may work to improve recovery of sensory and locomotor function not only by attenuating nociception and excitotoxicity, but perhaps by rescuing the capacity for adaptive plasticity in the injured spinal cord (Genovese et al., 2006; Ferguson et al., 2008b; Marchand et al., 2009). Together these data provide a potential molecular mechanism for observed commonalities between spinal learning deficits and nociceptive sensitization. Further work is needed to provide further mechanistic support.

It is important to recognize the potential negative consequences of inappropriate training and nociceptive input for individuals with SCI. Even in complete SCI where a patient does not consciously experience pain below an injury, nociceptive input to the spinal cord may promote a maladaptive plasticity that undermines future spinal cord training and rehabilitation potential. Perhaps even more troubling, the present findings and prior studies suggest that inappropriate training of the spinal cord can generate a lasting central sensitization of nociceptive reactivity. Caudle et al. recently demonstrated that passive stretch therapy in rats after SCI hindered locomotor recovery, as did limb immobilization in a wheelchair (Caudle et al., 2011). Likewise, Petruska et al. found that in step-trained transected rats, the introduction of noxious stimuli (due to cutaneous hindpaw lesions) undermined the beneficial locomotor effects of step-training (Petruska et al., 2007). Thus, it is possible that impairment of recovery after SCI may reflect nociceptive plasticity induced by the uncontrollable afferent input, arising from numerous possible sources. Care must be taken in designing rehabilitative strategies for SCI patients to avoid therapies that might produce uncontrolled nociceptive input. One must be aware of the injured spinal cord's vulnerability to central sensitization, so that rehabilitation strategies that are intended to promote recovery do not end up creating new problems. Central sensitization can lay down an enduring pain memory in the spinal cord that interferes with the re-acquisition of adaptive behavior and fosters the development of neuropathic pain (Woolf, 1983). The supraspinal experience of neuropathic pain could be unveiled if future regenerative therapies successfully reconnect the brain to a lumbar spinal cord containing sensitized nociceptive circuitry. Further research is needed to ensure therapies that promote adaptive spinal plasticity while limiting central pain.

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APPENDIX

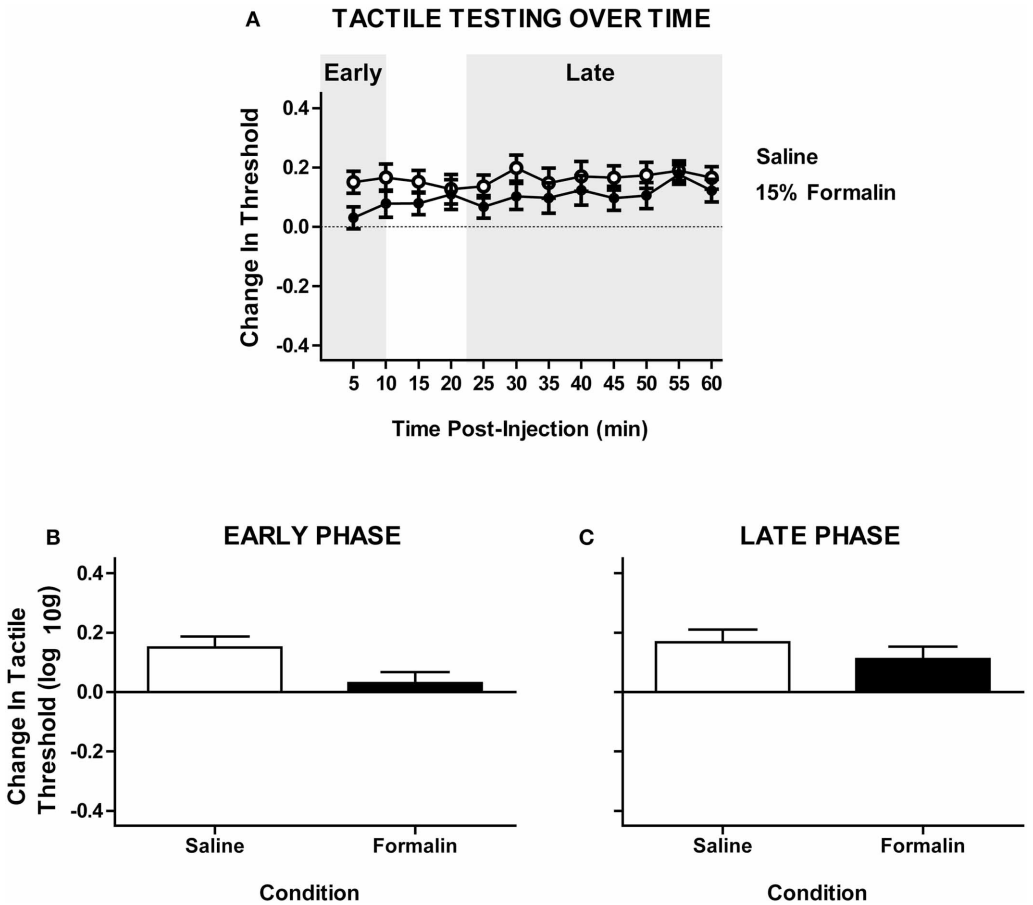

FIGURE A1 | Intradermal formalin does not produce a significant contralateral hyper-reactivity in untrained subjects with complete SCI. **(A)** Time-course of tactile response with repeated von Frey testing on the plantar surface of ipsilateral hindpaw after intradermal formalin injection. **(B)** Non-significant trend toward hyper-reactivity on the early phase formalin response contralateral to injection [$F_{(1,12)} = 2.17$, $p = 0.166$, $n = 7$] rats/formalin condition. **(C)** Lack of hyper-reactivity in the late phase response, $P \gg 0.05$. Points and bars represent group means (±SEM).

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Impact of behavioral control on the processing of nociceptive stimulation

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How nociceptive signals are processed within the spinal cord, and whether these signals lead to behavioral signs of neuropathic pain, depends upon their relation to other events and behavior. Our work shows that these relations can have a lasting effect on spinal plasticity, inducing a form of learning that alters the effect of subsequent nociceptive stimuli. The capacity of lower spinal systems to adapt, in the absence of brain input, is examined in spinally transected rats that receive a nociceptive shock to the tibialis anterior muscle of one hind leg. If shock is delivered whenever the leg is extended (controllable stimulation), it induces an increase in flexion duration that minimizes net shock exposure. This learning is not observed in subjects that receive the same amount of shock independent of leg position (uncontrollable stimulation). These two forms of stimulation have a lasting, and divergent, effect on subsequent learning: controllable stimulation enables learning whereas uncontrollable stimulation disables it (learning deficit). Uncontrollable stimulation also enhances mechanical reactivity. We review evidence that training with controllable stimulation engages a brain-derived neurotrophic factor (BDNF)-dependent process that can both prevent and reverse the consequences of uncontrollable shock. We relate these effects to changes in BDNF protein and TrkB signaling. Controllable stimulation is also shown to counter the effects of peripheral inflammation (from intradermal capsaicin). A model is proposed that assumes nociceptive input is gated at an early sensory stage. This gate is sensitive to current environmental relations (between proprioceptive and nociceptive input), allowing stimulation to be classified as controllable or uncontrollable. We further propose that the status of this gate is affected by past experience and that a history of uncontrollable stimulation will promote the development of neuropathic pain.

Keywords: plasticity, instrumental conditioning, learning, spinal cord injury, nociception, BDNF, allodynia, recovery of function

INTRODUCTION

In the absence of spinal injury, the processing of afferent pain (nociceptive) signals within the spinal cord is regulated by the brain through descending pathways (Sandkühler and Liu, 1998; Gjerstad et al., 2001). In the presence of prolonged nociceptive stimulation, these descending brain pathways can exert a protective effect that dampens neural excitability and, thereby, prevents the sensitization of nociceptive mechanisms (central sensitization) and the development of neuropathic pain (Davies et al., 1983; Faden et al., 1988; Eaton et al., 1997; Hains et al., 2002). Spinal cord injury (SCI) removes this protective effect, allowing spinal systems to react in an unbridled way to on-going afferent input. In the absence of the brain's oversight, how nociceptive signals impact spinal systems will depend upon intrinsic mechanisms. We will show that these intraspinal systems are tuned to detect whether the nociceptive signal is related to the performance of a particular response (controllable stimulation) and that allowing behavioral control can engage processes that exert a protective/restorative effect that helps to ameliorate the effect of spinal injury. Conversely, a lack of behavioral control can enhance the adverse effect of nociceptive stimulation and promote the development of neuropathic pain, an issue that is discussed in our companion paper (Ferguson et al., under review). Here we focus on the processes that underlie the abstraction of behavioral control and the mechanisms that underlie its long-term benefit.

Because this work relies on concepts developed within the field of learning, we will first provide an overview of some essential concepts in learning and their application to spinal cord plasticity and behavioral rehabilitation. We will then discuss evidence that a nociceptive stimulus has divergent effects depending upon whether it is controllable (response-contingent) or uncontrollable (non-contingent). We will present evidence that a history of behavioral control can reduce the adverse effects of nociceptive stimulation and counter the development of neuropathic pain. We will conclude by reviewing evidence that the beneficial effect of controllable stimulation depends on brain-derived neurotrophic

factor (BDNF) and will present a model that integrates these observations. We propose that behavioral control acts to gate how afferent nociceptive signals are processed, and that this determines whether the stimulus has an adaptive or maladaptive effect.

LEARNING AND REHABILITATION

Our work is guided by an understanding of how systems adapt (i.e., how they learn) in the intact organism. Learning from this perspective represents a form of plasticity, where the effect of a stimulus (*S*), a response (*R*), or an outcome (*O*), depends upon whether the event (the *S*, *R*, or *O*) has previously occurred and its relation to other events (Domjan, 2010). Within this structure, learning is thought of as a process, a mechanism that detects and encodes on-going events and their relation to past experience. Memory represents the preservation of this information over time.

Within this rubric, we use the term outcome to refer to stimulus events that follow a *R*. If instituting a relationship between a particular *R* and an *O* brings about a change in the *R*, the underlying process is sometimes referred to as reinforcement and the *O* a reinforcer. For example, if a rat is placed in a situation wherein pressing a bar yields a food pellet, the bar-press corresponds to the *R* and the food is the *O*. If this contingency brings about an increase in responding, it is commonly said that the presentation of food *reinforced* bar pressing behavior.

A potential source of confusion stems from the fact that an *O* is a stimulus event and, when its stimulus properties (e.g., intensity, duration) are of concern, may be referred to as such. But more often, the term *S* is used to refer to events that signal whether a particular *R*-*O* relation is in effect or to stimulus events that occur irrespective of any particular behavioral *R*. For example, presentation of a *S* alone might bring about a reduction (habituation) or increase (sensitization) in the behavioral *R* elicited by the *S*. Alternatively, interposing a relationship between two stimuli [usually called the conditioned stimulus (CS) and unconditioned stimulus (US)] can bring about a change in the response elicited by the CS [the conditioned response (CR)], a phenomenon known as Pavlovian (classical) conditioning. Finally, a *S* can indicate whether a particular *R*-*O* relation is in effect, in which case the *S* may be referred to as a discriminative stimulus (S^D).

Past work has shown that spinal mechanisms exhibit habituation, sensitization, and are sensitive to CS-US relations (for reviews, see Patterson, 2001; Patterson and Grau, 2001). Here we focus on an alternative form of learning, instrumental conditioning. Learning theorists have traditionally classified behavioral phenomena on the basis of methodology (Grau and Joynes, 2005a). From this view, single stimulus learning (habituation and sensitization) and Pavlovian conditioning depend solely upon the history of stimulus events encountered; a behavioral response may be used as an index of learning, but is not relevant to the environmental relations that produce the learning. In contrast, instrumental learning depends upon the temporal relationship between a behavioral response and an environmental outcome, the *R*-*O* relation (Grau, 2010). For instrumental learning, the response is central – if establishing a contingency between a particular *R* (whether simple or complex) has a lasting, neurally mediated, effect on behavior, the methodology involves instrumental learning. We focus on this form of learning because, from past work, it was not clear whether isolated spinal mechanisms could exhibit this type of learning and because instrumental learning would seem especially relevant to behavioral rehabilitation after SCI.

A key question at this juncture is: why focus on learning? How is this relevant to the recovery of function after SCI? To understand the importance of learning, consider the primary aim of behavioral rehabilitation – to "retrain" the injured system. At its heart, behavioral rehabilitation involves a set of tasks designed to promote the performance of behaviors that will enhance function and the patient's well being. To the extent that these procedures yield a lasting effect, they involve a form of learning, and to the extent this learning depends on having experienced a particular *R*-*O* relation, they involve instrumental conditioning. The import of these observations is enhanced by the recognition that behavioral rehabilitation remains the most effective treatment for the restoration of function after injury.

Learning will likely also prove essential to medical treatments designed to foster neural growth to bridge an injury, because encouraging axon elongation is only part of the story. Once the injury is spanned, the pattern of synaptic connectivity must be tuned to promote adaptive processes and avoid maladaptive outcomes (e.g., neuropathic pain). Just as experience helps to shape the pattern of connectivity during development, rewiring spinal circuits will require procedures that promote adaptive learning.

SPINALLY MEDIATED LEARNING

Our claim is that behavioral rehabilitation has a lasting effect because it encourages a form of learning and that this process occurs, in part, within the spinal cord. In subsequent sections, we bolster this claim with physiological and pharmacological studies examining the underlying mechanisms. As we will see, this work suggests that behavioral control may gate nociceptive signals within the dorsal spinal cord and thereby determine whether stimulation has an adaptive or maladaptive effect. But before we get there, we need to reinforce our central claim – that spinal mechanisms can support learning. Addressing past issues has required a detailed behavioral analysis, providing evidence of learning and uncovering some key features of the underlying processes. Indeed, our work turns the usual analysis of instrumental behavior on its head, shifting the focus from the behavioral response (the consequence of learning) to processes related to the sensory cues. Along the way, we will note the implications of this analysis for behavioral rehabilitation and address some issues in terminology that have led to confusion and controversy.

HABITUATION, SENSITIZATION, AND PAVLOVIAN CONDITIONING

It is well recognized that spinal systems can exhibit some basic forms of learning (Patterson and Grau, 2001). The focus in these studies has typically been on the functional capacities of the lower (lumbosacral) spinal cord and the central issue is: To what extent can neurons within this region support learning in the absence of input from the brain? To address this issue, researchers typically sever neural communication with the brain by means of a mid-level (thoracic) transection. After this spinal injury, spinally mediated learning can be studied using stimuli applied to the hind limbs or tail. Because nociceptive reflexes remain intact, and provide a means for monitoring the behavioral consequences

of stimulation, many studies use stimuli that engage nociceptive fibers. Of course, because ascending sensory fibers have been cut, subjects perceive no pain.

Research using spinally transected animals has established that spinal systems can support single stimulus learning and provided the foundation for the dual-process model of habituation and sensitization (Groves and Thompson, 1970). Though motivated by different concerns, recent work has extended these observations to demonstrate that afferent nociceptive signals can cause a lasting increase in neural excitability within the spinal cord (central sensitization). This sensitization enhances reactivity to tactile stimulation and is thought to contribute to the development of neuropathic pain (Woolf, 1983; Willis, 2001; Latremoliere and Woolf, 2009). The neurochemical systems that support this plasticity have much in common with the machinery that underlies brain-dependent learning and memory within the hippocampus (Sandkühler, 2000; Ji et al., 2003).

There is also considerable evidence that spinal mechanisms are sensitive to *S–S* (Pavlovian) relations (Patterson, 2001). In these studies, the stimuli are applied to dermatomes below a complete spinal transection. A common finding (Fitzgerald and Thompson, 1967; Patterson et al., 1973; Beggs et al., 1985; Durkovic, 1986, 2001; Grau et al., 1990; Illich et al., 1994; Joynes and Grau, 1996) is that the physiological/behavioral response elicited by one stimulus (CS) depends upon whether it has been paired with a noxious input (the US) generated using electrical stimulation at an intensity that engages nociceptive fibers. These studies highlight a common feature within this literature – that spinal learning is often studied using nociceptive stimulation/reflexes. For this reason, the work details a form of nociceptive plasticity.

It has been known for decades that spinal mechanisms exhibit single stimulus (a.k.a. non-associative) learning and Pavlovian conditioning (Fitzgerald and Thompson, 1967; Groves and Thompson, 1970). Yet, the initial acceptance of this work was tempered by an intellectual climate that saw "true" learning as associative in nature – as reflecting the *de novo* linking of two arbitrarily paired events. Much has changed in the ensuing years. Researchers found that invertebrates, with neural assemblies far simpler than that found within the spinal cord, also exhibit a range of learning phenomena (Sahley and Crow, 1998). This behavioral work laid the foundation for uncovering the neurobiological mechanisms involved in learning, in both invertebrates and vertebrates (Kandel and Schwartz, 1982; Pittenger and Kandel, 2003). Concurrent studies revealed that learning is often biologically prepared, tuned by the organism's evolutionary history (Timberlake and Lucas, 1989; Timberlake, 1999). If both a taste and a visual cue (the CSs) are paired with illness (the US), rats acquire an aversion to the taste but not the visual cue (Garcia et al., 1989). If shock is used as the US, these relations are reversed. Recognizing that learning is often prepared is important because demonstrations of learning within the spinal cord, and in invertebrates, routinely take advantage of pre-existing response tendencies. At the same time, our view of what constitutes learning expanded to include non-associative effects (e.g., Domjan, 2010). Indeed, on closer analysis, we now recognize that true associative learning may be the exception, rather than the rule (Grau and Joynes, 2005a,b); in most Pavlovian paradigms, the CS has some capacity to elicit a CR-like response prior to its being paired with the US. Within this broader modern context, evidence of habituation, sensitization, and Pavlovian conditioning demonstrate that spinal systems can learn.

INSTRUMENTAL CONDITIONING

What has proven more controversial is whether spinal neurons are sensitive to *R-O* (instrumental) relations (discussed in Grau et al., 1998, 2006). To explore this issue, researchers have typically used a variant of the Horridge (1962) procedure. Rats undergo a spinal transection and, after a recovery period, are placed in an opaque tube where they can comfortably rest with their hind limbs hanging freely (**Figure 1A**). With this apparatus, leg position can be monitored by taping a contact electrode to one hind paw. When the tip of this electrode contacts an underlying salt solution, it completes a circuit, providing a binary measure of whether the leg is extended or flexed. Shock is applied through electrodes that stimulate the tibialis anterior muscle at an intensity that elicits a flexion response. With this apparatus, a *R-O* relation can be instituted by administering leg-shock whenever the leg is extended, and terminating shock when the leg is flexed.

To examine whether the *R-O* relation matters, researchers often include a second group that receives shock independent of leg position. This is accomplished by experimentally coupling (yoking) a subject that has behavioral control (the master rat) to a second subject (the yoked rat) that receives shock at the same time and for the same duration as the master. For the yoked rat, shock occurs in a non-contingent (uncontrollable) manner.

Using this paradigm, early researchers showed that stimulation of the tibialis anterior muscle yielded different behavioral outcomes in master versus yoked subjects, and from this it was suggested that spinal systems are capable of instrumental conditioning (Buerger and Fennessy, 1970; Buerger and Chopin, 1976; Chopin and Buerger, 1976). This claim was soon challenged (Church and Lerner, 1976; Church, 1989) and, as a result, the standard dogma remained – that instrumental learning requires a brain. In retrospect, the difficulties here stemmed from two sources. The first concerned some methodological issues. The second concerned an over-statement of the results based, in part, on some confusion in terminology (e.g., operant versus instrumental conditioning).

Regarding methodology, some of the issues arose because the research crosses interdisciplinary boundaries. Those performing the studies were generally trained in physiology and neuroscience while the critics were typically trained in experimental psychology and learning theory. Each area naturally brings field-specific concerns regarding the relative importance of different experimental variables. Having demonstrated the basic phenomenon, the physiologists sought to study the underlying neurobiological mechanisms whereas the learning theorists sought a more thorough analysis of the phenomenon. The latter raised concerns regarding group size, experimental controls, non-standardized training regimes, and statistical analyses. While we acknowledge the merit of these criticisms, they can be readily addressed.

More problematic than these methodological issues was the realization that the master-yoke paradigm could generate behavioral differences in the absence of instrumental learning (Church and Lerner, 1976). The difficulty is that a *reactive model*, a mechanical (robotic) system that does not encode the *R-O* relation, can

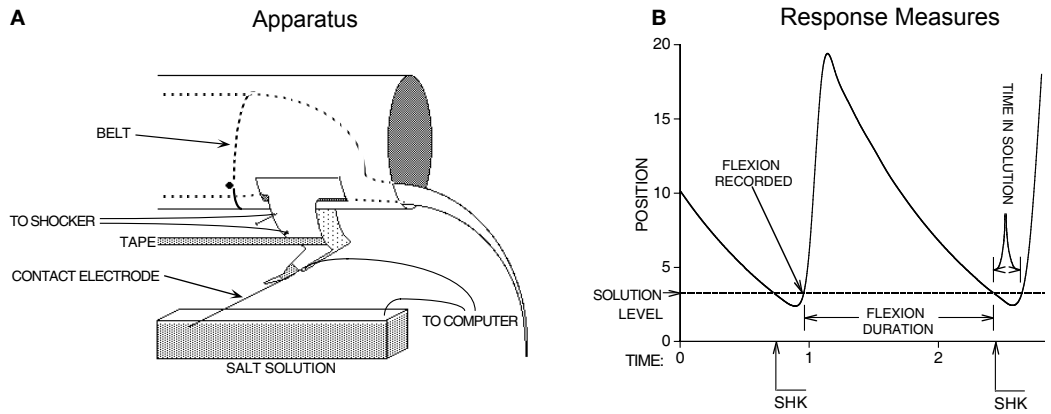

FIGURE 1 | Apparatus and measures used to study instrumental learning in spinally transected rats. (A) Leg position is monitored by means of a contact electrode that is taped to the rat's paw. When the electrode touches the underlying salt solution, it completes a circuit that is monitored by a computer. Applying leg-shock elicits a flexion response that lifts the contact electrode and breaks the circuit. **(B)** The response measures derived from leg position over time. Rats given controllable shock receive a shock when the contact electrode falls and touches the underlying solution. This elicits a flexion response that lifts the contact electrode and breaks the circuit, whereupon shock is terminated, the duration of solution contact is recorded, and response number is incremented by one. Yoked animals receive shock independent of leg position and the same criteria are used to monitor time in solution and response number. Adapted from Grau et al. (1998).

produce differential behavior in master and yoked subjects. To see the problem, consider the performance of the yoked rat. If we assume some variability in the rate at which the shocked leg falls, the yoked rat's leg would reach the underlying solution first roughly half the time. On these trials, the leg will remain extended (touching the solution) until the master rat's leg is extended, whereupon both subjects receive a shock that elicits a flexion response. Notice that the behavioral contingency effectively drives the master rat's leg up whenever it is extended, minimizing solution contact relative to the yoked subject and, as a result, a master-yoke difference would emerge in the absence of any learning (for additional details, see Grau et al., 1998, 2006). Because earlier results could be generated by a reactive model, the claim that spinal neurons can support instrumental learning was rejected.

Recognizing these pitfalls, we adopted an alternative measure of learning: flexion duration (Grau et al., 1998). Imagine that, while standing, you experienced a shock to one leg whenever that leg was extended. You would soon learn to maintain your leg in a flexed position, recognizing that, if you allowed it to fall, you would be punished by the presentation of another shock. Likewise, if spinal neurons are sensitive to the *R* (extension)-*O* (shock) relation, subjects should exhibit an increase in flexion duration. In our laboratory, we quantify changes in flexion (response) duration by breaking the 30 min training session into 1 min bins. Within each time bin (i), mean response (flexion) duration is computed for each subject using the following formula:

$$\text{R esponse duration}_i = \frac{(60\,\text{s} - \text{time in solution}_i)}{\text{flexion number}_i + 1}.$$

Importantly, the reactive model suggested by Church and his colleagues does not anticipate that training with controllable stimulation will lead to an increase in flexion duration (Grau et al., 1998). Indeed, if anything, the higher response rate observed in a mechanical master rat should generate shorter flexion duration scores (relative to the yoked control).

Using response duration as our measure of learning, we examined whether spinal neurons are sensitive to response-outcome relations. Rats underwent a spinal transection and were set-up in the apparatus illustrated in **Figure 1A**. The behavioral response was monitored as illustrated in **Figure 1B**. In an effort to standardize the training protocol, and because preliminary data suggested that failures to learn were related to variation in initial flexion force, we adjusted shock intensity to equate flexion force across subjects at the start of training. To address other methodological issues, we standardized other aspects of the training regime (e.g., the duration of training/testing session), used adequate and equal sample sizes, full factorial designs coupled and rigorous statistical techniques. Under these conditions, we found that master, but not yoked, rats exhibited a progressive increase in flexion duration (**Figure 2**). Interestingly, whether learning was observed depended upon the intensity of the nociceptive stimulation (Grau et al., 1998). If the stimulation was too weak, subjects soon habituated. If the stimulus was very intense, the master rats responded in a mechanical (robotic) manner and generated data that was consistent with a reactive model. These intensity-dependent effects suggest that, in clinical application, training parameters will need to be individually adjusted to an intensity sufficient to maintain behavioral performance without over-stimulating the system.

The claim of instrumental learning implies a form of memory – that the experience has a lasting effect on behavior. If a behavioral contingency simply drives performance to a particular endpoint, and its effect disappears as soon as the contingency is removed, there is no learning. To demonstrate learning, we must show that the experience has an effect that is preserved over time and is evident when subjects are tested under common

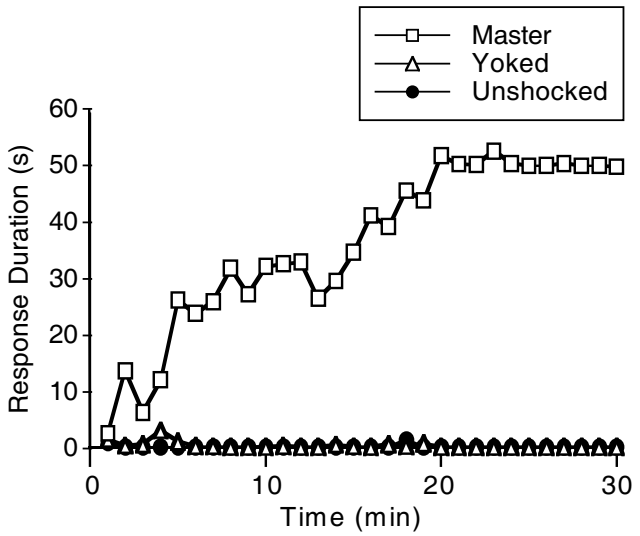

FIGURE 2 | Impact of training on response duration. Spinally transected rats received controllable shock (Master), uncontrollable shock (Yoked), or nothing (Unshocked). Master rats exhibited a progressive increase in response (flexion) duration across the 30 min of training. Yoked rats, that received an equal amount of shock independent of limb position, did not exhibit an increase in response duration. Adapted from Grau et al. (1998).

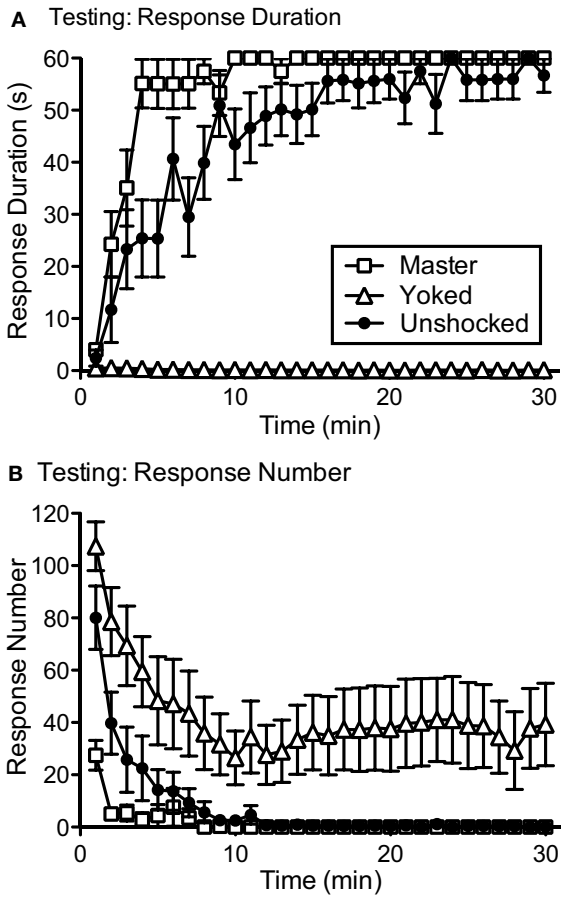

FIGURE 3 | Testing under common conditions. (A) Subjects that had previously received training with controllable shock (Master), uncontrollable shock (Yoked), or nothing (Unshocked), were tested for 30 min with response-contingent leg-shock. Previously trained rats (Master) exhibited a savings effect that facilitated learning relative to the Unshocked controls. Subjects that had previously received uncontrollable shock (Yoked) did not exhibit an increase in flexion duration (our index of learning) when tested with controllable shock. **(B)** This learning deficit was not due to a failure to respond. Yoked rats exhibited the highest rate of responding and, as a result, repeatedly experienced the response-outcome (*R-O*) relation. Adapted from Grau et al. (1998).

conditions (Rescorla, 1988). We addressed this issue by re-equating flexion force [to minimize the contribution of peripheral factors (e.g., muscle fatigue) and single stimulus learning (e.g., habituation)] and re-tested subjects with response-contingent shock (Grau et al., 1998). Previously untreated animals (Unshocked) exhibited a progressive increase in flexion duration when tested with controllable stimulation (**Figure 3A**). Rats that had undergone training with controllable shock (Master) exhibited some savings and re-acquired the behavior somewhat faster. Surprisingly, rats that previously received uncontrollable shock (Yoked) failed to learn when tested with controllable stimulation, exhibiting a learning deficit reminiscent of the behavioral phenomenon learned helplessness (Maier and Seligman, 1976). Importantly, this learning deficit was not due to a failure to respond. Indeed, rats that had previously received uncontrollable shock exhibited the highest rate of responding (**Figure 3B**). Thus, yoked rats repeatedly experienced the response-outcome contingency, but failed to exhibit an increase in response duration. It seems that prior exposure to uncontrollable stimulation *disabled* an essential component of the learning process.

SHOCK ONSET REINFORCES LEARNING: MECHANISTIC IMPLICATIONS

The data presented thus far support the contention that spinal mechanisms are sensitive to *R-O* relations. To bolster this conclusion, we sought converging evidence that the *R-O* relation matters. According to Church (1964), this issue can be addressed by experimentally manipulating the temporal relationship between the *R* and the *O*. If the *R-O* relation matters, then degrading this relationship by inserting a temporal gap should disrupt learning. The implicit assumption here is that learning depends on *R-O* contiguity. As we will see, addressing this issue not only uncovers the effective reinforcer, it also informs our model of the underlying process.

To disrupt response-outcome contiguity, we simply delayed both the onset and offset of shock (Grau et al., 1998). For example, for subjects assigned to the 100 ms delay condition, shock did not come on until 100 ms after the contact electrode touched the solution and the shock remained on for an additional 100 ms after the leg was lifted (**Figure 4A**). Other groups received training with a 0, 50, or 200 ms delay. We found that delaying shock onset and offset by 100 ms or more eliminated learning (**Figure 4B**).

Next, we examined whether learning was reinforced by shock onset or offset (Grau et al., 1998). Do subjects exhibit an increase in response duration because a downward movement initiates the shock (in behavioral terms, a form of punishment) or because an

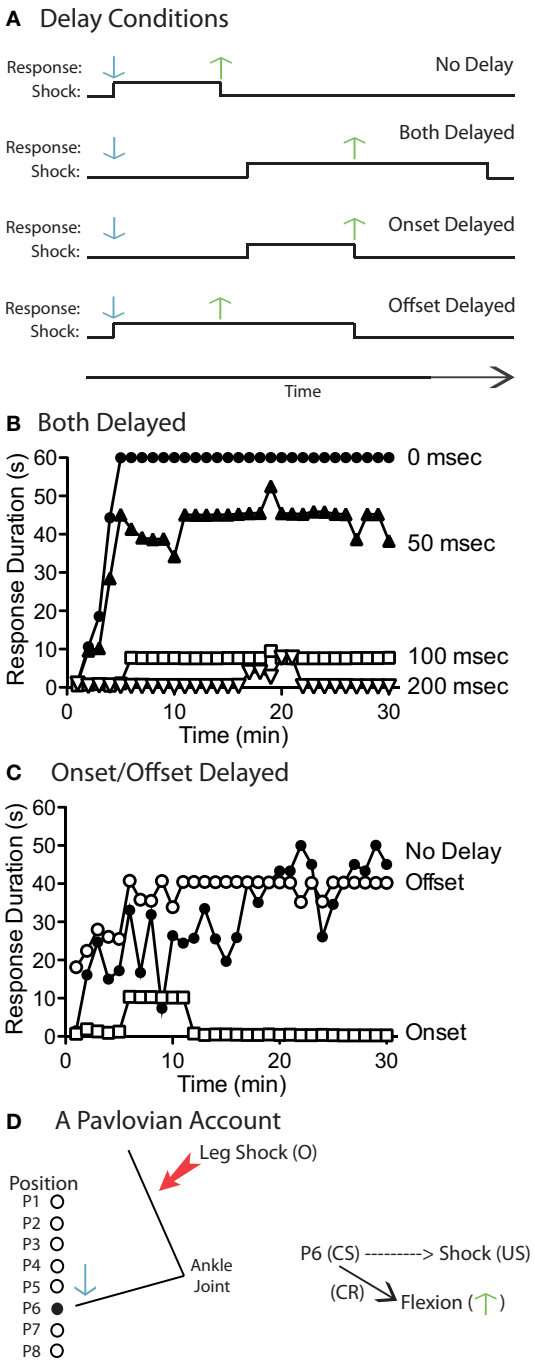

FIGURE 4 | Relative contribution of shock onset versus offset to learning. (A) Illustration of the manipulations used to explore the impact of delaying shock onset or offset. For each training condition, the onset (up-tick) and offset (down-tick) of shock are indicated over time. It was assumed that, in the absence of a delay (No Delay), shock duration would be approximately 80 ms (Crown et al., 2002b). A downward response (solution contact) and up response are indicated by the down (blue) and up (green) arrows, respectively. The panel illustrates the effect of delaying both onset and offset (Both Delayed), or delaying just onset (Onset Delayed) or offset (Offset Delayed). **(B)** Delaying both shock onset and offset by 100 ms *(Continued)*

FIGURE 4 | Continued
disrupted instrumental learning. **(C)** Delaying onset, but not offset, by 100 ms disrupted learning. **(D)** A theoretical account of the underlying processes. It is assumed that proprioceptive cues (P) provide an afferent signal of limb position. In instrumental training, shock onset (the effective reinforcer) always occurs at the same position (e.g., P6). We suggest that the index of limb position (P6) can function as a Pavlovian conditioned stimulus (CS) and that shock onset may act as an unconditioned stimulus (US). As a result of the CS-US pairing, the CS (P6) may acquire the capacity to elicit a flexion response (the conditioned response, CR). **(B,C)** Adapted from Grau et al. (1998).

upward movement turns off the shock (escape) (Domjan, 2010)? To examine these issues, we independently delayed shock onset and offset by 100 ms (**Figure 4A**). When offset alone was delayed, it had no effect on learning (**Figure 4C**). When onset was delayed, learning was disrupted. What this suggests is that it is a misnomer to refer to the behavior observed in this paradigm as "escape learning." Indeed, it is tempting to speculate that escape learning may require more sophisticated (brain-dependent) neural systems.

What we did not fully appreciate when we first described these results is that they have implications regarding the mechanisms that underlie the detection of behavioral control. The findings suggest that the abstraction of behavioral control is linked to events that occur at the onset of the nociceptive stimulus. To see why this is important, consider how the master and yoked rats differ. Only the master rat receives shock when the leg reaches a specific position. The yoked rat receives the same shock, but it occurs independent of leg position. For this difference to matter, spinal systems must register more than shock onset. The system must also have an index of leg position, which we assume is provided by proprioceptive cues. For master rats, shock onset always occurs in the presence of the same cue (leg angle) and our behavioral data suggest that this has special significance – it generates a limb specific increase in flexion duration. Further, for a nociceptive stimulus to have a greater impact when it is given in the presence of a constant proprioceptive cue, the system must have a way of tracking the regularity of this relationship. The system must have a way of encoding (tagging) the leg angle/position at which shock occurred on the previous trial.

An issue that arises at this point is whether the effective code (the index of leg position) is within the animal or built into our apparatus. Have we effectively "tuned" our apparatus (Timberlake and Lucas, 1989), so that all subjects are set-up with a common angle, one that has special biological significance and supports a shock-induced enhancement of flexion duration? Two observations argue against this possibility. First, there is considerable variability across subjects in resting position (i.e., initial foot angle). Second, as we will see later, it is possible to train rats using a different (higher) leg position.

If controllability is tied to the relationship between an index of leg position and shock onset, a lack of control would arise when there is variability in this relationship. For yoked rats, a master generated shock might occur while the yoked rat's leg is up on one trial and down on the next. Similarly, for master rats, interposing a delay in shock onset would introduce *R-O* variability and

potentially entrain an inappropriate response (linked to a more extended leg position that maintains solution contact).

These observations have important implications for how we characterize the mechanisms that underlie instrumental learning. While it is natural to assume that *R-O* learning involves a motoric effect, our analysis suggests that much of the work may be accomplished on the sensory side – that behavioral control is registered, based on the proprioceptive context in which the stimulus occurs. If that context is constant, the stimulus is encoded as controllable. If it varies, the stimulus is encoded as uncontrollable. From this view, early sensory systems may allow us to directly perceive whether or not a stimulus is response-contingent or non-contingent.

In introducing a cue (proprioceptive feedback), we open the door to a seemingly new account of how spinal systems could support instrumental learning. A signal indicative of leg position could act like a Pavlovian CS which, when paired with the onset of a nociceptive stimulus, acquires the capacity to drive a flexion response (the CR; **Figure 4D**). From this view, after a shock-elicited flexion is generated, the leg will begin to fall back to a relaxed position. As the ankle approaches the angle at which shock occurs, proprioceptive cues drive a motor response (flexion) that slows the rate of descent, yielding an increase in response duration. Of course, we are not the first to suggest that Pavlovian conditioning may contribute to instrumental learning. Indeed, decades ago Konorski recognized that Pavlovian mechanisms could contribute to instrumental behavior in a flexion paradigm (Konorski and Miller, 1937; Konorski, 1948).

As a result of instrumental training, an active behavioral response (increased flexion) is established. As we will see below, we have established that intraspinal mechanisms mediate the process of learning. We have not, however, specified how this process produces an increase in flexion duration (the memory); it could reflect an intraspinal modification of motor neuron activity within the ventral horn or a selective enhancement of the efferent output. Nor do we know what constitutes the presumed proprioceptive signal; it could be mediated by an index of the static angle or a vector that describes a movement toward that angle. In either case, our results suggest that a passive leg movement is sufficient to generate the requisite signal, because an external force (gravity) draws the leg downward. From the subject's perspective, it should not matter whether the leg was moved by gravity, the experimenter (or therapist), or a mechanical device – all that should matter is that the onset of the nociceptive stimulus is regularly paired with movement toward a particular leg position. This suggests that, within the clinic, new instrumental behavior could be established through a form of guided therapy, wherein movement of the patient's limb is regularly paired with the onset of biologically significant (nociceptive) cue. Our work suggests that the success of training will be modulated by temporal regularity (i.e., strong response-outcome contiguity), the extent to which the learning is biologically prepared, and whether prior experience has engaged an intraspinal system that opposes (disables) new learning.

We have suggested that a form of Pavlovian conditioning contributes to instrumental behavior, and in so doing, have seemingly blurred the distinction between these two forms of learning. Indeed, the reader may wonder, if common mechanisms are at work, why maintain separate terms? Here, and elsewhere, it is clear that biological systems often rely on common elements to subserve distinct functions. While this commonality simplifies our analysis, more molar (behaviorally relevant) descriptions of how the system operates retain explanatory value. At the level relevant to behavioral rehabilitation, only instrumental conditioning depends on the relationship between a particular response and an outcome. The fact that *R-O* and *S–S* relations may be encoded using similar biological machinery simplifies our analysis and may suggest novel treatments. But from the experimenter's and patient's perspective, the triggering events differ (a behavioral *R* versus an external CS), and for this reason, the distinction still holds sway.

RELATION TO OPERANT BEHAVIOR AND PASSIVE AVOIDANCE (PUNISHMENT)

There is another theoretical implication of our analysis of spinal learning that speaks to an earlier issue and criticisms of this line of work. As noted above, the idea that spinal mechanisms can support instrumental learning has been challenged. Yet, if given the mechanistic account provided above, we expect few would question the claim. Why such a disconnect? At the heart of the problem, we believe, was a casualness in the use of terms that mistakenly implied a form of over-generalization. To see this, it is useful to consider Skinner's (1938) distinction between respondent and operant behavior. Skinner suggested that respondent behavior is "elicited" (reflexive in nature) whereas operant responses are "emitted." In the latter case, the organism could operate on its environment in many ways and performance may be affected by a variety of reinforcers. Ideally, such behavior is relatively unprepared and flexible. To the extent that this is true, we can arbitrarily decide to train any one of a range of responses using a variety of reinforcers. On these criteria, spinal learning will likely fail. We cannot arbitrarily train an extension or flexion using the same outcome. Nor can we train a given behavior using a variety of reinforcers. These limits arise because spinal learning occurs within a highly prepared system, in which the outcome elicits a defined response and our theoretical account evokes the language of Pavlovian conditioning. In Skinner's terminology, this represents a form of respondent conditioning. We mention this because the terms instrumental conditioning and operant learning are sometimes used as synonyms. For both, performance depends on the *R-O* contingency, but the historical roots (and presumed mechanisms) differ. While the term operant was coined by Skinner to describe emitted behavior, the term instrumental conditioning has its roots in the reflexive tradition of Thorndike and Hull (Hillgard and Marquis, 1940), who assumed reinforcers act by modifying *S-R* reflexes. What is important here is that the term instrumental conditioning includes examples of learning that involve a modification of reflexive behavior, which Skinner would classify as a kind of respondent. The implication is that instrumental conditioning represents a broader term, that includes cases of *R-O* learning that are biologically prepared (based on pre-existing reflexes) as well as instances that are relatively unprepared. From this view, the term operant behavior refers to a subcategory of instrumental conditioning and is best applied to examples that seem non-respondent (non-reflexive) in

nature (see Grau, 2010). Because spinally mediated instrumental conditioning involves the modification of a pre-existing reflex, it would not (from our view) constitute an example of operant behavior (Grau et al., 1998, 2006).

Various forms of instrumental conditioning can be classified depending upon the nature of the *O* (appetitive versus aversive) and whether the behavioral response causes the *O* to occur or be omitted (Domjan, 2010). Above, we showed that the effective *O* in our spinal preparation is shock onset. In behavioral terms, this suggests that learning occurs because the initiating response (a leg extension) is followed by shock, a form of punishment. Punishment is a kind of *passive avoidance*, in which the onset of a nociceptive stimulus brings about a decrease in a behavioral response (the leg extending). In behavioral terms, this seems true. But the description misses the fact that this learning must involve more than an inhibition of a behavior (extension). It must also involve an active process, in which an increase in flexion magnitude reduces net shock exposure. This view mirrors a popular account of punishment in intact subjects (Estes, 1944, 1969). Consider a common paradigm in which rats are placed in a two-sided chamber, with one side brightly lit while the other side is painted black and dimly illuminated. Whenever the rat enters the dark side, it receives a shock. Subjects soon learn not to enter the dark chamber and, in behavioral terms, this reflects a kind of passive avoidance. However, at a mechanistic level, an active process is likely at work. Rats innately prefer the dark side of a chamber and, as a result, have a tendency to enter that context. The dark context (the CS) is then paired with shock (the US), establishing a conditioned fear to the shocked environment that acts like an invisible fence to repel the subject (Domjan, 2010). Here too, what appears to reflect a behaviorally passive process (avoiding the shocked chamber) is maintained by an active, stimulus-elicited, physiological response (conditioned fear elicited by the shocked context).

In summary, we have shown that spinal neurons are sensitive to a *R*-*O* relation and provided evidence that this learning involves, at a behavioral level, a form of passive avoidance (punishment) in which the onset of a nociceptive cue reduces the probability of a specific response (leg extension). At a mechanistic level, we suggest that this process reflects the development of an active process in which an afferent signal indicative of leg position acquires the capacity to drive a flexion response. We assume that the system is built to quickly detect such relations and, in this way, is biased (biologically prepared) in favor of detecting control. Registering the relationship between an index of position/movement and external stimulation would allow the organism to, in a sense, directly perceive control. This position is analogous to a Gibsonian account of depth perception (Gibson, 1979), which showed how information available within the two-dimensional signal detected by the retina (e.g., texture gradients) could provide a cue for depth. In many cases, no down-stream processing (e.g., the computation of binocular disparity) is needed – depth can be directly perceived. Likewise, our account ties the detection of behavioral control to sensory, rather than motor, systems. In terms of spinal anatomy, our analysis suggests a shift in focus, from the ventral to the dorsal horn.

UNCONTROLLABLE STIMULATION

The present review focuses on the consequences of controllable stimulation – how behavioral control is detected and how it affects spinal plasticity. A lack of behavioral control could theoretically have no effect, beyond the unconditioned consequences of stimulation *per se*. Our results suggest otherwise, that uncontrollable stimulation engages an active cellular process that has a lasting effect on spinal plasticity. This process is not neutral with respect to instrumental learning, but instead, actively opposes it. The mechanisms that underlie this inhibitory effect are discussed in detail in our companion article (Ferguson et al., under review; also see Baumbauer et al., 2009b). Here, we provide a short overview focusing on concepts relevant to the interaction between controllable and uncontrollable stimulation.

To study the consequences of uncontrollable stimulation, and the underlying neurobiological mechanisms, Crown et al. (2002b) simplified our paradigm by developing a computer program that emulated the variable shock sequence produced by a typical master rat during the first 5–10 min of training. This program generates brief (80 ms) shocks that occur at a variable interval with an average inter-stimulus interval (ISI) of 2 s. Using this program, Crown et al. (2002b) showed that just 6 min of stimulation (approximately 180 shocks) inhibits instrumental learning for up to 48 h. Additional studies showed that the induction of this effect requires protein synthesis (Patton et al., 2004; Baumbauer et al., 2006).

Interestingly, uncontrollable intermittent shock to one hind leg inhibits learning independent of whether subjects are tested on the same (ipsilateral) or opposite (contralateral) leg (Joynes et al., 2003). Indeed, uncontrollable intermittent shock applied to the tail is just as effective (Crown et al., 2002b). These observations suggest that a common system, within the spinal cord, underlies the induction and maintenance of the learning deficit. Further evidence for spinal mediation was obtained by cutting the sciatic nerve prior to intermittent leg-shock (Joynes et al., 2003). When sensory transmission was disrupted in this manner, leg-shock had no effect on learning when subjects were tested on the contralateral leg. Likewise, inactivating the spinal cord (using the Na^+ channel blocker lidocaine) prior to intermittent shock blocks the induction of the deficit. The induction of the deficit can also be blocked by the spinal application [an intrathecal (i.t.) injection] of an NMDA receptor (NMDAR) antagonist (MK-801), a mGluR1 antagonist (CPCCOEt), or a $GABA_A$-R antagonist (bicuculline; Joynes et al., 2004; Ferguson et al., 2006, 2008). Non-neuronal systems (glia and cytokines) also contribute to the induction of the learning deficit (Young et al., 2007; Vichaya et al., 2009; Huie et al., 2012a). The expression of the deficit is blocked by both bicuculline and pretreatment with an opioid antagonist (naltrexone or nor-BNI; Ferguson et al., 2003; Joynes and Grau, 2004; Washburn et al., 2008).

The fact that an opioid antagonist given prior to testing blocks the expression of the learning deficit led us to hypothesize that intermittent shock might inhibit learning because it induces a lasting, opioid-dependent, inhibition of nociceptive processing (antinociception). Indeed, we had previously shown that a long-continuous tail-shock can induce a robust antinociception [inferred from the inhibition of tail-withdrawal from a noxious

thermal stimulus (the tail-flick test)] (Meagher et al., 1993). To explore this possibility, Crown et al. (2002b) examined whether exposure to 6 min of intermittent shock induces antinociception. As a positive control, other spinally transected rats received 6 min of continuous tail-shock. Continuous shock induced a robust antinoception, but intermittent shock had no effect. Moreover, when we then tested the capacity for instrumental learning, we found that only intermittent stimulation impaired learning. Continuous shock to the tail not only failed to induce a deficit, it exerted a protective effect that prevented the induction of the learning deficit by intermittent leg-shock (Crown et al., 2002b).

To further explore how intermittent shock affects behavioral reactivity, Ferguson et al. (2006) assessed responsiveness to mechanical stimulation (von Frey stimuli) applied to the mid-plantar surface of the hind paw. We found that intermittent shock enhanced mechanical reactivity (EMR). EMR is of clinical interest because it is generally assumed that the sensitization of nociceptive circuits within the spinal cord affects *both* motor reactivity and the signal relayed to the brain, causing a previously innocuous stimulus to be "perceived" as painful (the clinical definition of allodynia). While this working model has proven valuable, it must be remembered that it is based on an assumed relation and that further work will be needed to determine whether manipulations that affect motor reactivity within an animal model have a parallel effect on human pain. For these reasons, when describing an increase in motor reactivity to tactile stimulation in spinally transected rats, we will refer to it in behavioral terms as EMR.

EMR is often observed after treatments that induce peripheral inflammation (e.g., intradermal application of formalin or capsaicin) and has been linked to a lasting NMDAR-dependent increase in neural excitability within the spinal cord (central sensitization) and the development of neuropathic pain (Woolf and Thompson, 1991; Coderre et al., 1993; Herrero et al., 2000). Perhaps intermittent, uncontrollable, shock induces a similar effect. If so, this could also explain the disruption in instrumental learning. Within the hippocampus, diffusely saturating NMDAR-dependent plasticity can block the induction of long-term potentiation (LTP) within a selective pathway (Moser and Moser, 1999). Likewise, inducing central sensitization could saturate NMDAR-mediated plasticity within the spinal cord and thereby disrupt the acquisition of selective response modifications (instrumental learning). If this hypothesis is true, then treatments that produce central sensitization should inhibit instrumental learning. Supporting this, Hook et al. (2008) showed that peripheral application of capsaicin produces a dose-dependent inhibition of instrumental learning (**Figure 5**).

In the uninjured state, descending systems normally exert a protective effect that inhibits the induction of the learning deficit (Crown and Grau, 2005). Supporting this, if intermittent shock is given prior to a spinal transection, it has no effect on spinal learning. This protective effect appears to depend on serotonergic fibers that descend through the dorsolateral funiculus (DLF). Crown and Grau (2005) also demonstrated that bilateral lesions limited to the DLF remove the brain-dependent protective effect. So too does i.t. application of the serotonin 5-HT_{1A} antagonist (WAY 100635). Conversely, spinally transected animals given 5-HT, or the 5-HT_{1A} agonist 8-OH DPAT, prior to intermittent shock do not develop a learning deficit.

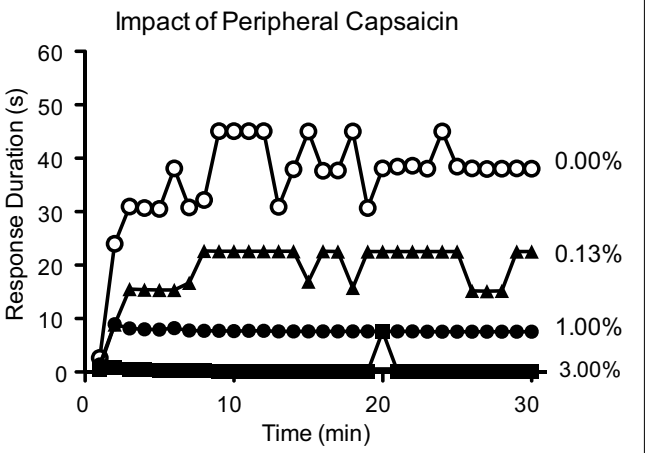

FIGURE 5 | Peripheral inflammation from capsaicin treatment induces a dose-dependent disruption in instrumental learning. Adapted from Hook et al. (2008).

Interestingly, this brain-dependent protective effect is not observed in anesthetized rats. Supporting this, intact rats given intermittent tail-shock while anesthetized with pentobarbital, and then transected, exhibit a learning deficit (Washburn et al., 2007). This suggests that noxious stimulation during surgery can adversely affect spinal systems, to inhibit adaptive plasticity and potentially promote the development of neuropathic pain.

In summary, we have shown that intermittent uncontrollable shock induces a lasting inhibition of instrumental learning. This deficit involves a NMDAR-dependent form of plasticity that may be related to the induction of a central sensitization-like process. Spinal injury allows this maladaptive process to develop by releasing lower neural systems from a brain-dependent process that counters the consequences of uncontrollable nociceptive stimulation, possibly by dampening the development of over-excitation and the induction of central sensitization.

CONTROLLABLE STIMULATION

Returning to the focus of the present paper, we will present evidence that controllable stimulation engages a spinally mediated process that has a protective/restorative effect and provide evidence that this process depends on the neurotrophin BDNF.

BEHAVIORAL PROPERTIES

Recognizing that peripheral changes could contribute to instrumental performance, we first sought evidence that the change in flexion duration (our index of learning) depended upon spinal neurons. Again, we assessed the impact of disrupting the afferent signal (by cutting the sciatic nerve) and inactivating the cord (through i.t. application of lidocaine). After both manipulations, subjects failed to learn (Crown et al., 2002a). Stimulation of the tibialis anterior muscle still elicited a flexion response, but response-contingent shock did not produce an increase in flexion duration. Instead, subjects responded in a mechanical manner,

with shock eliciting a robotic like response that often varied little over time. The consistency of responding was, in some cases, remarkable [e.g., varying less than 10% across consecutive training bins midway (min 16–20) through testing], seemingly affected only by motor fatigue.

An interesting feature of the learning deficit is that uncontrollable stimulation applied to one leg impairs learning when subjects are tested on the contralateral limb, an observation that suggests that uncontrollable stimulation induces a general change within the lumbosacral spinal cord that undermines (disables) the capacity for instrumental learning. Given this observation, Crown et al. (2002a) looked at whether controllable stimulation might have the opposite effect and act to enable learning. To examine this issue, subjects received 30 min of training using our usual response criterion, which submerged the contact electrode by 4 mm. We then tested subjects on either the same or opposite leg with a higher (8 mm) response criterion. Raising the criterion made the task so difficult that untrained subjects failed to learn (**Figure 6**). But subjects that had previously been trained with controllable shock learned at this high criterion and this was true independent of whether they were tested on the trained (ipsilateral) or untrained (contralateral) leg. It appears that training with controllable stimulation has an enabling effect that generally promotes instrumental learning.

Given that controllable and uncontrollable stimulation appear to impact spinal cord plasticity in an opposing manner, Crown and Grau (2001) explored whether the two forms of stimulation interact. Would, for example, prior training with controllable stimulation have a protective effect that prevents the induction of the learning deficit? To test this (see **Table 1Ai**), spinally transected rats received 30 min of training with controllable shock (Master), uncontrollable shock (Yoked), or nothing (Unshocked). Subjects then received 6 min of variable intermittent tail-shock, which we had previously shown produces a learning deficit (Crown et al., 2002b). Finally, subjects were tested with response-contingent shock applied to the untrained leg. As usual, subjects that had received intermittent tail-shock alone failed to learn. This learning deficit was not observed in rats that received controllable stimulation prior to non-contingent tail-shock, suggesting that training with controllable shock blocked the induction of the learning deficit.

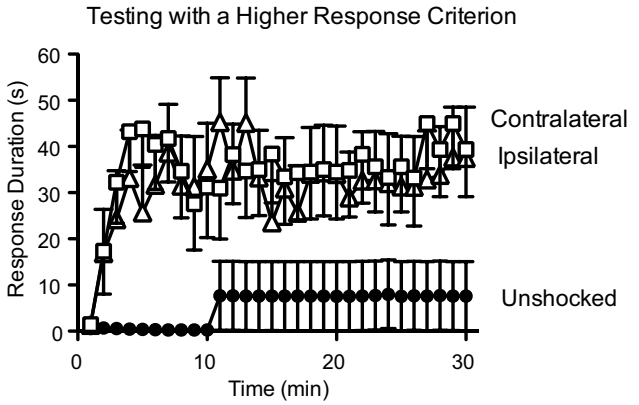

FIGURE 6 | Prior training with controllable shock enables learning. Spinally transected rats received instrumental training using a moderate (4 mm contact electrode depth) and were then tested with response-contingent shock applied to the pretrained (ipsilateral) or opposite (contralateral) leg. Prior to testing, the task was made more difficult by raising the response criterion to an electrode depth of 8 mm. Under these conditions, previously untrained rats (Unshocked) failed to learn. Rats that had received instrumental training were able to learn and this was true irrespective of whether they were tested on the ipsilateral or contralateral leg. Adapted from Crown et al. (2002a).

Crown and Grau (2001) also explored whether training with controllable stimulation could have a restorative effect that reinstates the capacity for learning after the deficit has been induced. But how can we test this if uncontrollable stimulation disrupts subsequent learning? To explore the therapeutic potential of controllable stimulation, we needed a way of temporarily blocking the expression of the learning deficit. Concurrent studies had revealed that i.t. administration of an opioid antagonist (naltrexone) blocked the expression of the learning deficit (Joynes and Grau, 2004). Perhaps, if we trained rats while the deficit was pharmacologically blocked, behavioral training would have a long-term restorative effect that would be evident 24 h later (after the drug had cleared the system). The experimental design (**Table 1Bi**) was roughly the mirror image of the one used to examine the protective effect of controllable stimulation (**Table 1Ai**). First, we induced a learning deficit by exposing rats to variable intermittent tail-shock. Subjects then received an i.t. injection of naltrexone, followed by 30 min of training with controllable shock (Master), uncontrollable shock (Yoked), or nothing (Unshocked). The next day, rats were tested with controllable shock applied to the untrained leg. Rats that received uncontrollable shock alone failed to learn, confirming that non-contingent shock induces a lasting deficit and that the drug treatment *per se* had no long-term beneficial effect on performance. Importantly, rats that received non-contingent shock were able to learn when controllable stimulation was applied immediately after naltrexone treatment, confirming that the drug blocks the expression of the learning deficit. The critical question was whether this training would have a lasting therapeutic effect that would be evident the next day when subjects were tested in a drug-free state. We found that it did, suggesting that training with controllable stimulation can reverse the learning deficit.

Earlier, we described how manipulations that induce central sensitization also impair instrumental learning. For example, intradermal capsaicin (a TRPV1 receptor agonist) produces both EMR and a lasting impairment of instrumental learning that is observed when subjects are tested 24 h later on the untreated (contralateral) leg (Hook et al., 2008). If capsaicin treatment and uncontrollable stimulation impact spinal plasticity in the same way, training with controllable stimulation should attenuate the capsaicin-induced learning deficit. To examine this, spinally transected rats received 30 min of training with controllable leg-shock (Master), uncontrollable leg-shock (Yoked), or nothing (Unshocked). Immediately after, they received an intradermal injection of 3% capsaicin or its vehicle to the same leg. Hook et al. (2008) then assessed mechanical reactivity (**Figure 7A**). In vehicle treated rats, uncontrollable, but not controllable, shock-induced EMR on the treated leg. Capsaicin produced a robust EMR in both the Unshocked and Yoked groups on both the treated and

Table 1 | Impact of instrumental training (i, ii, and iv), or an extended exposure to fixed spaced shock (iii and v), on the learning deficit and the enhanced mechanical reactivity (EMR) induced by variable shock (i and iii) or capsaicin (iv and v) treatment.

A. PROTECTIVE EFFECT					
	Pretreatment	**Treatment**	→	**Learning**	**Tactile**
i	Unshocked	Variable shock		Deficit	EMR
	Master			Normal	–
	Yoked			Deficit	–
ii	Master + TrkB-IgG	Variable shock		Deficit	–
	BDNF	Variable shock		Normal	Normal
iii	Fixed spaced shock	Variable shock		Normal	–
	Fixed spaced shock + TrkB-IgG			Deficit	–
iv	Unshocked	Peripheral capsaicin		Deficit	EMR
	Master			Normal	Normal
	Yoked			Deficit	EMR
v	Fixed spaced shock	Peripheral capsaicin		Normal	Normal

B. RESTORATIVE EFFECT						
	Treatment	**Post treatment**		→	**Learning**	**Tactile**
i	Variable shock	Naltrex.	Unshocked		Deficit	–
			Master		Normal	–
			Yoked		Deficit	–
ii	Variable shock	Naltrex.	Master + TrkB-IgG		Deficit	–
	Variable shock	BDNF			Normal	–
iii	Variable shock	Fixed Spaced Shock			Normal	–
iv	Peripheral capsaicin	Naltrex.	Unshocked		Deficit	EMR
			Master		Normal	Normal
v	Peripheral capsaicin	Fixed spaced shock			Normal	Normal

Behavioral treatments have both a protective (A) and restorative (B) effect that reduces the learning deficit and EMR. Both the protective and the restorative effect of instrumental training have been linked to the release of BDNF (ii). To assess the restorative effect of instrumental training (B: i, ii, iv), naltrexone (Naltrex.) was given prior to training to block the expression of the learning deficit. Untested cells are indicated with a "–."

untreated leg. As noted by Ferguson et al. (under review), inflammatory agents can induce peripheral effects that contribute to the EMR observed on the ipsilateral leg. For this reason, the EMR observed on the contralateral (untreated) leg is often viewed as a purer measure inflammation-induced central sensitization. Given this, it is informative that prior training with controllable shock eliminated the EMR observed when subjects were tested on the untreated leg, but had no effect on reactivity when subjects were tested on the treated leg. A similar pattern was observed when peripheral inflammation was induced with intradermal formalin (Ferguson et al., under review). The next day, instrumental learning was tested using the untreated limb. Hook et al. (2008) found that a high concentration of capsaicin induced a robust learning deficit. Prior training with controllable stimulation appeared to lessen this deficit, but the effect was not robust. We reasoned that a small effect may have been observed because capsaicin produced such a strong learning impairment (**Figure 5**). To evaluate this possibility, we repeated the experiment using a lower concentration of capsaicin (1%). Subjects that received capsaicin alone (Unshk → 1%) failed to learn when tested on the contralateral leg 24 h later (**Figure 7B**). More importantly, prior training with controllable shock (Train → 1%) completely blocked the deficit.

Hook et al. (2008) then examined the converse issue, whether training with controllable stimulation could restore the capacity for learning if given *after* capsaicin treatment. Again, we faced a dilemma, because our behavioral rehabilitation depends on the capacity for learning, yet that was disrupted by capsaicin treatment. If uncontrollable shock and inflammation impair learning through a common mechanism, we should be able to block the expression of the learning deficit by pretreating subjects with naltrexone. To test this, rats received an intradermal injection of 1% capsaicin or its vehicle. Six hours later, half the subjects in each condition received an i.t. injection of naltrexone, followed by 30 min of training with response-contingent shock applied to the treated leg. We found that capsaicin induced a learning deficit and that the expression of this deficit was blocked by naltrexone. Hook et al. (2008) then tested the subjects 24 h later with response-contingent shock applied to the contralateral leg. We found that training with controllable stimulation had a therapeutic effect that restored the capacity for learning in capsaicin-treated rats (**Table 1Biv**).

Our results demonstrate that training with controllable stimulation induces a spinally mediated alteration that enables instrumental learning and exerts a protective effect that counters the learning deficit induced by either uncontrollable stimulation or peripheral inflammation.

NEURAL MECHANISMS

We next consider the neural mechanisms that underlie instrumental learning and its protective/restorative effect. The first key

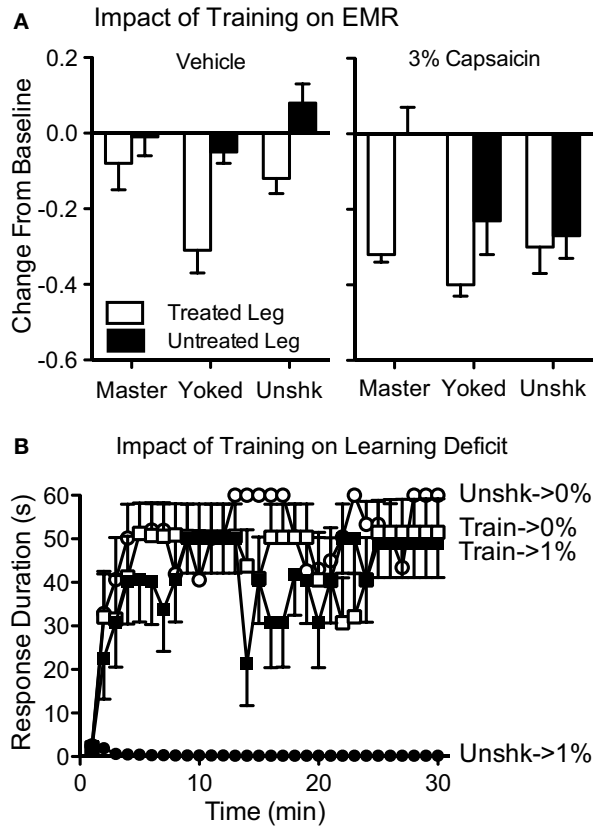

FIGURE 7 | Training with controllable shock attenuates the EMR and learning deficit observed after peripheral capsaicin. (A) Spinally transected rats received controllable shock (Master), uncontrollable shock (Yoked) or nothing (Unshk). They then received an intradermal injection of capsaicin (3%) or its vehicle into the paw of the pretreated leg. Mechanical reactivity was tested using von Frey stimuli applied to the treated, or untreated, leg. In vehicle treated rats, uncontrollable shock enhanced reactivity on the treated leg. Capsaicin induced a robust bilateral EMR in both the Yoked and Unshk groups. Training with controllable stimulation (Master) eliminated the EMR observed on the contralateral (Untreated) leg. **(B)** Transected rats received instrumental training (Train) or nothing (Unshk), followed by a peripheral injection of capsaicin (1%) into the paw of the same leg. The next day, rats were tested with response-contingent shock applied to the contralateral leg. Untrained rats that received capsaicin (Unshk → 1%) failed to learn. Prior training with controllable shock (Train → 1%) eliminated this learning deficit. Adapted from Hook et al. (2008).

question is: where does the learning occur? Liu et al. (2005) addressed this issue using a combination of techniques. We began by microinjecting fluorescent tracers (DiI and Fluoro-Gold) into the tibialis anterior muscle, at the site and depth of the needle electrode used to induce a flexion response. We found that the dyes labeled motoneurons in the lower L4-L5 region, an area implicated in the production of hind-limb stepping behavior (Nishimaru and Kudo, 2000). Next, separate groups of T2 transected rats received a slow infusion of lidocaine through an i.t. cannula positioned at T10/11, L3/4, S2, or Co1. Using India ink, we showed that this injection procedure impacted a region that extended approximately 0.1–0.2 cm rostral and 0.8–0.9 cm caudal to the cannula tip. When lidocaine was slowly infused, it disrupted performance when infused at L3-L4, but not at T10/11 or Co1 (with a partial effect when given at S2).

Liu et al. (2005) then examined the impact of selective knife cuts that transected the cord at different levels between L1 and S1, reasoning that spinal learning should remain intact as long as the knife cut is rostral to the essential circuit, while a transection at the site of learning would have a disruptive effect. We found that knife cuts between L1 and L4 had little effect on instrumental learning and that a more caudal cut, at L6-S1, disrupted learning. Finally, we combined a transection at L4 with a second more caudal transection, at S2, S3, or Co1. Learning was observed when the second transection occurred at S3 or lower, but not at S2, implying that the essential neural circuit lies between L4 and S3. These experiments both localize the essential neural circuit and laid the groundwork for future studies designed to identify the underlying neurochemical systems.

Given that NMDA-mediated plasticity has been shown to play an important role in a variety of learning phenomena (e.g., Morris et al., 1986; Collingridge and Bliss, 1987; Morris, 1994), and the discovery that spinal neurons support NMDAR-mediated plasticity (Dickenson and Sullivan, 1987; Coderre et al., 1993), we examined whether spinally mediated instrumental learning depends on the NMDAR. Using the competitive NMDAR antagonist AP5, Joynes et al. (2004) showed that learning was disrupted in a dose-dependent manner. Ferguson et al. (2006) subsequently extended this observation, demonstrating that learning is also disrupted by pretreatment with the non-competitive antagonist MK-801.

Further work showed that AP5 not only disrupts the acquisition of spinal learning, it also undermines the maintenance of instrumental behavior (Joynes et al., 2004). A similar outcome was reported for another example of NMDAR-dependent plasticity, wind-up (the enhancement in neural excitability observed with repetitive electrophysiological stimulation at an intensity that engages C-fibers; Mendell, 1966; Ma and Woolf, 1995). In this way, spinally mediated forms of NMDAR-mediated plasticity appear to differ from traditional preparations, such as hippocampal LTP, where it is generally held that NMDAR-dependent plasticity contributes to the induction, but not the maintenance, of LTP (Staubli et al., 1989).

In collaboration with Fernando Gómez-Pinilla and Reggie Edgerton (Gómez-Pinilla et al., 2007), we conducted cellular assays that targeted genes implicated in plasticity. This study was motivated, in part, by the hypothesis that controllable stimulation might enable learning by engaging processes related to the release of BDNF. Research suggested that BDNF is essential to the development of LTP (Kang and Schuman, 1995; Patterson et al., 1996; Bekinschtein et al., 2008) and that this neurotrophin potentiates plasticity in spinal neurons (Heppenstall and Lewin, 2001; Baker-Herman et al., 2004; Zhou et al., 2008). To examine whether instrumental training affects the expression of BDNF, spinally transected rats were given controllable shock (Master), an equal amount of uncontrollable stimulation (Yoked), or nothing (Unshocked). After training, the L4-S1 spinal cord was removed and real-time RT-PCR was performed. We found that training with controllable stimulation produced a significant increase in BDNF mRNA expression, while uncontrollable stimulation produced a

decrease (relative to the unshocked controls). Two down-stream targets, calcium/calmodulin activated protein kinase II (CaMKII) and the gene transcription factor cAMP-response element binding protein (CREB) showed the same pattern of results. We then examined whether instrumental performance predicted mRNA expression. Reasoning that expression may be most related to performance during the learning phase, we computed the mean response duration observed during the first 10 min of training. Independent analyses revealed that BDNF, CaMKII, and CREB were well-correlated with instrumental performance in master rats (all r's > 0.93, $p < 0.005$). No significant relations were observed in the yoked controls. *In situ* hybridization showed that training with controllable shock enhanced BDNF mRNA expression throughout the spinal central gray (Huie et al., 2012b). Protein assays (Western blotting), showed that training with controllable shock increases the expression of both BDNF and its receptor, the trypomyosin receptor kinase TrkB. Immunohistochemical analyses revealed that controllable stimulation enhances TrkB protein expression within neurons of the dorsal horn (**Figure 8**), a modification that may provide a form of synaptic tag (Lu et al., 2011).

Given that the production of new protein will require some time, we hypothesized that the increase in BDNF and CaMKII expression may mediate the consequences of training, rather than instrumental learning *per se*. Supporting this, pretreatment with either a BDNF inhibitor (TrkB-IgG) or a CaMKII inhibitor (AIP) did not have a significant impact on instrumental learning (Gómez-Pinilla et al., 2007). After training, we tested subjects on the contralateral leg with a higher response criterion. As described above, in the absence of pretraining, subjects could not learn. Pretrained rats were able to learn when tested with a higher response criterion and this effect was blocked by pretreatment with either TrkB-IgG or AIP. Further evidence that BDNF contributes to the enabling effect was derived by administering BDNF (i.t.) prior to testing with a high response criterion. As a positive control, Gómez-Pinilla et al. (2007) also included a group that received instrumental training instead of drug treatment. As usual, these subjects were able to learn when tested with a higher criterion. Pretreatment with BDNF (0.1–0.4 μg i.t.) also enabled learning and did so in a dose-dependent manner.

Huie et al. (2012b) then examined whether BDNF contributes to the protective/restorative effect of instrumental training. To evaluate whether BDNF was necessary to the protective effect, subjects were given TrkB-IgG or its vehicle. Subjects then received instrumental training, or nothing (Unshk), prior to 6 min of intermittent tail-shock (Int Shk). The next day, subjects were tested on the contralateral leg using the usual response criterion. In vehicle treated rats, prior training with controllable stimulation blocked the induction of the learning deficit. Pretreatment with TrkB-IgG eliminated this protective effect (**Figure 9A**).

If instrumental learning has a protective effect because it induces the release of BDNF, then i.t. BDNF should substitute for instrumental training and inhibit the induction of the learning deficit. To explore this possibility, Huie et al. (2012b) administered a low dose of BDNF (0.4 μg i.t.) or its vehicle 30 min before they received 6 min of intermittent tail-shock (Int Shk). The next day subjects were tested with response-contingent shock. As usual, uncontrollable shock impaired learning. No learning impairment

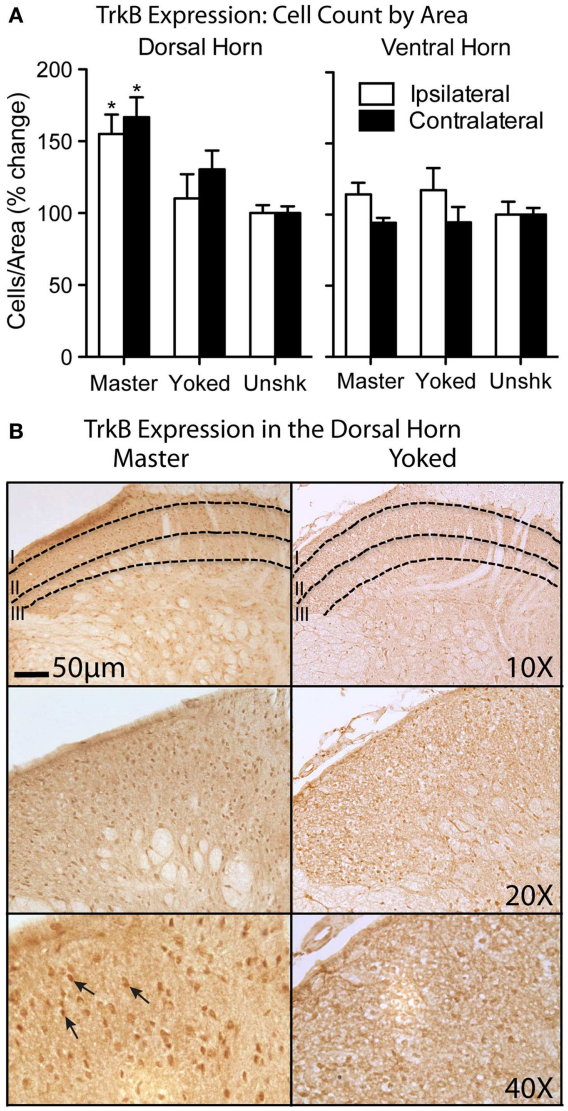

FIGURE 8 | Controllable shock increases TrkB expression within the dorsal horn. (A) Number of cells labeled within the dorsal and ventral horn. Master rats exhibited greater TrkB expression within dorsal horn on both the treated (shocked; ipsilateral) and untreated side (contralateral). No differences were observed within the ventral horn (*$p < 0.05$). **(B)** Light immunohistochemistry showed increased TrkB immunolabeling in the dorsal horn of Master, but not Yoked, rats. Adapted from Huie et al. (2012b).

was observed in rats that received BDNF prior to uncontrollable shock (**Figure 9B**).

Above we noted that exposure to uncontrollable stimulation enhances tactile reactivity. Huie et al. (2012b) replicated this finding and showed that pretreatment with BDNF also attenuates shock-induced EMR (**Figure 9C**). This observation contrasts with other studies that implicate BDNF in the induction of central sensitization (Kerr et al., 1999; Garraway et al., 2003; Merighi et al., 2008; Lu et al., 2009), a finding that suggests that BDNF should have, if anything, enhanced EMR. This apparent discrepancy is not an isolated instance. For example, Pezet et al. (2002) showed

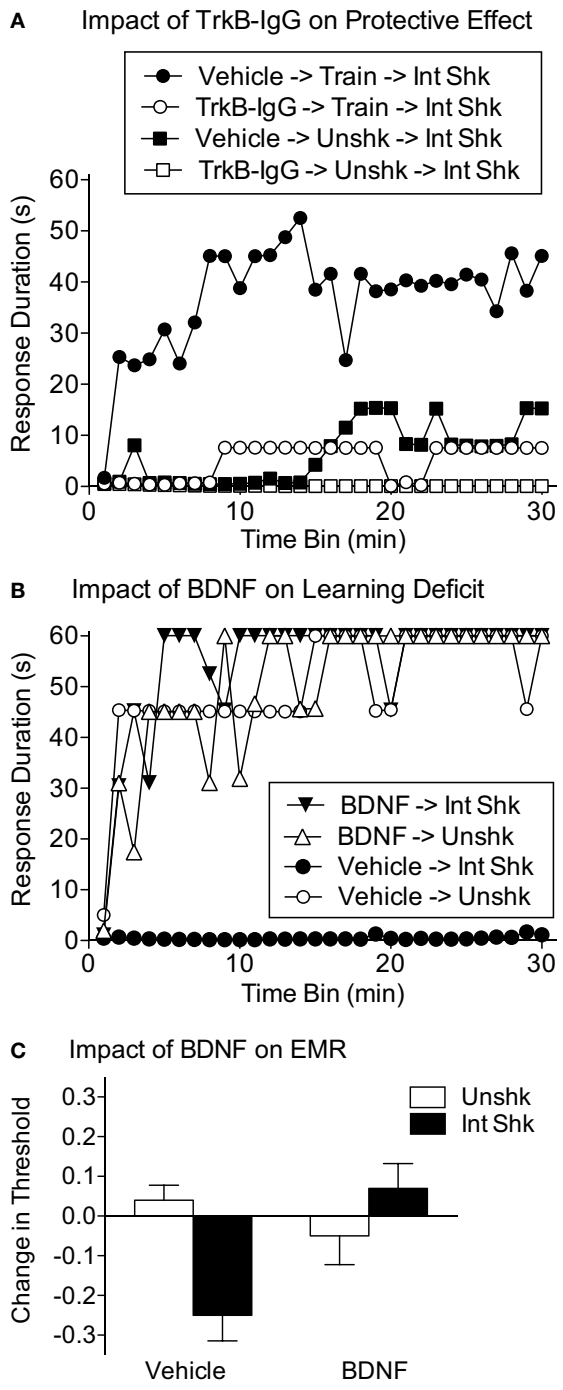

FIGURE 9 | Evidence BDNF contributes to the protective effect of controllable stimulation. (A) Spinally transected rats received the BDNF inhibitor TrkB-IgG (i.t.) or its vehicle, followed by instrumental training (Train) or nothing (Unshk). Subjects then received variable intermittent tail-shock (Int Shk). The next day, subjects were tested with response-contingent shock applied to the previously untrained leg. Intermittent shock induced a learning deficit in the untreated subjects (Vehicle → Unshk → Int Shk). Prior training with controllable shock (Vehicle → Train → Int Shk) prevented the learning deficit. Pretreatment with TrkB-IgG (TrkB-IgG → Train → Shk) eliminated this protective effect. **(B)** Rats received BDNF (0.4 μg, i.t.) or its *(Continued)*

FIGURE 9 | Continued
vehicle and 30 min later variable intermittent tail-shock (Int Shk) or nothing. Subjects were tested 24 h later. Vehicle treated rats that had received intermittent shock (Vehicle → Int Shk) failed to learn. Pretreatment with BDNF (BDNF → Int Shk) blocked the induction of this learning deficit. **(C)** Spinally transected rats received BDNF (0.4 μg, i.t.) or its vehicle followed by 6 min of variable intermittent shock (Int Shock) to one hind leg or nothing (Unshk). Mechanical reactivity was then tested using von Frey stimuli. Because comparable results were observed on both the shocked and unshocked leg, the data were collapsed across this variable. Intermittent shock-induced EMR in vehicle treated rats, but not rats pretreated with BDNF. Adapted from Huie et al. (2012b).

that treatment with BDNF can induce a thermal antinociception, an effect they attributed to BDNF inhibiting (via a GABAergic interneuron) substance P release within the dorsal horn. In a model of neuropathic pain (spinal nerve ligation), Lever et al. (2003) showed that i.t. BDNF attenuated the ligation-induced thermal hyperalgesia. Nerve injury was associated with a reduction in GABA, which was restored by BDNF treatment. In a similar vein, Cejas et al. (2000) showed that application of BDNF secreting cells a week after sciatic nerve injury attenuated both the injury-induced mechanical allodynia and thermal hyperalgesia. Importantly, both effects were observed for weeks after treatment. As noted in a recent review (Merighi et al., 2008), there is also ample evidence that BDNF can enhance pain. For example, Coull et al. (2005) showed that a high dose of BDNF (20 μg) can induce tactile allodynia. Conversely, Kerr et al. (1999) reported that treatment with TrkB-IgG attenuates the nociceptive responses elicited by intraplantar treatment with formalin or carrageenan. Similarly, mice that are BDNF deficient in nociceptive neurons exhibit diminished formalin-induced pain behavior (second phase) and attenuated thermal hyperalgesia after carrageenan (Zhao et al., 2006). These latter studies suggest that the induction of pain behavior after peripheral inflammation depends on endogenous BDNF.

Brain-derived neurotrophic factor likely yields a wide range of effects because it can influence neural processing within the spinal cord in multiple ways. First, it can act postsynaptically to enhance neural excitability through a NMDAR-mediated process. This action has been observed within nociceptive neurons in lamina II (Garraway et al., 2003) and in motoneurons of the ventral horn (Arvanian and Mendell, 2001). When coupled with response-contingent stimulation, we assume that this type of mechanism contributes to the BDNF-dependent enabling of instrumental learning (Gómez-Pinilla et al., 2007). Second, BDNF can act presynaptically to inhibit transmitter release, and this effect too has been observed within both the dorsal (Pezet et al., 2002) and ventral (Arvanian and Mendell, 2001) spinal cord. As noted above, this inhibitory effect has been attributed to a BDNF-dependent activation of GABAergic interneurons (Pezet et al., 2002). Given these observations, we suggest that the outcome observed depends upon at least three factors: (1) the dose of BDNF used [low concentrations appear to have an antinociceptive effect (Miki et al., 2000; Huie et al., 2012b) while a high concentration can enhance pain behavior (Miki et al., 2000; Coull et al., 2005)]; (2) the model of pain behavior employed; and (3) whether subjects have received a spinal injury (Garraway and Mendell, 2007). The third variable

may be especially important because injury releases spinal mechanisms from sources of tonic inhibition (e.g., 5-HT), can alter levels of GABA, and increase the intracellular levels of Cl^- which can cause GABA to have a depolarizing effect (Millan, 2002; Diaz-Ruiz et al., 2007; Gwak and Hulsebosch, 2011). In this compromised state, we posit that BDNF may generally benefit spinal function, to curb over-excitation and promote adaptive plasticity.

A final complexity stems for the realization that the precursor (proBDNF) to the mature form of BDNF (mBDNF) is biologically active and can induce cellular effects that are antagonist to the action of mBDNF (Bothwell, 1996; Lee et al., 2001; Lu et al., 2005; Cunha et al., 2010), leading others to suggest a yin-yang model of proBDNF-mBDNF function (Lu et al., 2005). Though speculative, it is possible that the opposing effects of controllable and uncontrollable are related to the relative balance of proBDNF to mBDNF. At the least, some caution is warranted in clinical applications, because a physiological manipulation designed to increase BDNF protein expression could inadvertently lead to a maladaptive outcome if the conversion of proBDNF to mBDNF is down-regulated.

Huie et al. (2012b) also asked whether BDNF release plays an essential role in the therapeutic effect of controllable shock. Subjects were given 6 min of non-contingent tail-shock. To temporarily block the expression of the learning deficit, all subjects then received an i.t. injection of naltrexone. To examine whether the therapeutic effect of training depends on BDNF, half the subjects also received the BDNF inhibitor TrkB-IgG. Finally, half the subjects in each drug condition received 30 min of training with response-contingent shock. The next day, subjects were tested for 30 min with response-contingent shock applied to the contralateral leg. As usual, uncontrollable shock impaired learning. Subjects that received 30 min of instrumental training after uncontrollable shock did not exhibit a learning deficit and this therapeutic effect of training was blocked by pretreatment with TrkB-IgG (**Table 1Bii**). Interestingly, a follow-up experiment showed that administering TrkB-IgG after instrumental training also blocked the therapeutic effect of training, suggesting that higher levels of BDNF must be maintained after training for it to have a lasting effect.

If training has a therapeutic effect because it increases BDNF release, then administration of BDNF should substitute for instrumental training and restore the capacity for learning. Huie et al. (2012b) examined this issue in two ways. In both experiments, subjects received uncontrollable tail-shock and were tested with response-contingent shock 24 h later. In the first experiment, BDNF was administered immediately after subjects received uncontrollable shock. In the second experiment, BDNF was given the next day, 30 min before testing. In both cases, BDNF treatment eliminated the learning deficit, suggesting that this neurotrophin can both reverse, and restore, the capacity for learning.

In summary, we have shown that instrumental learning depends on neurons that lie within the L4-S2 spinal tissue. Learning depends on a form NMDAR-mediated plasticity and engages the expression of a number of plasticity related genes, including BDNF, CaMKII, and CREB. We further showed that training with controllable stimulation increases the expression of both BDNF and its receptor, TrkB. The latter effect was localized to the dorsal horn. Finally, evidence was presented that the beneficial/restorative effect of instrumental training is related to the release of BDNF; a BDNF inhibitor (TrkB-IgG) blocked the protective/therapeutic effect of instrumental training and i.t. administration of BDNF substituted for instrumental training to both prevent, and reverse, the learning deficit.

PREDICTABILITY

Our focus has been on behavioral control and how it can engage an adaptive, BDNF-dependent, process that exerts a protective/restorative effect. Recently, Baumbauer et al. (2008, 2009a) discovered that temporal predictability can have a similar effect and may do so using some of the same neurobiological mechanisms. The original aim of these experiments was to identify the stimulus conditions that produce a learning deficit. Using electrophysiological stimulation of the sciatic nerve, Baumbauer et al. (2008) showed that 180 shocks at 0.5 Hz (an ISI of 2″) produces a deficit when shock intensity is increased to a level that engages C-fibers. Moreover, stimulation induced a deficit independent of whether it occurred in a variable (0.2–3.8 s apart, rectangular distribution) or regular (fixed spaced) manner. What was surprising is that, when shock number was increased fivefold (900 shocks), only variable shock impaired subsequent learning. Because we had previously shown that 180 fixed spaced shocks induce a deficit, the fact 900 fixed spaced shocks does not implies that the additional (720) stimuli engaged a restorative process that eliminated the learning deficit.

In a subsequent paper, Baumbauer et al. (2009a) showed that an extended exposure [24–30 min (720+ shocks)] to fixed spaced shock has a protective/restorative effect that parallels the beneficial effect of instrumental control (see **Tables 1iii,v**). Specifically, we found that 720 fixed spaced shocks given before, or after, 180 variably spaced shocks eliminates the learning deficit. Likewise, the learning deficit and EMR induced by peripheral capsaicin was attenuated by exposure to fixed spaced shock (Baumbauer et al., 2010; Baumbauer and Grau, 2011).

Other studies showed that an extended exposure to fixed spaced shock has a lasting protective effect that prevents the induction of the learning deficit by variably spaced shock given 24 h later (Baumbauer et al., 2009a). Rats given the NMDA antagonist MK-801 prior to fixed spaced stimulation do not exhibit the protective effect 24 h later, when challenged with variably spaced shock. The long-term protective effect is also eliminated by administering the protein synthesis inhibitor cycloheximide immediately after exposure to fixed spaced shock. Like behavioral control, pretreatment with the BDNF inhibitor TrkB-IgG eliminated the protective effect.

The observation that fixed and variably spaced shock have divergent effects on spinal function suggests that they are somehow discriminated; that introducing a regular (predictable) temporal relation engages distinct neural processes. Here, we need not take a stand on whether this discrimination involves a sensory filter or a central integrative process, possibly linked to the central pattern generator (CPG) assumed to organize stepping (Grillner and Wallen, 1985). What is important for present purposes is that the results imply that the consequences of intermittent stimulation depend on *both* controllability and predictability: controllable/predictable stimulation engages a BDNF-dependent process

that appears to have a protective/restorative effect whereas uncontrollable/unpredictable stimulation engages processes that inhibit learning and enhance mechanical reactivity.

The fact that the long-term consequences of fixed spaced stimulation require extended training, are NMDA-dependent, and involve protein synthesis, suggests that a kind of learning may be engaged. In intact animals, there is ample evidence that elapsed time can act as a Pavlovian CS and, with a regularly presented US, elicit a CR that is timed to the occurrence of the US (temporal conditioning). This type of learning may underlie the fixed spaced shock effects described by Baumbauer et al. (2008, 2009a) and Baumbauer and Grau (2011).

In terms of clinical application, fixed spaced stimulation may provide an attractive alternative in situations where instituting behavioral control is not possible. There is a caveat, however, because far more training is needed to establish the fixed spaced shock effect; whereas the behavioral effects of controllable stimulation are evident within minutes of training (with fewer than 180 shocks), the beneficial effect of fixed spaced shock only emerges after extended training (e.g., 720 stimulus presentations or more). The spinal learning system appears to be better equipped (biologically prepared) to learn about behavioral controllability (i.e., contingent vs. non-contingent stimulation) than to learn about temporal predictability (i.e., fixed-space stimulation).

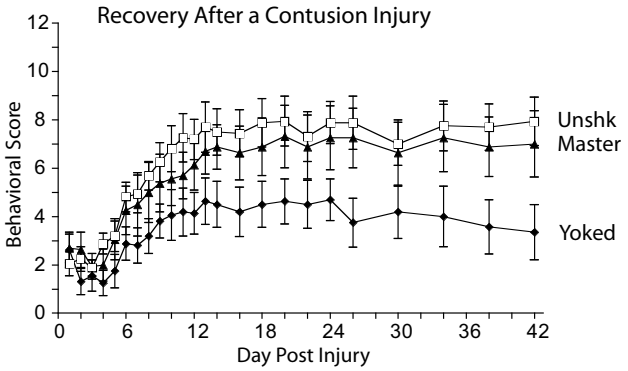

FIGURE 10 | Only uncontrollable stimulation impairs recovery after a contusion injury. Rats received a moderate contusion injury and, 24 h later, 30 min of training with controllable shock (Master). Other groups received shock independent of leg position (Yoked) or nothing (Unshk). These treatments were repeated the next day and locomotor recovery was monitored for the next 6 weeks using a modified version of the BBB locomotor scale (Basso et al., 1995; Ferguson et al., 2004). Exposure to uncontrollable shock (Yoked) impaired recovery. Nociceptive stimulation had no adverse effect when it was given in a controllable manner (Master). Adapted from Grau et al. (2004).

CLINICAL IMPLICATIONS

We have begun to explore some of the clinical implications of our work and have shown that the same shock schedule (6 min of intermittent tail-shock) that impairs spinal plasticity also disrupts recovery after a contusion injury (Grau et al., 2004). A key question is whether this effect is also modulated by instrumental control. To explore this possibility, we administered a moderate contusion injury in the lower thoracic region. The next day, master rats received 30 min of response-contingent leg-shock (Master), while yoked subjects received an equal amount of shock given independent of leg position. A third group served as the unshocked controls. These treatments were repeated the next day and locomotor recovery was monitored over the next 6 weeks. We found that uncontrollable stimulation impaired recovery (**Figure 10**). Master subjects, that received the same amount of shock but could control its presentation, exhibited normal recovery. Thus, introducing instrumental control can blunt the adverse effect of nociceptive stimulation.

Other recent data suggest that the adverse effect of uncontrollable stimulation may be related to a down-regulation of BDNF. In these studies, rats again received a moderate contusion injury and uncontrollable shock 24 h later (Garraway et al., 2011). A day after shock treatment, subjects exhibited a decrease in BDNF mRNA and protein expression within the dorsal horn. Shock also down-regulated TrkB and CaMKII protein within the dorsal, but not the ventral, cord.

These observations suggest that our work using spinally transected rats has implications for recovery after a contusion injury. Our hope is to show that introducing instrumental control not only counters the effect of nociceptive stimulation, but also engages a BDNF-dependent process that promotes recovery. We suspect that demonstrating such an effect will require training parameters that minimize the unconditioned (unlearned) adverse effects of nociceptive stimulation. Accomplishing this may require a procedure in which shock intensity is titrated downward to the lowest level that supports learning.

Learning-like adaptations also impact stepping after injury (Edgerton et al., 2004). A particularly good example of this was reported by Edgerton et al. (1997), who showed that spinally transected animals can exhibit a training-induced alteration in hind-limb stepping. After subjects were trained to step on a treadmill, an obstacle was introduced – a bar that one paw struck during the swing phase. Over time, subjects exhibited a stronger flexion response during the swing phase, which reduced the force with which the paw hit the bar. Here too, the onset of a biologically significant stimulus (hitting the bar), in the presence of cues that signal a particular leg position, engenders a change in on-going behavior. Conversely, stand training appears to induce an effect that inhibits learning; rats that received 7 weeks of stand training exhibit impaired learning on a spinally mediated instrumental learning task (Bigbee et al., 2007). Our work also fits nicely with studies demonstrating that up-regulating BDNF expression can promote locomotor behavior in spinally transected rats (Boyce et al., 2007, 2012).

As discussed in Hook and Grau (2007), learning can also contribute to the behavioral changes elicited by the functional electrical stimulation (FES) used to prevent foot drop and/or muscle atrophy. Importantly, the stimulation used in FES is generally applied in a response-contingent manner (e.g., to drive cycling). Our work suggests that, if it was not, the stimulation could adversely affect spinal function.

Finally, Harkema et al. (2011) found that coupling epidural stimulation of the L5-S1 region with sensory stimulation related

to bilateral extension and loading fostered standing behavior in a paraplegic patient (Harkema et al., 2011). In addition, when combined with task-specific sensory cues, epidural stimulation generated locomotor-like behavior. The researchers hypothesized that the stimulation was effective because it engaged populations of interneurons that integrate load-bearing related proprioceptive input to coordinate motor pool activity, and thereby enables use-dependent plasticity.

A SUMMARY MODEL

We have provided evidence that spinal neurons are sensitive to response-outcome relations and that this learning has a lasting effect, demonstrating that training with controllable stimulation enables learning whereas training with uncontrollable stimulation has a disabling effect that inhibits learning. We have further shown that these effects depend on NMDAR-dependent alterations within the spinal cord and that both effects have a general impact on plasticity. As discussed in Ferguson et al. (under review), because these effects concern factors that regulate the plasticity of plasticity, they can be considered forms of metaplasticity (Abraham and Bear, 1996).

Our behavioral analysis sought to both clarify the nature of the learning and its relevance to rehabilitation and suggested that the key events are tied to the onset of nociceptive stimulation. Based on this observation, we suggested that the detection of control must be linked to proprioceptive signals and hypothesized that the system is biased in favor of control. This process can be envisioned as a kind of physiological gate, in which the relationship between the nociceptive stimulus and proprioceptive signals determines how stimulation affects spinal systems (**Figure 11**). If the nociceptive signal is tied to a particular proprioceptive signal (controllable), it engages an adaptive behavioral response (that reduces net exposure to the nociceptive signal) and enlists down-stream (BDNF-dependent) processes that exert a protective/restorative effect. If the stimulus occurs in a manner that is unrelated to a particular proprioceptive cue (uncontrollable), it engages an opponent-like process that inhibits new learning, induces EMR, and impairs recovery. The induction and expression of this deficit has been linked to a GABA-dependent process. Opioids and NMDAR/mGluR-mediated plasticity have also been shown to play a role. Finally, evidence suggests that in uninjured subjects descending 5-HT systems exert a protective effect that acts to counter the adverse effect of uncontrollable stimulation. Within this hypothetical system, NMDAR-mediated plasticity could contribute to long-term retention in a variety of ways. One possibility is that it acts as a kind of latch, locking the hypothetical gate in one mode or the other.

The model illustrated in **Figure 11** was designed to illustrate the functional relations that underlie spinally mediated learning and how this affects nociceptive processing. The aim was to describe a system that could provide an interface between clinical application and the analysis of the underlying neurobiological mechanisms. While we believe that the switching metaphor provides a useful heuristic, it should be recognized that many details remain to be specified. Further, in seeking parsimony, we have likely consolidated functions that are mediated by distinct neuroanatomical systems. For example, we assume that abstracting the relation between proprioceptive and nociceptive inputs requires a form of neural convergence and reflects a local effect. Where might this convergence occur? Proprioceptive afferents that carry information regarding muscle length/velocity (A-alpha fibers) project to lamina VI, as well as deeper laminae (Watson et al., 2008). Nociceptive fibers (A-delta and C) project to laminae I and II. In addition, lamina V receives input from A-delta fibers and polysynaptic inputs from C-fibers. These anatomical considerations suggest that the abstraction of the response-outcome (proprioceptive-nociceptive) relation may occur in laminae V/VI. Alternatively, an interneuronal projection could relay proprioceptive signals to regions within the superficial dorsal horn that receive nociceptive input.

While we assume that learning the relation between a particular leg position and the onset of a nociceptive stimulus is mediated by a local interaction, our results suggest that some consequences of this learning have a more general effect that promotes learning and counters the adverse effects of uncontrollable stimulation. We have shown that this process depends on an up-regulation of BDNF and *in situ* hybridization suggests that BDNF mRNA expression is diffusely increased throughout both the dorsal and ventral horn (Huie et al., 2012b). Likewise, the consequences of uncontrollable stimulation have been likened to the induction of a diffuse state of over-excitation that generally saturates plasticity and enhances mechanical reactivity (Ferguson et al., 2006). This diffuse state has been tied to a GABA-dependent process and the cytokine TNF-alpha (Ferguson et al., 2003; Huie et al., 2012a). Thus, while we illustrate the consequences of these processes on a local effect (influencing the state of the hypothetical gate), we envision the metaplastic effects as having a more global influence on neural processing that extends across multiple laminae. Further, distinct components of the nociceptive signal may be important for learning and the induction of the learning deficit. Because learning depends on strong *R-O* contiguity, fast (myelinated) A-delta fiber input may be critical to abstracting the relation between proprioceptive and nociceptive inputs. At the same time, research indicates that C-fiber input is essential to the induction of the learning deficit (Baumbauer et al., 2008, 2009b).

Two additional details that need to be elucidated concern the mechanisms that underlie the dissemination of the metaplastic effects and the role of GABA. Our behavioral and cellular studies suggest that controllable/uncontrollable stimulation can affect neural processing of afferent signals from remote dermatomes. What process allows the functional spread of the cellular effect across distinct regions of the spinal cord? One possibility is that a cytokine (e.g., TNF-alpha) released from glia has a diffuse effect (Huie et al., 2012a; Vichaya et al., 2009). A second, and potentially related, question concerns the role of GABA. While it is clear that a GABA-dependent process can disrupt learning, it is not clear whether this is due to neural inhibition or a paradoxical excitatory effect linked to an injury-induced shift in intracellular chloride levels that causes GABA to have depolarizing effect (which could contribute to the saturation of NMDAR-mediated plasticity; Diaz-Ruiz et al., 2007; Gwak and Hulsebosch, 2011).

Our model is consistent with an emerging view of motor function. Postural control and adaptation to changing loads requires

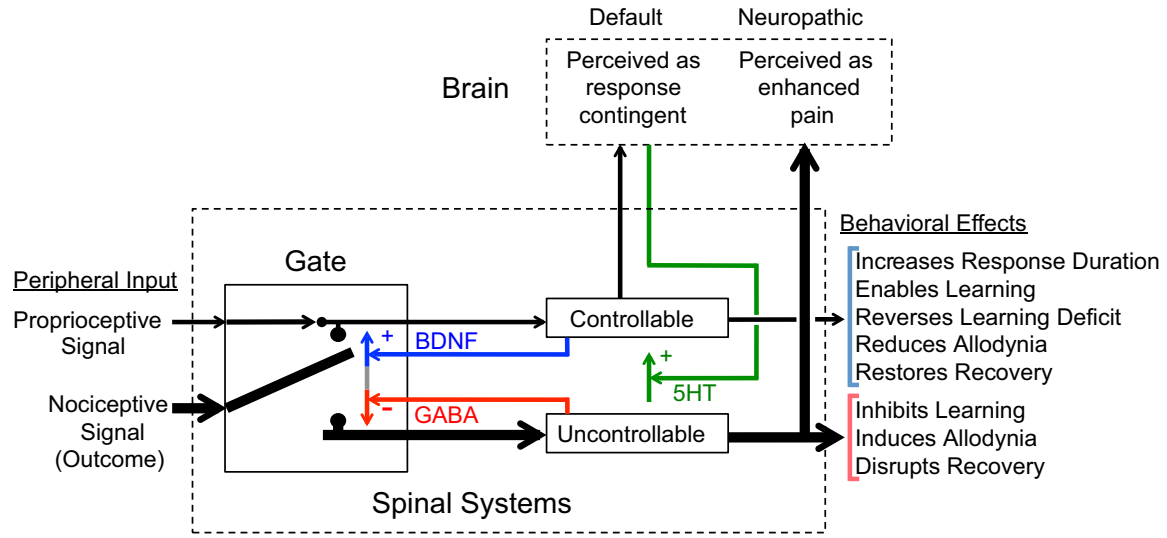

FIGURE 11 | A model of the processes that underlie the spinal consequences of controllable versus uncontrollable stimulation. It is assumed that proprioceptive signals provide an indication of current limb position and that the system is biologically prepared to detect the relationship between this cue and the onset of a nociceptive stimulus (preparedness is represented by initial position of the nociceptive input, which is tilted in favor of behavioral control). When a relation is detected, the stimulation is encoded as controllable. This process promotes adaptive behavior (e.g., an increase in response duration), enables learning, prevents and reverses the learning deficit, attenuates the allodynia elicited by uncontrollable shock or peripheral inflammation, and prevents nociceptive stimulation from adversely affecting recovery. Findings reported above suggest that these adaptive processes are linked to BDNF (blue), which could enable learning and attenuate the consequences of uncontrollable stimulation by biasing the gate in favor of controllability. Uncontrollable stimulation appears to have the opposite effect, engaging a process (red) that inhibits learning, induces allodynia, and undermines recovery after a contusion injury. Psychologically, these maladaptive effects could lead to enhanced (neuropathic) pain. GABAergic systems have been shown to play an important role in both the induction and expression of the learning deficit (Ferguson et al., 2003). Recent data also implicate the cytokine TNF-alpha (Huie et al., 2012a). For both controllable and uncontrollable stimulation, NMDAR-mediated plasticity may provide a kind of physiological latch that maintains these states over time, yielding a form of metaplasticity that enables (controllable) or disables (uncontrollable) adaptive learning. In the uninjured state, descending serotonergic (5-HT) systems (green) counter the effects of uncontrollable stimulation, which we assume helps to maintain the default state (biased in favor of adaptive plasticity).

an internal model of limb dynamics that encodes proprioceptive information (Windhorst, 2007). Researchers have traditionally assumed that this model is mediated by supraspinal structures. However, data collected over the last 20 years has shown that spinal mechanisms can organize well-behaved dynamic limb movements in the absence of input from the brain. Given this, Windhorst (2007) has suggested that spinal systems must also build/maintain a motor map that is linked to proprioceptive/cutaneous input. It is further suggested that learning can occur within this system through a form of back-propagation in the dendritic tree of motoneurons, which could support NMDAR-dependent/Hebbian synaptic plasticity. From our view, the dynamic updating of an internal map could be seen as a form of instrumental learning.

It is also recognized that the model described above incorporates features of the gate control theory of pain (Melzack and Wall, 1965), which proposed that non-nociceptive input conducted by large myelinated fibers can inhibit pain. Our proposal extends this view by suggesting nociceptive inputs can also be modulated by proprioceptive cues. We further suggest that the consequences of non-nociceptive input will depend upon whether it is correlated with the onset of nociceptive stimulation. We also propose that the gate can be latched in one state or the other, providing a kind of sensory memory that will influence how subsequent nociceptive stimuli are processed.

Our research shows that procedures and constructs derived from the field of learning can help us understand how spinal neurons process neural signals. Our behavioral analyses uncovered the events that support learning and thereby shifted our view of how response-contingent stimulation is encoded, to see behavioral control as a form of sensor processing. Behavioral analyses further revealed how factors such as controllability and predictability can engage modulatory (metaplastic) effects that regulate adaptability and the development/maintenance of central sensitization. Our behavioral observations were reinforced by neurobiological studies that linked these effects to the neurotrophin BDNF and NMDAR-mediated plasticity. The studies suggest that behavioral factors can determine whether nociceptive signals lead to neuropathic pain and adversely affect recovery.

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Altered patterns of reflex excitability, balance, and locomotion following spinal cord injury and locomotor training

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Spasticity is an important problem that complicates daily living in many individuals with spinal cord injury (SCI). While previous studies in human and animals revealed significant improvements in locomotor ability with treadmill locomotor training, it is not known to what extent locomotor training influences spasticity. In addition, it would be of considerable practical interest to know how the more ergonomically feasible cycle training compares with treadmill training as therapy to manage SCI-induced spasticity and to improve locomotor function. Thus the main objective of our present studies was to evaluate the influence of different types of locomotor training on measures of limb spasticity, gait, and reflex components that contribute to locomotion. For these studies, 30 animals received midthoracic SCI using the standard Multicenter Animal Spinal cord Injury Studies (MASCIS) protocol (10 g 2.5 cm weight drop). They were divided randomly into three equal groups: control (contused untrained), contused treadmill trained, and contused cycle trained. Treadmill and cycle training were started on post-injury day 8. Velocity-dependent ankle torque was tested across a wide range of velocities (612–49°/s) to permit quantitation of tonic (low velocity) and dynamic (high velocity) contributions to lower limb spasticity. By post-injury weeks 4 and 6, the untrained group revealed significant velocity-dependent ankle extensor spasticity, compared to pre-surgical control values. At these post-injury time points, spasticity was not observed in either of the two training groups. Instead, a significantly milder form of velocity-dependent spasticity was detected at postcontusion weeks 8–12 in both treadmill and bicycle training groups at the four fastest ankle rotation velocities (350–612°/s). Locomotor training using treadmill or bicycle also produced significant increase in the rate of recovery of limb placement measures (limb axis, base of support, and open field locomotor ability) and reflex rate-depression, a quantitative assessment of neurophysiological processes that regulate segmental reflex excitability, compared with those of untrained injured controls. Light microscopic qualitative studies of spared tissue revealed better preservation of myelin, axons, and collagen morphology in both locomotor trained animals. Both locomotor trained groups revealed decreased lesion volume (rostrocaudal extension) and more spared tissue at the lesion site. These improvements were accompanied by marked upregulation of BDNF, GABA/$GABA_b$, and monoamines (e.g., norepinephrine and serotonin) which might account for these improved functions. These data are the first to indicate that the therapeutic efficacy of ergonomically practical cycle training is equal to that of the more labor-intensive treadmill training in reducing spasticity and improving locomotion following SCI in an animal model.

Keywords: spasticity, ankle torque, EMG, locomotor training, spinal cord injury, rat

INTRODUCTION

Spinal cord injury (SCI) produces a number of complicated challenges to the recovery of locomotor function, particularly, retraining the residual nervous system to overcome obstacles posed by the loss of connectivity diminished by injury or enhanced by non-adaptive plasticity. The fundamental locomotor disabilities span a wide range and include spasticity and balance instability. Spasticity is a disability that occurs in a high proportion of SCI patients. While often complicated in form and content, the clinical hallmark of spasticity is a significant exaggeration of the velocity-dependent lengthening resistance of the affected muscles (Lance, 1980; Young, 1989a,b). Inappropriate resistance to movement, painful spasms, and movement interference associated with spasticity complicate the quality of life and contribute

barriers to locomotor recovery (Katz and Rymer, 1989). In addition to spasticity, there are additional factors in the post-SCI setting that may influence limb use; postural instability associated with changes in descending modulation of balance may produce compensatory changes in base of support and limb axis. Profound atrophy of locomotor skeletal muscle is a potentially serious complication that may further challenge locomotor recovery as well as contribute to metabolic changes following SCI (Houle, 1999; Hutchinson et al., 2001; Gregory et al., 2003; Haddad et al., 2003; Stevens et al., 2006; Liu et al., 2008, 2010; Shah et al., 2008).

Rehabilitation strategies utilizing locomotor activity to direct constructive plasticity of spinal cord locomotor circuits have revealed encouraging breakthroughs in the potential for locomotor recovery using treadmill and, less frequently, stationary bicycle, training programs. Recent evidence indicates that individuals with complete and incomplete SCIs improve their ability to step on a treadmill, to cycle or walk overground following specific locomotor training (Visintin and Barbeau, 1989; Wernig and Muller, 1992; Dietz et al., 1994; Wernig et al., 1995; Harkema et al., 1997; Behrman and Harkema, 2000; and also see reviews, Barbeau et al., 1999; Basso, 2000; Wolpaw and Tennissen, 2001; Dietz and Harkema, 2004). Such locomotor training uses principles derived from animal and human studies showing that stepping can be generated by virtue of the neuromuscular system's responsiveness to phasic, peripheral sensory information associated with locomotion (Lovely et al., 1986, 1990; Barbeau and Rossignol, 1987; Edgerton et al., 1997, 2004; Harkema et al., 1997; de Leon et al., 1998; Behrman and Harkema, 2000). This experimental regimen may promote the recovery of walking by optimizing the activity-dependent neuroplasticity of the nervous system produced by task-appropriate locomotor training (Edgerton et al., 1992; Muir and Steeves, 1997; Gomez-Pinilla et al., 2002). Neuronal circuits, stimulated by the proper activation of peripheral afferents via training, may reorganize by strengthening existing and previously inactive descending connections and local neural circuits (Edgerton et al., 1992; Dietz et al., 1997; Muir and Steeves, 1997; Barbeau et al., 1998; Basso et al., 2002). However, it is not known to what extent locomotor training influences lower limb spasticity and limb use parameters, or if there are significant differences in outcomes relative to the type of locomotor training used. Most of the rehabilitation studies (both clinical and laboratory) are largely accommodated by treadmill training programs. While treadmill training has been demonstrated to be effective, there are personnel, equipment, and space considerations associated with its use. By comparison, the stationary bicycle is spatially compact, economical to acquire, and can be safely accessed with minimal assistance. Therefore, it would be of importance to understand how bicycle locomotor training compares with treadmill training since there are several practical factors that make use of the bicycle training more accessible to SCI-individuals, particularly even in the home setting.

Studies in our laboratory have demonstrated the appearance of significant velocity-dependent lower limb spasticity, changes in limb placement during gait, and significant changes in excitability of stretch reflex pathways of lower limb muscles (Thompson et al., 1992, 1998; Bose et al., 2002). Collectively these changes represent robust and comprehensive changes consistent with a clinical definition of spasticity. The purpose of the present studies was to utilize these biomechanical, behavioral, and neurophysiological measures in this model of SCI to (1) provide preclinical data on quantitative assessment of the influence of locomotor training on lower limb spasticity, (2) to correlate these changes with neurophysiological processes that regulate reflex excitability, and (3) to compare potential benefits of treadmill vs. bicycle locomotor training.

MATERIALS AND METHODS

ANIMAL SUBJECTS

Thirty Sprague Dawley specific pathogen free (SPF) rats (12 weeks old, weighing 220–260 g at the start of this study; Charles Rivers Laboratory, USA) were used in this project. All procedures were performed in accordance with the U.S. Government Principle for the Utilization and Care of Vertebrate Animals and were approved by the Institutional Animal Care & Use Committee at the North Florida/South Georgia Veterans Health System and the University of Florida.

SURGICAL PROCEDURE – CONTUSION INJURIES

The contusion injuries were produced using a Multicenter Animal Spinal cord Injury Studies (MASCIS) impactor and protocol. Briefly, the MASCIS impactor, 10 g weight, was dropped from a 2.5 cm height onto the T_8 segment of the spinal cord exposed by laminectomy under sterile conditions. Each animal received Ampicillin (s.q.) twice each day starting at the day of surgery for a total of 5 days. The procedure was performed under ketamine (100 mg/kg)-xylazine (6.7 mg/kg) anesthesia (details in Reier et al., 1992; Thompson et al., 1992, 1993; Bose et al., 2002). The animals were kept under vigilant post-operative (po) care which included daily examination for signs of distress, weight loss, dehydration, and bowel and bladder dysfunction. Manual expression of bladders was performed 2–3 times daily as required, and the animals were monitored for the possibility of urinary tract infection. Animals were housed in pairs (except for a brief po recovery period). At po day 7, velocity-dependent ankle torque (Bose et al., 2002) and open field locomotion were assessed by the Basso, Beattie, Bresnahan (BBB) scoring scale (Basso et al., 1995) to obtain measures of spasticity and injury severity, respectively. If any animal does not fall within certain preset scores (ankle torque at 612°/s angular rotation, 160–220 kdyne, and BBB scores >5 at po day 7), it was considered too mildly injured and eliminated from the study to reduce the variability. Out of a total of 42 animals, 30 animals were qualified for the preset criteria. The animals ($n = 30$) were then randomly divided into three equal groups ($n = 10$ each). Two groups were assigned for treadmill and cycling locomotor trainings, and, the third group did not receive training (contused control for both groups), however, was checked routinely.

LOCOMOTOR TRAINING

A three-runway treadmill (Columbus Instrument, OH, USA) and two custom made bicycles were used in this study for locomotor training.

RAT BICYCLE

A rodent motorized rat bicycle was designed and custom built to promote locomotion following SCI (University of Florida patent

pending; Application number: 61698752). The bicycle is composed of a direct drive gear box, adjustable foot pedals, and a support harness. Two pedal guide-wires are placed to maintain proper pedal orientation. The drive shaft ends are keyed to allow multiple bikes to operate in series on a single drive motor. The assembly is mounted on a thick aluminum base plate for added stability and strength (see **Figure 1**).

EXERCISE PROTOCOL

The animals were trained over the course of 3 months. The training schedule was performed 5 days a week using two 20 min trials/day, starting from po day 8 in both training paradigms. On the first day of training, the rats were given 5 min to explore the treadmill and then encouraged to walk on the moving treadmill (11 meter per minute, mpm, Kunkel-Bagden et al., 1993) for a series of 4, 5-min bouts of walking. The rats were given a minimum of 5 min rest between bouts. On the second day of training the rats walked for two bouts of 10 min each, twice a day, and then day 3–90, the rats were trained to walk for 20 min without a rest with a at least 2 h interval between trails. First 7 days bodyweight was supported as needed using support pole and harness attached with the support pole as seen in **Figure 1**. This allowed us to suspend the rat over the treadmill and provided similar bodyweight supported training like bicycle training. Moreover, this design of bodyweight support allowed us to assist limb locomotion with hands to promote normal walking during treadmill locomotion. The body support sling was positioned at a height such a way so that this could provide the desire body weight support/load during both types of exercise. After 7 days, the bodyweight was supported as needed. The bicycle exercise regimen involves suspending the rats on the rat harness (**Figure 1**) with the hindlimbs hanging down and hind feet strap onto the pedals with cotton tapes. The exercise consists of a pedaling motion, which fixed one limb while extending the other without overstretching the limbs. The cycling speed was 31 rotations/minutes (around 11 mpm distance wise). The first 2 days, the bicycle training period and protocol were same those of treadmill training. During the first week of training, the rat tail was attached with the aluminum support boom by surgical tape to maintain the trunk stability during exercise. However, following second week of training, gradually the load was increased by positioning the body harness toward the chest, so that the hind portion of the body falls over the pedal.

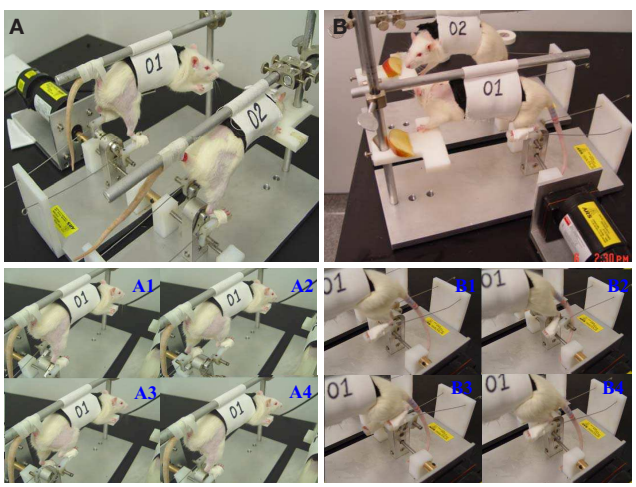

FIGURE 1 | A custom made motorized rat bicycle (A,B), and a series of sequential video images of spinal cord-contused rats cycling on this apparatus. Note, right hindlimb was shaved for EMG recording. During the first week of training, the tail was taped **(A, A1–A4)** to provide maximum body support. However, the body support was gradually reduced to maximize the loading during training after first week **(B, B1–B4)**.

ANKLE TORQUE AND EMGS MEASUREMENT

The lengthening resistance of the triceps surae muscles was measured indirectly by quantifying ankle torque and EMGs during 12° dorsiflexion ankle rotation (see detail in Thompson et al., 1996; Bose et al., 2002; Wang et al., 2002). This measurement is a standard procedure in our laboratory and detail is published, Bose et al. (2002) and Thompson et al. (1996). Prior to data acquisition, the animals was given a brief pre-recording period to adjust to the recording procedure by providing them with 12° ankle rotations produced at 3 s intervals at eight different velocities (49, 136, 204, 272, 350, 408, 490, and 612°/s). Rats were immobilized in a custom designed trunk restraint, without trauma or apparent agitation. All recordings were performed in awake animals. The proximal portion of the hind limbs to the midshank, were secured in a form-fitted cast that immobilized the limb while permitting normal range of ankle rotation (60–160°). The animals typically adjusted to the restraint device without detectable discomfort and were provided fruit to sniff or chew as a distraction. The neural activity of the triceps surae muscle was measured using transcutaneous EMG electrodes. The electrode was inserted in a skin fold over the distal soleus muscle just proximal to the aponeurotic convergence of the medial and lateral gastrocnemii into the tendonocalcaneousus. The reference electrode was placed in a skin fold over the greater trochanter. A xylocaine 2% jelly (Lidocaine HCl, Astra USA Inc.) was applied over the electrode insertion points to minimize pain during recording. The data recording session begins when the animal is relaxed and the protocol requires approximately 45 min. At each test velocity, five consecutive sets of waveforms, 10 waveforms per set (a total 50 waveforms/velocity), was recorded, signal averaged, and saved for subsequent analysis. A complete protocol for each animal was recorded during each of two separate recording sessions performed on separate days. Therefore, the data set for each animal for each test velocity was the signal average of 100 trials (50 per session × 2 sessions). The data were signal averaged upon acquired using a digital signal acquisition system and LabView graphic programming (Version 5.0, National Instrument).

RATE-DEPRESSION

Measurement of rate-depression is a well-established model in our laboratory (Thompson et al., 1992, 1993, 1998, 2001a). Rate-depression was assessed using a non-invasive procedure. The animals were anesthetized by i.p. injection of ketamine 100 mg/kg and immobilized in a prone position on the recording table using surgical tape. Ketamine was selected due to its minimal depression of monosynaptic reflex and because it does not alter the time course of presynaptic inhibition (Lodge and Anis, 1984). Core body heat

was maintained via heat lamp. The hair overlying the distal tibial nerve at the ankle was removed using a cosmetic hair removing gel. A bipolar stimulating electrode with 1 mm silver ball was applied to the ankle surface and just enough electrode gel to coat the tip of the electrode applied to the skin. A monopolar surface EMG recording electrode was applied to the plantar skin overlying the lateral plantar (digital interosseus) muscles. The reference was applied to the skin surface of the fifth digit. A ground electrode was applied to the skin surface intermediate between the stimulating and the recording electrode to minimize shock artifact. The distal tibial nerve was stimulated using 200μsec current pulses, according to a preset protocol to determine H-reflex threshold and H-max, M-wave threshold and M-max. An H-recruitment curve was then made to locate the minimum intensity for the maximal reflex amplitude. The frequency protocol was performed at this intensity and was adjusted slightly during the frequency series to maintain a constant M-wave amplitude (an assurance of a constant effective stimulus delivery to the distal tibial nerve). The frequency series was included 0.3 Hz as control with 7 test frequencies: 0.5, 1, 2, 3, 4, 5, and 10 Hz. The data set for each frequency was 32 consecutive waveforms that were signal averaged upon acquired using a digital signal acquisition system and LabView graphic programming (Version 5.0, National Instrument). Rate-depression at each test frequency was quantified by comparison of reflex amplitude and area to the 0.3 Hz control.

FOOTPRINTS

Graph paper was placed on the treadmill and the rats' hind limbs were inked. The rats were then placed on the treadmill (20×40 cm) surface at the practiced speed, 11 mpm. Axial angle of rotation, and base of support were analyzed from these footprints. Angle of rotation is the angle measurement found by drawing a line through the center of the third toe and the center of the heel of two consecutive paw prints. Base of support is the distance between two consecutive prints. Thus, hind limb gait abnormalities were measured from footprints obtained at preinjury, and 1–3 months flowing training using both trained and untrained contused animals.

OPEN FIELD LOCOMOTOR RECOVERY

Basso, Beattie, and Bresnahan open field locomotor scale was applied to score the early, intermediate, and late phases of recovery following locomotor training. The 21-point scale is based on the observation that after spinal cord contusion rats progressed through three general phases of recovery. The early phase is characterized by little or no hindlimb joint movement (scores 0–7). The intermediate phase includes bouts of uncoordinated stepping (scores 8–13), whereas the late phase involves fine details of locomotion such as dragging of the toes and tail, trunk instability, and rotation of the paws (scores 14–21). The animal was placed in a test apparatus, observed for 4-min, and scored in real time by two-blinded observers (Basso et al., 1995). All open field locomotor testing was video-taped for further analysis and review.

HISTOLOGICAL EVALUATION OF LESION

The contusion lesions were studied histologically to assess the severity and nature of the injury using 4% buffered paraformaldehyde fixed tissues. The portions of the SC that included the contusion epicenter, and segments extending 4 mm rostral and caudal to the injuries were dissected and saved, embedded in paraffin, and sectioned on a Microtome (10 μm thickness; $n = 6$ in each group; control, treadmill, and cycle trained). The sections were stained using conventional Luxol fast blue and cresyl violet staining techniques. To quantify injury lesion length, the number of sections containing the lesions was counted from rostral to caudal on serial sections. The total number of injured sections was multiplied by the individual section thickness of 10 μm, in order to obtain total injury lesion length. Further quantification involved volumetric measurement of the injury lesions. In order to obtain volume measurements, the lesion area was measured in every tenth SC section (Noble and Wrathall, 1985). After obtaining lesion area, a previously published mathematical formula (Rosen and Harry, 1990) was applied to calculate the volume. Lesion areas equally placed at 100 μm was used in the Cavalier's estimator of morphometric volume (Rosen and Harry, 1990): $V_C = d[\Sigma(y_i)] - (t)y_{max}$ where, "V_C" is the Cavalieri's estimator of volume; "d" is the distance between the sections being measured (100 μm); y_i is the cross-sectional area of the i-th section through the morphometric region; "y_{max}" is the maximum area in the series, and "t" is section thickness (10 μm). The amount of spared white matter, both dorsal and ventral quadrants of the cavity, was also measured on the serial sections. The proportion of the residual ventral white matter was expressed in relation to that observed in intact normal animal's tissue (100% spared ventral white matter). Light microscopic qualitative studies were also conducted to assess the morphology of the spared tissue. This goal was achieved by examining the extent of myelination, characteristics of remaining axons, and the degree of collagen infiltration. Normal-appearing gray matter was distinguished from damaged tissue by the presence of healthy neurons and normal cellular density (without the presence of numerous nuclei which is indicative of immune cell infiltration). Normal-appearing white matter was defined as being non-fragmented, darkly bluestained (not pale blue), and without immune cell infiltration (Pearse et al., 2004).

IMMUNOHISTOCHEMISTRY

Spinal cord segments caudal to the injuries, and lumbar spinal cord, L_3-L_6) were dissected and removed after perfusion and kept in the same fresh fixative mixture for 1 h and were cryoprotected for at least 2 days in 30% sucrose in phosphate buffer (PB). The specimens were cut serially (cross section) by cryostat (40 μm thickness) and processed by Avidine-Biotine Complex (ABC) and fluorescent immunohistochemistry (IHC). The immunoreactivity of GAD_{67}, $GABA_b$, and dopamine-beta-hydroxylase (DBH, for NE descending projections) and BDNF were identified in SC. The cryostat cut sections were incubated with primary antibodies generated against GAD_{67} (mouse mAb; 1:1,000, National Hybridoma Laboratory, St Louis, USA), $GABA_b$ (guinea pig mAb, 1:4,000; Chemicon International), DBH (mouse mAb, 1:7000; Chemicon International), a synthetic enzyme found in the neurotransmitter vesicles of noradrenergic fibers, and BDNF (rabbit Ab, Chemicon International) for 24–48 h at 4°C. The sections were then washed in PBS and incubated for 1.5 h in alexa fluor-conjugated appropriate anti-mouse, anti-guinea pig, or anti-rabbit IgG (1:1,000,

Molecular Probes). For ABC technique, anti-guinea pig (1:200; Chemicon), anti-mouse, and anti-rabbit (1:200; mouse and rabbit Elite kits, Vector Lab) secondary antibodies were used to bind with appropriate primary antibodies. Co-localization of GAD_{67}, BDNF, and DBH labeled cells were also performed by double labeling with the appropriate antibodies using the same procedure. Sections were washed again and mounted for microscopic analyses.

STATISTICAL ANALYSES

Analysis of variance (ANOVA) was used to detect differences in ankle torques and EMGs values obtained at each velocity from pre-contused, contused, treadmill, and cycle exercised animals. Ankle torques and corresponding EMG values were obtained at pre-injury time point from each group were also tested by ANOVA. In addition, a repeated measures ANOVA (RM ANOVA) was used to test the within group differences in ankle torque or EMGs across po weeks. Data from H-reflex, footprints, BBB, and histological experiments were analyzed by using ANOVA to assess treatment effects from contused and time-matched normal control groups. The level of significant difference was set for all analysis was $p \leq 0.05$. Significant differences are marked with asterisks (*) or ^ according to their respective *p*-values: *,compared with pre-injury values; ^,compared with contused controls. Values are expressed as the mean value ± standard error of the mean (SEM) in all graphs.

RESULTS

VELOCITY-DEPENDENT ANKLE TORQUE AND ASSOCIATED EMGS

Baseline measures of velocity-dependent ankle torques and extensor EMGs were obtained from all animals before injury (**Figures 2A,B**), at post-injury weeks 1 and 2, and then at alternate weeks up to po week 12. When tested at 1 week following injury, all three groups revealed significantly increased magnitudes of ankle torque during rotation at each of the eight ankle rotation velocities compared to the control values recorded before injury. There was no difference between these three groups, **Figure 2C** (ANOVA). The EMG-RMS magnitudes time-locked with increased ankle torques that were recorded at each of the test velocities were also significantly greater compared with those recorded before injury (**Figure 2D**).

At post-injury week 2, a pattern of significant hyporeflexia was observed in the untrained contused group, **Figures 2E,F**. However, ankle torque and triceps surae EMG magnitudes recorded from the two trained groups at the end of postcontusion week 2 did not demonstrate this pattern of hyporeflexia, but decreased from the week 1 values and were similar to data observed in precontusion animals (**Figures 2E,F**). Moreover, there was no difference between the data recorded from these two training groups at this post-injury time point.

By week 4 post-injury, a significant velocity-dependent ankle extensor spasticity re-appeared in the untrained contused group **Figures 3A,B**. However, this second appearance of spasticity occurred only during the faster ankle (dynamic) rotations and was no longer observed during the low velocity (tonic) rotations. Tests at all later time points revealed that this significant dynamic velocity-dependent increase in ankle torque was enduring. Surprisingly, at this post-injury time point, this re-emergent spasticity was not observed in either of the two training groups (**Figures 3A,B**). At this point, ankle torque and EMG magnitudes did not increase significantly at the four fastest ankle rotation velocities (350–612°/s) compared with the untrained contused animals (**Figures 3A,B**). Moreover, at the end of po week 6 (following 5 weeks of training), we did not observe increased ankle torque and EMG magnitudes at the four fastest ankle rotation velocities, as were clearly evident in the untrained contused animals (**Figures 3C,D**).

At postcontusion weeks 8–12, an increase in the velocity-dependent ankle torque was observed in both treadmill and bicycle training groups. This appeared at the four fastest ankle rotation velocities (350–612°/s), and was of lower magnitude compared with values recorded in the untrained contused control group (**Figures 3E,F** and **4A–D**). The mean torque and EMG values recorded at these rotation velocities from these trained groups were also increased and were intermediate in magnitude compared with corresponding values recorded from pre-injured normal and untrained contused groups. No significant increases in ankle torque or EMG magnitude was observed during ankle rotations at the slowest four velocities at postcontusion week 4–12 (**Figures 3** and **4**). It is important to note here also that there was no significant difference between the treadmill and bicycle group in ankle torques or EMGs recorded during post-injury week 4–12.

RATE-DEPRESSION OF TIBIAL/PLANTER H-REFLEXES

Rate-depression of the tibial/plantar H-reflexes was tested before injury and at 3 months post-injury in the trained and untrained injury groups. Compared with pre-injury controls, rate-depression was significantly reduced in the untrained group at each of the test frequencies from 1 to 10 Hz. By contrast, the rate-depression observed in the trained animals was intermediate in amplitude between the pre-injury controls and the untrained animals at 1–10 Hz, see **Figure 4**. Further, note that the rate-depression produced in the trained animals in response to test frequencies of 1–2 Hz, was similar to that recorded in the normal controls (**Figure 5**). Similar magnitudes of rate-depression were observed in bicycle and treadmill-trained animals.

GAIT AND OPEN FIELD LOCOMOTION

Footprint analyses

Footprint analyses were performed before injury, at 2 and 3 months post-injury. Before injury, limb axis and base of support were measured to be 30.25 ± 5.9 and 3.29 ± 0.51, respectively. By comparison, these measures were 55.78 ± 6.58 and 6.03 ± 0.42 in the untrained animals at 2 months post-injury. Similar values, 57.62 ± 6.84 and 6.52 ± 0.52, respectively, were measured at 3 months. Compared with the pre-injury control values, these measures revealed that limb axis and base of support were significantly increased in the untrained contusion-injured animals. In the 2–3 months locomotor training groups, limb axis and base of support were observed to be 41.37 ± 4.85 (at 2 months), 40.35 ± 5.87 (at 3 months) and 5.04 ± 0.32 (at 2 months), 4.52 ± 0.48 (at 3 months), respectively. These measures were significantly less altered than those observed in the untrained animals (**Figures 6A,B**). However, no significant difference in hind limb rotation or base of support was observed between the two

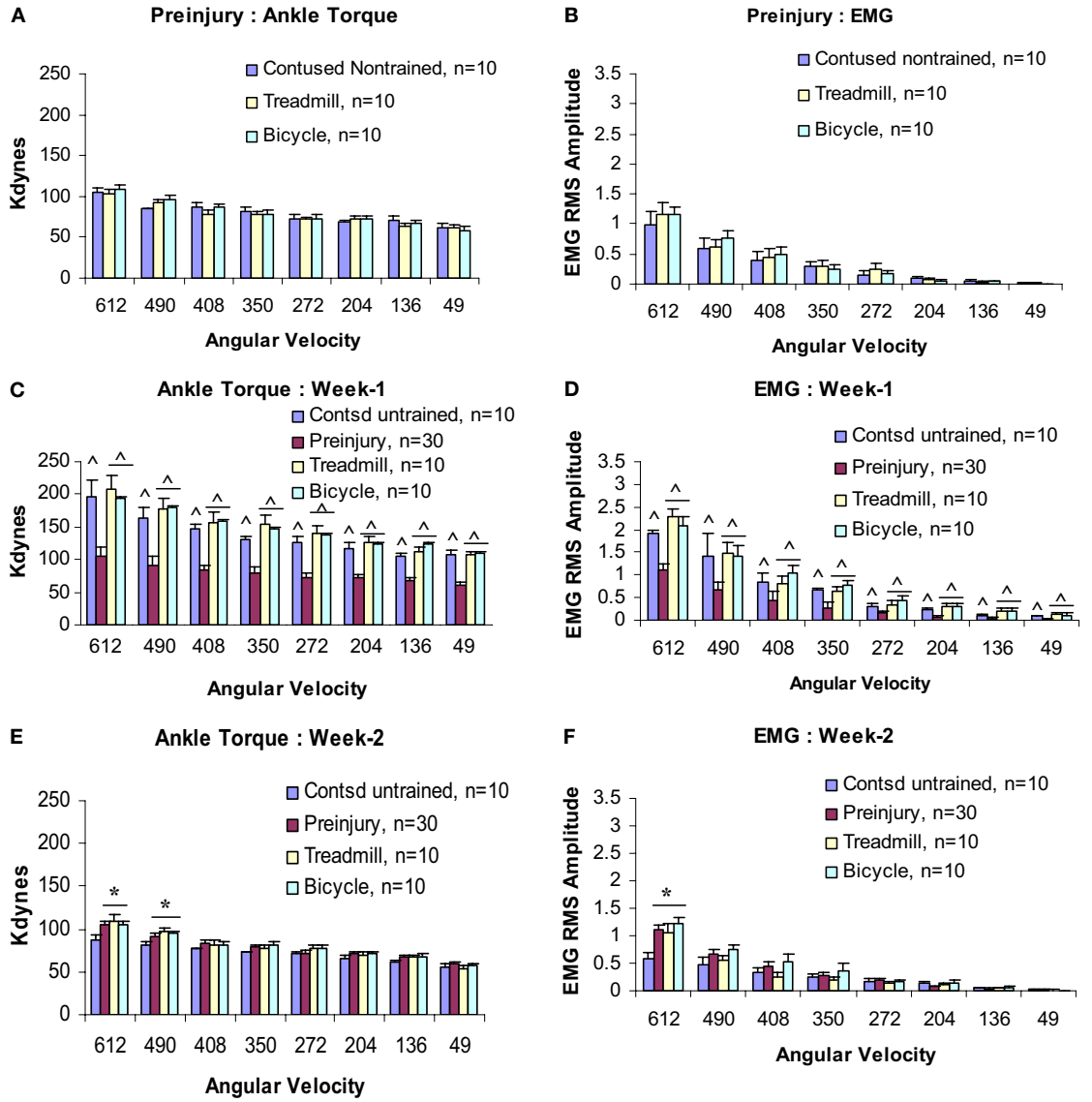

FIGURE 2 | Velocity-dependent ankle torque (A,C,E) and time-locked EMG-RMS magnitude (B,D,F) of precontusion and postcontusion weeks 1 and 2. Note, both ankle torque and EMG-RMS values of postcontusion week 1 in all eight velocities (612–49°/s) were significantly greater compared with those of pre-contused values **(C,D)**. Interestingly, only 1 week of exercise (training started at p.o. day 8) prevents hypotonia **(E,F**; see text for detail). $p < 0.05$; *compared with controls; ^compared with preinjury.

training groups (ANOVA) at 2 or 3 months following training (**Figures 6A,B**).

OPEN FIELD LOCOMOTOR RECOVERY

Open field locomotor behavior was scaled (BBB) in both trained and untrained animals before injury and at post-injury 4, 8, and 12 weeks to evaluate recovery during the early, intermediate, and late phases of recovery.

At week 4, untrained contused animals displayed extensive movement of all three joints of the hindlimb, however, these animals could not support their body weight (mean score, 7.8 ± 1.2). In contrast, treadmill-trained animals showed occasional to frequent weight supported plantar steps, however, no forelimb-hindlimb (FL-HL) coordination (mean score, 10.2 ± 1.07) was observed. Interestingly, bicycle-trained animals displayed frequent to consistent weight supported plantar steps and occasional to frequent FL-HL coordination (mean score, 12.2 ± 1.4). This BBB score in the bicycle trained group was significantly greater ($p < 0.05$, ANOVA) than observed in either the untrained and the treadmill-trained groups (**Figure 6C**).

At postcontusion week 8, untrained control animals showed occasional to frequent weight supported plantar steps without FL-HL coordination (mean score 10.0 ± 1.24), whereas, both treadmill and bicycle-trained animals showed consistent weight supported plantar steps and frequent to consistent

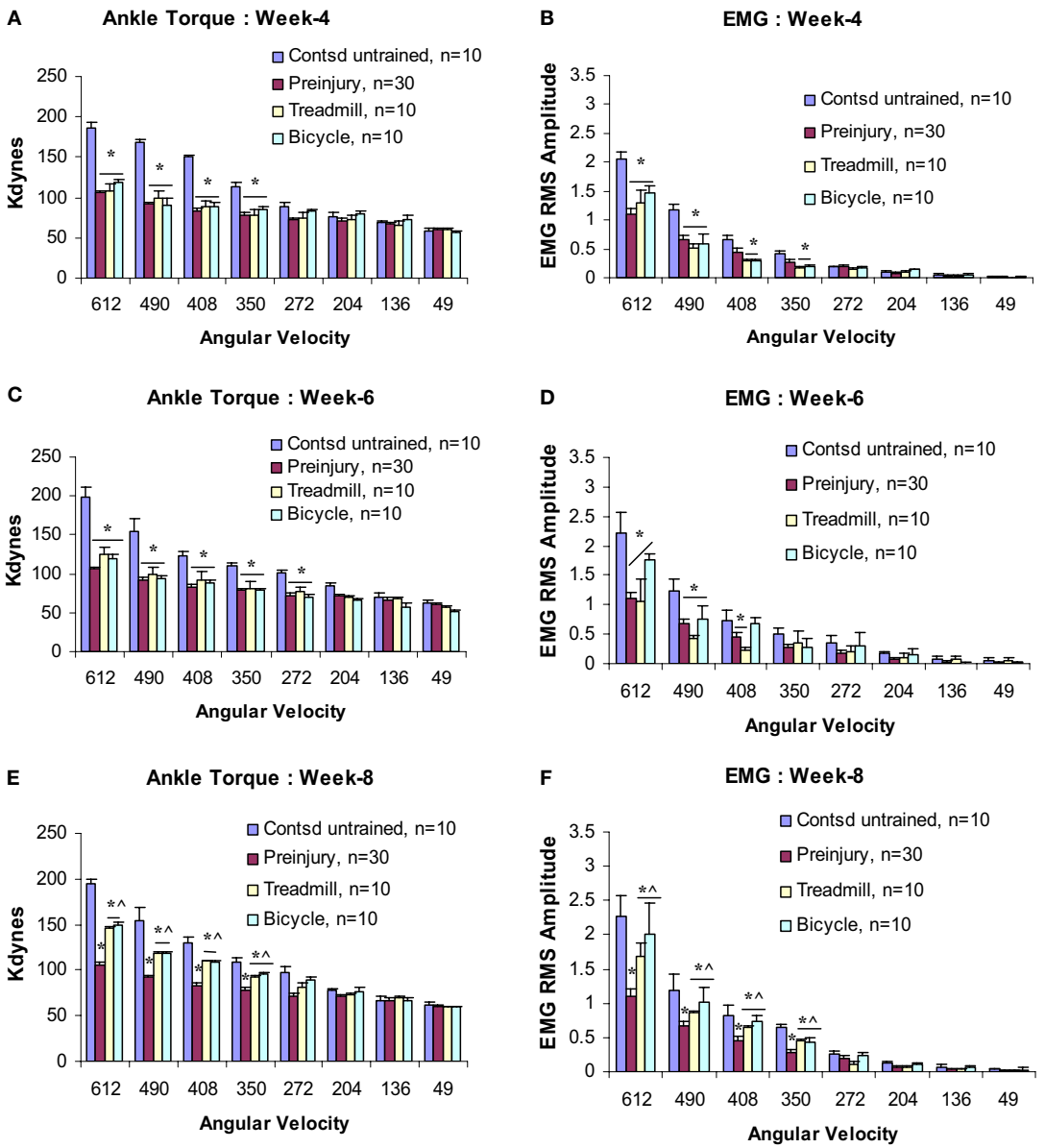

FIGURE 3 | Velocity-dependent ankle torque (A,C,E) and time-locked EMG-RMS magnitude (B,D,F) of postcontusion weeks 4–8. Note, both type of trainings prevent development of spasticity up to postcontusion week 6 **(A–D)**, however, a milder form of spasticity (42% less than control) has been detected at postcontusion week 8 compared with untrained contused controls **(E,F)**. $p < 0.05$; *compared with controls; ^compared with preinjury.

FL-HL coordination (mean score, bicycle, 13.8 ± 1.3, treadmill, 14.0 ± 1.4). Both of the BBB scores in the locomotor trained groups were significantly greater ($p < 0.05$, ANOVA) than the scores determined for the untrained group.

At the final stage of training (week 12), the contused untrained control animals showed frequent to consistent weight supported plantar steps, and no to occasional FL-HL coordination (mean score, 11.25 ± 1.25; **Figure 6C**). However, in this stage, animals of both trained groups showed consistent FL-HL coordinated and consistent weight supported steps (mean scores, bicycle, 14.25 ± 1.4, treadmill, 15.25 ± 1.7). Moreover, these trained animals showed occasional dorsal stepping and rotated paw positioning during their locomotion. Occasional toe clearance was also observed in some trained animals (in both groups). Please note, the terminologies, never (0%), occasional (less than or equal to half, ≤50%), frequent (more than half but not always, 51–94%), and consistent (nearly always or always, 95–100%) used above, are described in Basso et al. (1995).

In summary, open field locomotor recovery scores scaled at postcontusion weeks 4, 8, and 12 were significantly greater in both of the training groups compared with untrained controls (**Figure 6C**). The bicycle training group demonstrated the highest recovery score at post-injury 1 month, which was also significantly greater than the treadmill group (**Figure 6C**). However,

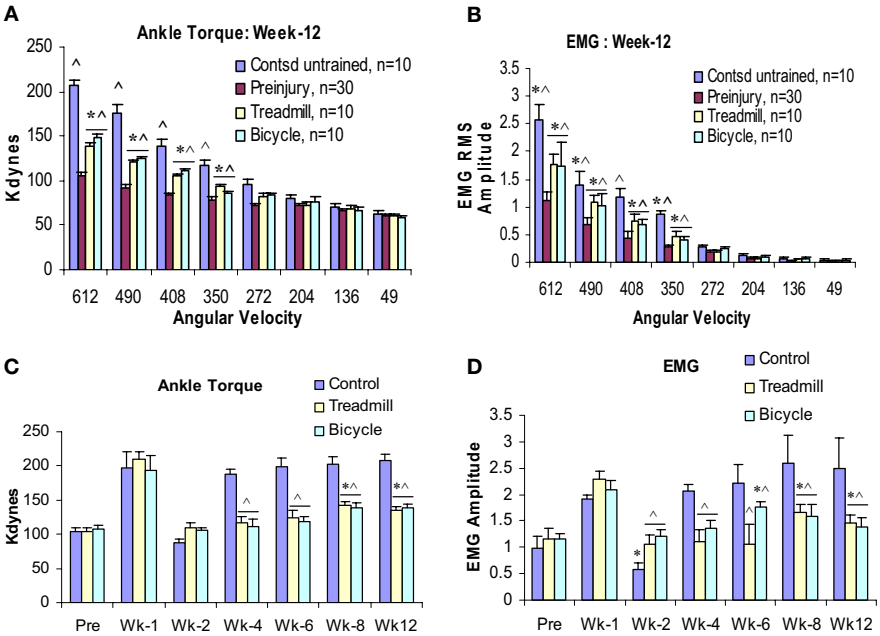

FIGURE 4 | Effects of 3 months locomotor training (treadmill and bicycle) on ankle torque (A), extensor muscle EMGs (B) in animals with midthoracic contusion injury. Three-month locomotor training showed significant reduction of spasticity (ankle torque and EMGs) in both training groups (49% reduction). The time course of velocity-dependent ankle torque **(C)** and time-locked EMG-RMS magnitude **(D)** over 12 weeks. Both treadmill and bicycle training prevented the initial hyporeflexic state (at week 2), prolonged the transition to develop a permanent hyperreflexic state (weeks 4–6) and attenuated the level of spasticity (weeks 8–12).

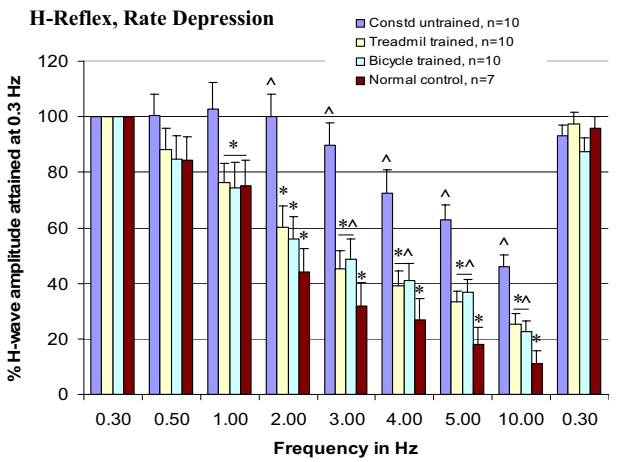

FIGURE 5 | Both types of locomotor training enhance rate-depression of H-reflex at 1–10 Hz test frequencies, ANOVA, *$p < 0.05$, compared with contused untrained controls, and ^$p < 0.05$, compared with normal intact animals.

at postcontusion 8 and 12 weeks, both training groups showed similar recovery scores (ANOVA).

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Both locomotor trained groups revealed decreased lesion volumes (rostro-caudal extension) and more spared tissue at the lesion site. Our histological studies indicated that both the injured-bicycle-trained group and the injured-treadmill trained group had shorter lesion lengths, and significant smaller lesion volume than the injured-untrained controls (**Figures 7D,E**). The measured lengths of lesion for the three different treatment groups showed bicycle-trained rats to have the shortest mean length ($5178.3 \pm 559.5\,\mu m$), followed by treadmill-trained rats ($5441.4 \pm 549.9\,\mu m$), and finally by the control rats ($6438.0 \pm 1019.1\,\mu m$). The difference in lesion lengths among the three treatment groups was not significant, but there was a noticeable trend. The lesion volumes for the three different treatment groups showed bicycle-trained rats to have significantly the shortest mean volume (mean ± SEM; $3.03 \pm 0.98\,mm^3$; $p < 0.001$ compared to control), followed by treadmill-trained rats ($3.33 \pm 0.55\,mm^3$; $p < 0.001$ compared to control), and finally by the control rats ($5.31 \pm 0.67\,mm^3$). Light microscopic qualitative studies of spared tissue revealed better preservation of myelin, axons, and collagen morphology in locomotor trained animals (**Figures 7A–C**). Importantly, these data indicate that the therapeutic efficacy of ergonomically practical cycle training was more effective in preserving spared tissue than more labor-intensive treadmill training.

We observed a robust increase in the immuno-expression of $GABA_b$ receptors and NE fiber sprouting throughout the lumbar spinal gray of both trained animals compared with tissues from untrained animals (**Figure 8**). Moreover IHC studies indicated upregulation of GAD_{67}, and BDNF immunoreactivity at T_{10}-T_{11} (immediately below the injury epicenter at T_8) especially areas

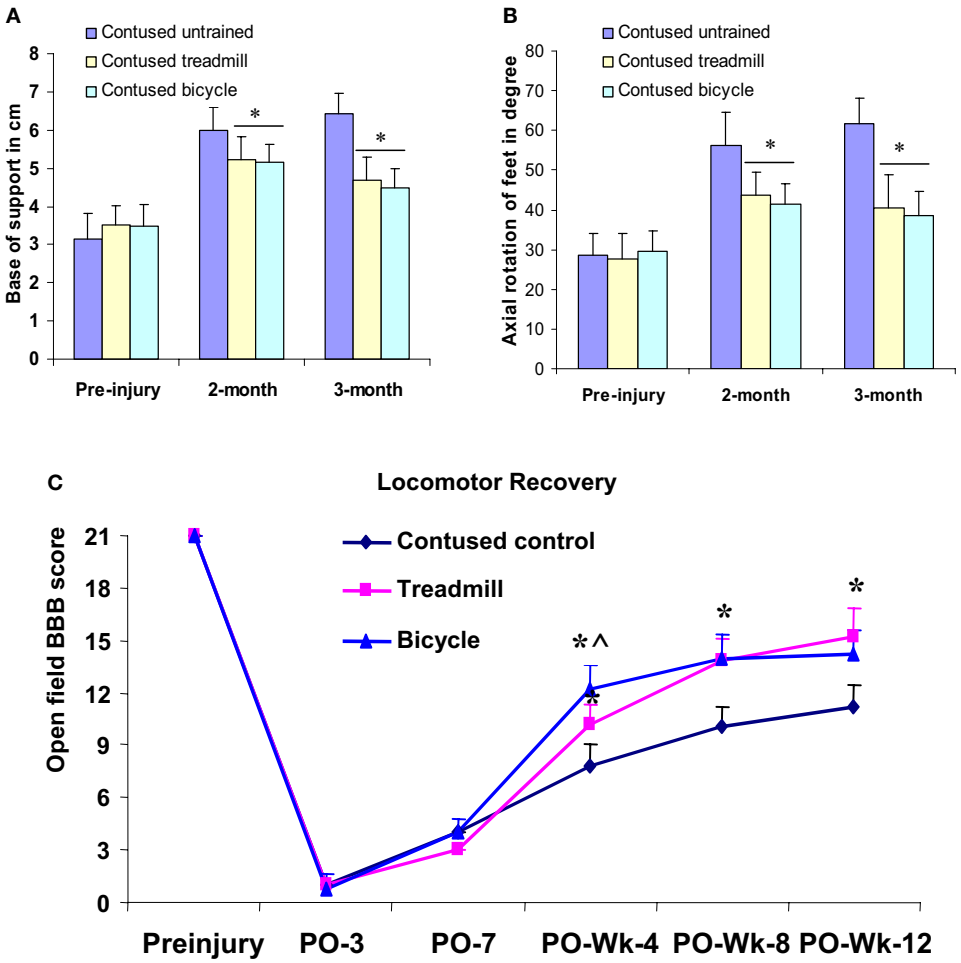

FIGURE 6 | Hind limb gait abnormalities and locomotor recovery measured from footprints and open field locomotor scores (BBB) and plotted as groups. Locomotor training significantly reduced the deviation of axial hind limb rotation **(B)** and base of support **(A)** in both treadmill and bicycle training groups compared with untrained contusion group at second and third month (*$p < 0.05$ compared with contused untrained group). Both types of training significantly improved locomotor recovery compared with untrained controls **(C)**. The bicycle training group demonstrated the greatest recovery at postop 4 weeks, which is significantly different even compared with treadmill group. However, at postop 8 weeks, both training groups showed similar recovery. *$p < 0.05$ compared with untrained contused group; ^compared with treadmill group (ANOVA).

adjacent to dorsal median septum and ventral horn (VH) following 1 week of bicycle locomotor training (PO 2 weeks; **Figure 9**). Interestingly, double IHC showed expression and co-localization of GAD_{67} and BDNF in the VH motoneurons (**Figure 10**). GAD_{67} showed a diffuse staining in cell bodies and fibers as well as punctate staining in the VH (**Figure 10**), and those GAD_{67} immunostained motoneuron cell bodies also stained with BDNF (**Figure 10C** and merged panels of **Figures 10A,B**).

DISCUSSION

The present study evaluated the influence of (two types of) locomotor training (treadmill and cycling) on measures of spasticity, reflex excitability, and limb use following injury in a laboratory model of traumatic SCI. These measures included assessment of ankle extensor spasticity; neurophysiological rate-depression of ankle extensor muscle monosynaptic reflexes, lower limb axis, base of support, and open field walking (BBB). Untrained animals with SCI revealed significant locomotor disabilities that were quantitated using these reflex measures. Compared with the untrained injured controls, each of these functional measures was significantly improved in the animals undergoing either of the two types of locomotor training. The three significant findings of these studies: (1) confirmed previous findings regarding significant changes in limb use, spasticity, and reflex excitability of lower limbs following experimental contusion injury of the midthoracic spinal cord, (2) demonstrated significant positive improvement in measures of limb use, spasticity, and reflex excitability by locomotor training, and (3) that the effectiveness of the ergonomically practical cycle training was comparable with treadmill locomotor training in influencing these specific measures.

SPASTICITY

In the present study, untrained contused animals revealed a pattern and time course for the development of spasticity that was

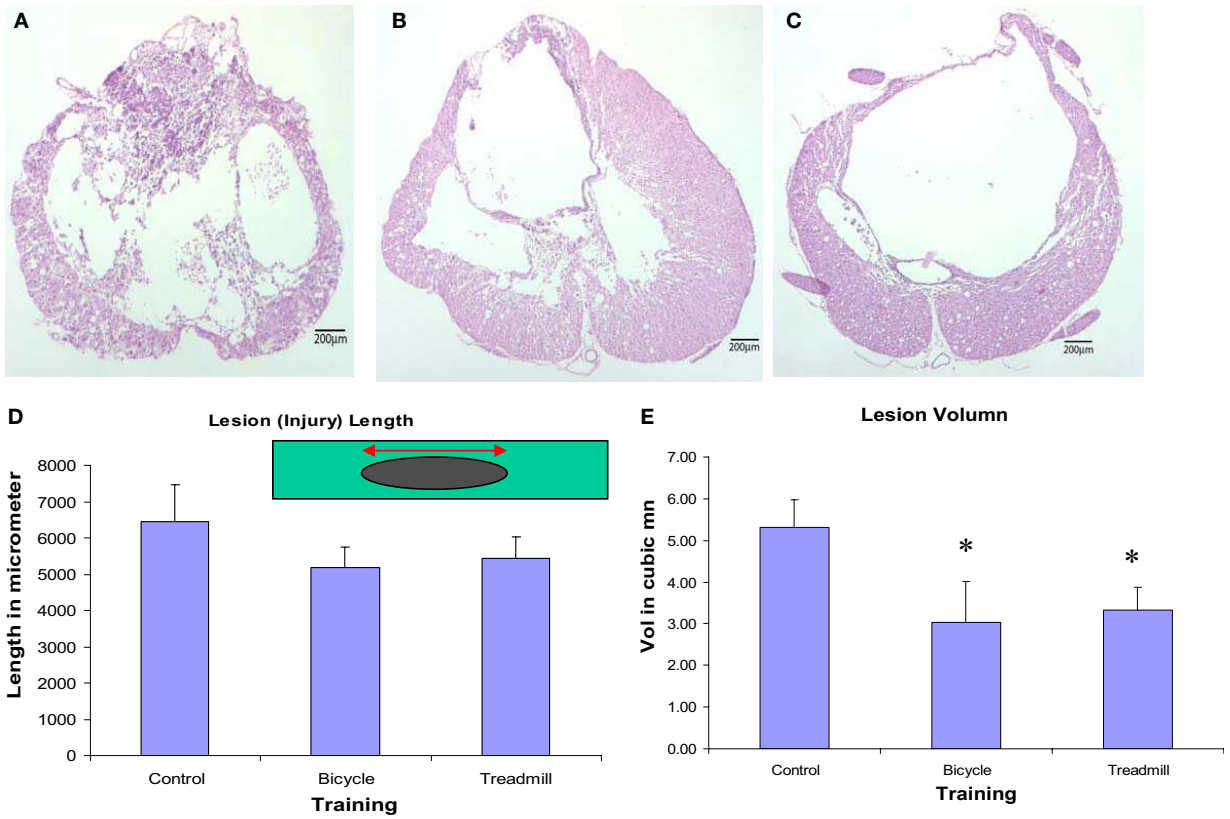

FIGURE 7 | Light microscopic qualitative studies of spared tissue revealed better preservation of myelin, axons, and collagen morphology in locomotor trained animals (B,C) compared to contused untrained control (A). Animals treated with either type of locomotor training revealed decreased lesion length (not significant) **(D)** and significantly decreased lesion volume **(E)** compared with contused untrained control ($p < 0.05$, $n = 6$ in each group; mean ± SEM).

similar to that we previously reported (Bose et al., 2002). Specifically, at postcontusion week 1, a "tonic" type of spasticity was observed (also in the other two groups, before locomotor training), whereas, at week 2, a suppressed velocity-dependent reflex excitability was detected in untrained contused animals. Finally, by week 4, an enduring, robust, velocity-dependent (dynamic) spasticity appeared. The ankle torques recorded at the lower test velocities (49–272°/s) were not significantly greater than observed in normal controls, nor were these correlated with synchronized EMG activity in the ankle extensor muscles. These low velocity ankle torques were, therefore, interpreted to be contributed by the passive properties of the muscle and joint tissues. By contrast, test rotations at the upper test velocities revealed increased stiffness of ankle rotation that was time-locked to stretch-evoked EMGs recorded from the ankle extensor muscles, indicating resistance contributions from activated ankle extensor muscle stretch reflexes (see also, Bose et al., 2002).

It has been suggested that the alterations in reflex excitability observed following SCI are associated with dynamic changes in the connectivity of the spinal neurons produced by injury-related disruption of descending fibers (Lance, 1981; Young, 1989b). Clinically, these changes in reflex excitability have been reported to include an initial period of hyporeflexia (spinal shock) followed by an enduring hyperreflexia (Kuhn and Mact, 1948; Hiersemenzel et al., 2000). Current evidence suggests that following the initial trauma, many secondary events include membrane damage, systemic and local vascular effects, altered energy metabolism, oxidative stress, inflammation, electrolyte imbalances, unregulated release of neurotransmitters, and a cascade of biochemical changes affect cellular survival, integrity, and excitability (Anderson and Hall, 1993; Tator, 1995; Faden, 1997; see also in, Velardo et al., 2000). Accordingly, alternating patterns of increased and decreased H-reflex excitability have been reported following midthoracic SCI in humans (Diamantopoulos and Olsen, 1967; Hiersemenzel et al., 2000), and in rats (Bose et al., 2002). This initial hyporeflexic state is suggested to be associated the sudden loss of tonic input and/or trophic support from supraspinal to spinal neuronal centers. In humans following midthoracic SCI, the initial period of hyporeflexia has been referred to as "spinal shock." A recent time course study of H-reflex excitability following human SCI interpreted this period of hyporeflexia as a period of transition that was followed by the appearance of a permanent hyperreflexia (Hiersemenzel et al., 2000). Hutchinson et al. (2001) and others (Gregory et al., 2003; Haddad et al., 2003; Stevens et al., 2006; Liu et al., 2008, 2010; Shah et al., 2008) reported a significant atrophy of the lower limb extensor muscles occurred in the rat during

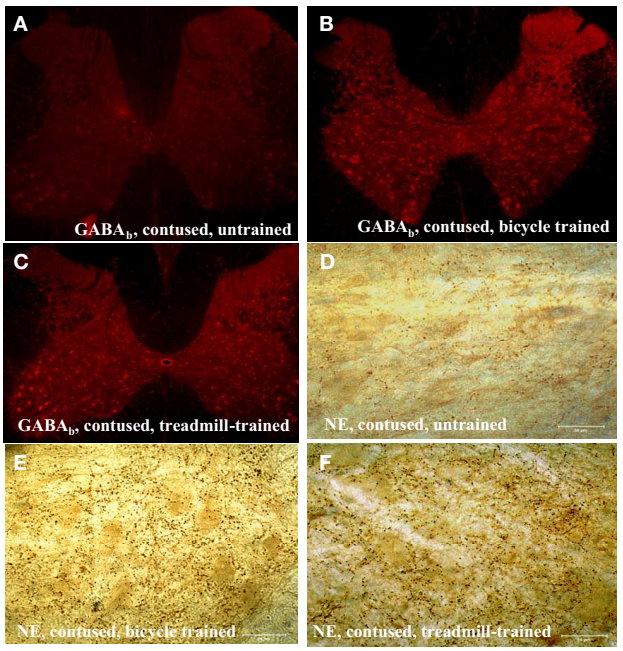

FIGURE 8 | Immunohistochemistry of lumbar spinal cord showing upregulation of GABA$_b$ receptors [5× magnification in (A–C)], and sprouting of dopamine-beta-hydroxylase (DBH) positive noradrenergic (NE) projecting fibers in the ventral horn of the lumbar spinal cord in both types of locomotor training (E,F) compared with contused untrained controls (D; scale bar, 50 μm).

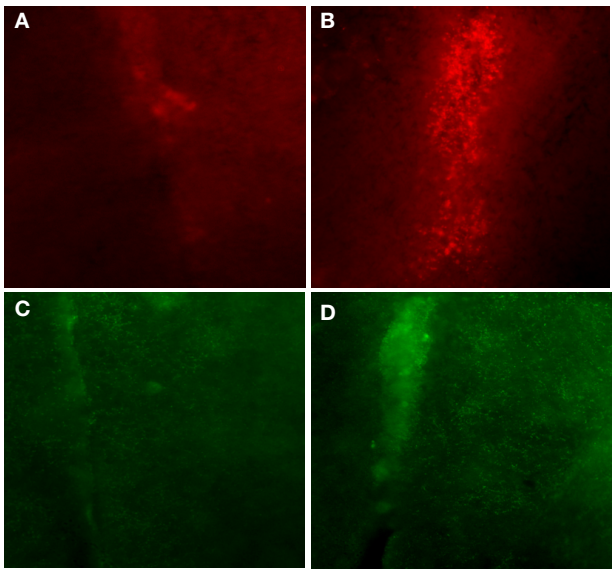

FIGURE 9 | Immunohistochemistry of thoracic (T$_{10}$) spinal cord (two segments below the injury epicenter), especially areas adjacent to dorsal median septum showed huge upregulation of BDNF (B) following 1 week of bicycle locomotor training compared with contused untrained control (A). The GABA synthetic enzyme, glutamic acid decarboxylase (GAD$_{67}$) showed also greater immunoreactivity **(D)** in the same area (1 week bicycle training) compared with untrained contused control **(C**; 20× magnification).

this initial 2 week period following midthoracic contusion SCI. Consistent with these observations, in the present study, the data recorded from the untrained contused animals revealed significant fluctuations in the excitability of lumbar reflexes over a time course of several weeks following midthoracic injury. Interestingly, only 1 week of training (both types) prevented the suppression of ankle extensor muscle stretch reflexes, as observed previously (Bose et al., 2002), and also recorded at postcontusion week 2 in untrained injured animals.

In addition, both treadmill and bicycle training not only prevented the initial hyporeflexic state, but also prolonged the transition to develop a permanent hyperreflexic state (spasticity; **Figures 4C,D**). At the end of postcontusion week 8–12, significant increases were observed in ankle torque and in the magnitude of the ankle extensor muscle EMGs burst discharge that was time-locked to the ankle rotation. Although, these values were greater compared with pre-injured normal controls, these increased ankle torque and EMGs were significantly lower than that observed in untrained contused controls. Since these increases in ankle torque and EMG were observed only at the higher rotation velocities, it was concluded that these were produced by increased velocity-dependent stretch reflexes of the ankle extensor muscles.

Following chronic SCI, several mechanisms might contribute to the increase in reflex excitability, although the principal common denominators include enhancement of excitatory synaptic input and a reduction of inhibitory control of synaptic input (Katz and Rymer, 1989; Katz, 1999). Excitatory interneurons may become more responsive to muscle or skin afferent activity due to collateral sprouting (Goldberger and Murray, 1988; Krenz and Weaver, 1998), denervation sensitivity (Curtis and Eccles, 1960), and/or changes in presynaptic inhibition (Burke, 1988), or a combination of these changes. In this regard it is noteworthy that our velocity-dependent spasticity measuring instrumentation incorporated both stretch reflex afferent input from the triceps surae muscles as well as input from the skin. The lengthening resistance of the triceps surae muscles was measured by quantitating ankle torque during 12° dorsiflexion rotations of the ankle from 90°. Contact with the foot was achieved using a form-fitted cradle attached with the force transducer and aligned with the dorsal edge of the central footpad 2.6 cm distal to the ankle joint. Therefore, during 12° dorsiflexion of the ankle, afferent input from lengthened muscles and skin afferent input from the footpad were activated. Therefore, the improvement in velocity-dependent ankle torque data represents improvement in spasticitic hyperreflexia elicited by muscle stretch and skin afferent input.

Descending fibers containing NE systems are considered to play important role in the regulation of spinal cord function and in the modulation of spinal reflexes (Fung and Barbeau, 1994; Hasegawa and Ono, 1995; Kobayashi et al., 1996; Li and Zhuo, 2001). Moreover, it has been reported that the synaptic effectiveness of group II afferents is modulated by NE descending neurons (Jankowska and Riddell, 1995). Although, it is not known specifically how locomotor training alters excitatory synaptic input and/or changes in presynaptic inhibition, recent studies have revealed that exercise dependent activity upregulates a host of factors that may

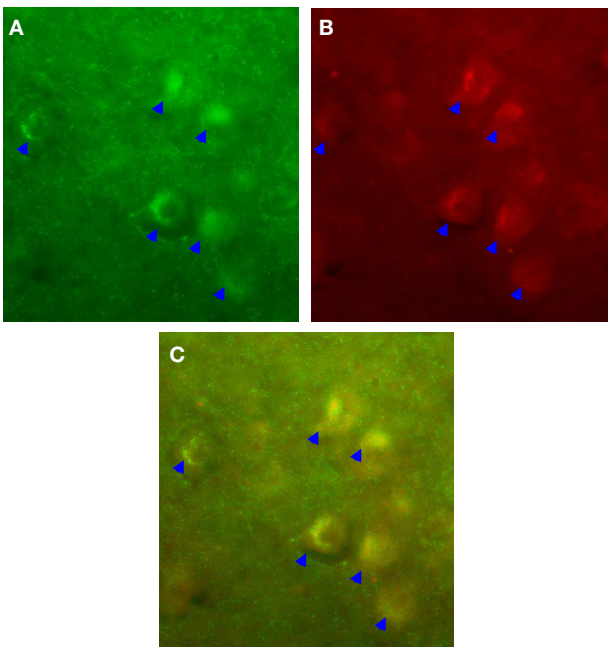

FIGURE 10 | IHC showed expression and co-localization of GAD_{67} and BDNF in the ventral horn (VH) motoneurons (arrows). GAD_{67} showed a diffuse staining in cell bodies and fibers as well as punctate staining in the VH **(A)**. Interestingly, those GAD_{67} immunostained motoneuron cell bodies also stained with BDNF **(B)**; **(C)** is the merged image of **(A,B)** (20× magnification).

contribute to these changes (Gomez-Pinilla et al., 2001; Bose et al., 2005). In addition to presynaptic mechanisms, changes in postsynaptic mechanisms that regulate the input/output gain of motoneuron discharge must also be considered (Kernell, 1979; Hounsgaard et al., 1984; Binder, 2003). Numerous studies have revealed that the gain of synaptic inputs can be amplified up to a factor of five by brainstem/monoaninergic inputs that regulate dendritic persistent inward currents (PICs) utilizing sodium and calcium channels (Schwindt and Crill, 1980; Bennett et al., 1998; Lee and Heckman, 1998a,b, 2000; Perrier and Hounsgaard, 2003; Harvey et al., 2006). The higher the PIC, the higher the synaptic gain and consequent burst rate of the motoneurons. Segmental regulation of PICs occurs through inhibitory mechanisms that regulate afferent inputs (Heckman et al., 2003). It has been proposed that the acute period of hyporeflexia that follows SCI can be attributed to a reduction in dendritic PICs; subsequently, after several weeks, motoneurons re-acquire PICs that can be easily initiated by segmental inputs (Lee et al., 2003). These unregulated PICs are proposed to significantly contribute to clonus and spasms, and associated amplified bi-stable properties of motoneurons. In this context, the segmental inhibitory processes, such as presynaptic inhibition, have an even more important role in the regulation of sensory transmission.

The delayed and milder form of spasticity development that accompanied locomotor training observed in the present studies, could be a result of improved inhibitory mechanisms that regulate afferent inputs. Activity related reorganization of segmental circuitry including descending inputs, segmental synaptic inputs, and local interneurons might contribute in this improvement. Neuronal circuits, stimulated by the proper activation of peripheral afferents via the training, may reorganize by strengthening existing and previously inactive descending connections and local neural circuits. Thus, optimization of neuroplasticity may be a viable foundation for developing rehabilitation strategies that facilitate recovery of locomotion following SCI.

REFLEX EXCITABILITY

Studies in rats with experimental spinal cord trauma have demonstrated the appearance of progressive changes in processes that regulate transmission in reflex paths to ankle extensor motoneurons (Thompson et al., 1992, 1993, 1998). Particularly evident was a robust decrease in rate-dependent depression tested in reflex pathways to ankle extensor muscles following midthoracic SCI that exhibited a clinical definition of spasticity (Bose et al., 2002). Similar changes have been observed in humans following injury (Ishikawa et al., 1966; Diamantopoulos and Olsen, 1967; Calancie et al., 1993; Schindler-Ivens and Shields, 2000). The loss of rate-dependent depression has been suggested to be associated with injury-induced plasticity of presynaptic processes that regulate afferent transmission to motoneruons (Thompson et al., 1992, 1998; Bose et al., 2002).

There are several important practical reasons for using the plantar H-reflexes (not soleus H-reflexes) in these studies. The distribution of tibial motoneurons innervating the soleus is anatomically continuous with those to the plantar muscles with considerable overlap in the fourth lumbar spinal cord segment (Crockett et al., 1987; Gramsbergen et al., 1996; Homonko and Theriault, 1997). The plantar H-reflexes are far more robust than soleus reflexes in anesthetized animals possible related to the high innervation ratio of plantar muscles (Crockett et al., 1987). Second, with regard to stimulation and recording, the plantar H-reflexes are far more accessible than soleus H-reflexes. Third, the short length of plantar muscles makes compound EMG recordings more robust than compound EMG recordings in the exceptionally long soleus muscle. Therefore, to accommodate weekly, non-invasive recordings (in anesthetized animals), the easily accessed, robust plantar/H-reflexes are far more practical. Further, it is presumed that since parallel changes occurred in both calf and foot muscles following SCI (Thompson et al., 1992), treatment-induced changes may similarly influence both the rostral and caudal portions of the tibial motoneuron pool that innervates these two muscle groups.

This reflex analysis has provided a quantitative assay of changes in inhibitory processes that regulate motoneuron excitability after experimental treatments in animals (Thompson et al., 1993; Skinner et al., 1996) and also in humans following experimental treatments using exercise (Trimble et al., 2001; Kiser et al., 2005) and transplantation (Thompson et al., 2001b). The influence of treatments on rate-sensitive depression has been suggested to be associated with treatment-induced changes in the influence of inhibitory interneurons. Skinner et al. (1996) proposed that continual depression of Ia afferents during cycling exercise in the spinalized rat promoted neural reorganization and preserved the local neural circuitry responsible for presynaptic inhibition, thus normalizing the values of rate-depression observed at rest.

A case study by Trimble et al. (1998) provided the first evidence for normalization of rate-sensitive depression following specialized locomotor training in a human after incomplete SCI (Trimble et al., 1998). The data from our studies indicate that the normalization of rate-sensitive depression is associated with an improvement of gait and open field locomotion, and velocity-dependent lengthening resistance of hindlimb extensor muscles. We propose that these task-specific trainings (bicycle and treadmill) provided patterned therapeutic activity in the injured and/or altered neural circuitry and this therapy decreased the maladaptive plasticity that contributes to spasticity and altered locomotor function. Two neurotransmitter systems that appear to play critical roles in the modulation of segmental reflex modulation are GABA and NE. We have observed that the rate-dependent inhibition and velocity-dependent ankle torque are profoundly influenced by $GABA_b$-specific agents (Wang et al., 2002; Thompson et al., 2005) and NE-specific lesions (Thompson et al., 1999; Bose et al., 2001). Specifically, L-baclofen (which acts upon $GABA_b$ segmental circuitry) increased rate-dependent inhibition and decreased velocity-dependent ankle torque, whereas selective neurotoxic lesions of NE fibers produced non-specific increase in reflex excitability. While the specific role of $GABA_b$ receptors is the topic of ongoing research, $GABA_b$-mediated synaptic depression using baclofen is currently the most potent and widely used drug for treating spasticity (Penn and Kroin, 1984, 1985; Penn, 1988). It is known that acute baclofen treatment reduces both the monosynaptic and the polysynaptic components of the stretch reflex (Capaday and Stein, 1986; Advokat et al., 1999). We propose that in this study locomotor activity-induced plasticity might up-regulate $GABA_b$ receptor and NE mediated inhibition which in turn result in improvement of reflex excitability.

LIMB AXIS AND BASE OF SUPPORT

Alteration of hind limb axis and base of support following spinal cord contusion have been reported with suggestions that these deficits were produced by injury-related dysfunction of long tracts, as well as injuries of the propriospinal system (Kunkel-Bagden et al., 1992, 1993; Bose et al., 2002). In addition, it has been shown that excitotoxic injury of the L_1-L_2 gray matter resulted in locomotor ataxia in rats (Magnuson et al., 1999). That balance deficits can be associated with long tract injury is also suggested by our recent work that reported that specific lesion of the NE descending neurons (using i.t. anti-DBH saporin toxin), resulted in external deviation of the hind limb axis and base of support (Bose et al., 2001). In animals and humans with SCI, previous studies have shown improvements in gait parameters following locomotor training using body weight support on the treadmill and manual assistance, but have not concurrently evaluated effects of bicycle locomotor training following animal SCI. In the present studies, 2–3 months of locomotor training significantly reduced outward deviation of the hind limb and base of support in both treadmill and bicycle training groups compared with untrained contusion group. Interestingly, no significant difference in hind limb rotation or base of support was observed between the two training groups following 2–3 months training. This study so far is the first comparable investigation of two rehabilitation strategies in an animal SCI spasticity model.

OPEN FIELD LOCOMOTION

Studies progressing over the last two decades have revealed that spontaneous locomotor recovery following SCI is contingent upon the preservation of fibers diffusely located in the ventral caudal – ventro-lateral funiculi of the rat spinal cord (Das, 1987; Brustein and Rossignol, 1999; Basso et al., 2002) or gray matter of the T_{13}-L_2 spinal segments (Magnuson et al., 1999). By contrast, animals with surgical lesions of the dorsal spinal cord at T_8 that preserved ventral funiculi, demonstrated sufficient self-training that no detectable difference was observed in their locomotor recovery compared with animals that were systematically trained using a treadmill (Fouad et al., 2000). However, following moderate midthoracic contusion injury, injured animals in the present study that received locomotor training revealed greater BBB scores than untrained animals. Longitudinal testing at 4, 8, and 12 weeks following injury revealed that training increased both the rate and magnitude of recovery. It is interesting that recovery scores tested at postcontusion week 4 indicated a particularly robust recovery in the cycle trained group, that was significantly greater than both the untrained and the treadmill trained group. Although the specific reason for this result at this time point is not clear, it is possible that the efficient and uniform pattern inherent to the nature of the cycle training was particularly effective during the early training period. By contrast, animals in the treadmill groups had more freedom to vary limb placement and weight support that could have contributed more variability in the training pattern during this initial period. Moreover, the loading was minimal during the treadmill training, especially in the first 4 weeks. In animals and humans with SCI, previous studies have shown improvements in gait parameters following locomotor training using body weight support on the treadmill and manual assistance (Harkema et al., 1997; Barbeau et al., 1998; Behrman and Harkema, 2000; Dietz and Harkema, 2004; Timoszyk et al., 2005) but have not concurrently evaluated effects of bicycle locomotor training following animal with SCI.

HISTOLOGY AND IHC

The ventral and lateral WM subserve most of the important hindlimb locomotor function and contain important descending pathways including the rubrospinal tract in the dorsolaeral funiculus and more importantly the reticulospinal tract that is more diffusely distributed in the ventral and lateral WM. Moreover, stride length and base of support have been associated with preservation of the reticulospinal and vestibulospinal pathways for maintenance of posture and trunk stability (Goldberger, 1988).

It is known that transection of the rat spinal cord reduced the binding of [^{3}H]GABA by 80% (Chuang, 1989). The decrease in GABA binding below the level of SCI suggests that a decrease occurs in the number of GABA receptors. Most of $GABA_b$, a metabotropic receptor, in the spinal cord is presynaptic and located on descending axons, although some of the $GABA_b$ receptors are on incoming dorsal root afferent axons (Bowery et al., 1980). Normally, incoming dorsal root information is subject to presynaptic GABA inhibition, which can reduce the amount of excitatory neurotransmitter release (Bowery et al., 1980). Possibly these areas are a source of GABAergic afferents which might participate in the upregulation of BDNF in response to training as seen elsewhere

in the CNS following exercise (see review, Cotman and Berchtold, 2002). We suggest that an increase in GAD_{67} leads to increased GABA production in spinal neurons below the injury site, resulting in altered inhibition and trophic support during posttrauma recovery and adaptation. Moreover, locomotor training induced hyperexpression of GAD_{67} might inhibit excitotoxic effects mediated by excitatory neurotransmitters at the site of injury. Increased GABA synthesis around the central canal, in the vicinity of ependymal cells, has been reported as a regenerative process in the mammalian spinal cord (Tillakaratne et al., 2000). This data is important and can be argued that locomotor training induced inhibitory neurotransmitters (GABA/GAD_{67} and NE) and BDNF's availability could be crucial in reducing the lesion length and volume by optimizing the excitotoxic effects, strengthening neuronal structure, stimulate neurogenesis and increase resistance to further injury. Although exercise mobilizes many gene expression profiles (Cotman and Berchtold, 2002), increased levels of BDNF and inhibitory molecules like GABA and NE could be related to spinal cord plasticity related to post-training improvement of spasticity.

In the present studies, locomotor training-related improvements in spasticity and locomotor recovery were correlated with the decreased lesion volume and more spared white matter. In addition, immunohistochemical studies of these tissues, compared with untrained SCI controls, revealed marked upregulation of BDNF, GABA, and norepinephrine which might account for these decreased lesion volume and more spared tissue.

The findings of the present study are consistent with the suggestions that as therapy, the locomotor training regimen using either treadmill or cycle, promotes the recovery of walking by optimizing the activity-dependent neuroplasticity of the nervous system (Edgerton et al., 1992; Muir and Steeves, 1997; Bose et al., 2005a). Neuronal circuits, stimulated by task-appropriate activation of peripheral and central afferents via the locomotor training, may also reorganize by strengthening existing and previously inactive descending connections and local neural circuits (Edgerton et al., 1992; Dietz et al., 1997; Muir and Steeves, 1997; Barbeau et al., 1998; Basso, 2000). These studies indicate that a locomotor training regimen using either treadmill or cycling significantly enhanced several issues related to locomotor recovery. It is interesting that similar results were obtained with therapeutic locomotor training using either treadmill or bicycle. Although the precise similarities and differences between the two modes of locomotor training have not been systematically quantitated in this animal model, there are some general observations that are relevant. While locomotor exercise is common to both modes of therapy, treadmill walking has an advantage of imposing a higher load, but also, has the disadvantage of a greater variance of locomotor form. On the other hand, cycling offers a lower level of loading, but provides opportunity for greater precision of systematic locomotor form. We propose that this locomotor exercise of either type, benefits from activity-dependent neuroplasticity of the locomotor circuitry, and that the distinct advantages of each mode sufficiently engage the circuitry to induce a positive therapeutic benefit.

The finding presented here is highly significant in terms of translational potential of less labor intensive cycle exercise into clinical use in treatment of human SCI patients in clinic as well as in home setting. This is due to the fact that cycling exercise requires much fewer support personnel and less expensive equipment than does treadmill walking. While treadmill exercise in humans has been shown to decrease the excitability of lower limb reflexes, cycling exercise in humans with both legs has not been tried as much but is promising (Trimble et al., 2001; Kiser et al., 2005). Conceivably, cycling devices for human SCI patients will need to be engineered incorporating appropriate physiological parameters to increase its clinical use.

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A neuroanatomical correlate of sensorimotor recovery in response to repeated vaginocervical stimulation in rats

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Gentle probing against the cervix via the vagina (vaginocervical stimulation, VCS) increases tail flick latency (TFL) to radiant heat; greater force abolishes the tail flick response and other withdrawal responses. This effect occurs in spinal cord-transected rats and in intact rats. On the basis of our earlier finding that VCS releases vasoactive intestinal peptide (VIP) into the spinal cord, and others' reports of neurotrophic effects of VIP *in vitro*, we hypothesized that repeated VCS would stimulate sprouting and sensorimotor function of terminals of genital nerve primary afferents in the sacral spinal cord. To test this hypothesis, in the present study, we denervated the genital tract *only unilaterally*, which significantly reduced the TFL-elevating effect of VCS. Then we applied repeated daily VCS for 1 week and compared the subsequent effectiveness of acute VCS in elevating TFL. The rats that received the repeated daily VCS showed a significantly greater elevation in TFL in response to acute VCS than control rats that did not receive the repeated stimulation. Then, to test whether daily repeated VCS stimulates sprouting of genital primary afferents in such unilaterally genital tract-denervated rats, we transected the contralateral remaining intact pelvic nerve, applied horseradish peroxidase (HRP) to its proximal cut end for 1–2 h, and 2–3 days later counted HRP particles in its terminal zone (L6–S1) in the spinal cord. There were significantly more HRP particles in the rats that received the daily repeated VCS than in the control rats. In the context of these findings, we conclude that VCS in rats can produce a functional sensorimotor recovery via a neurotrophic effect on compromised primary afferents in the spinal cord.

Keywords: recovery of function, genital stimulation, sprouting, primary afferents, HRP, analgesia

INTRODUCTION

Vaginocervical stimulation (VCS) in rats acts at the spinal cord to block withdrawal reflexes to noxious stimulation. Hind leg and tail withdrawal reflexes, which persisted after surgical removal of a segment of the spinal cord at the mid-thoracic level, were still completely blocked by VCS (Komisaruk and Larsson, 1971). A possible mechanism for this effect is that foot-shock-induced release of substance P into spinal cord superfusate is inhibited by concurrent VCS (Steinman et al., 1994). The pelvic nerves, which provide sensory innervation of the vagina and cervix (Komisaruk et al., 1972; Peters et al., 1987; Berkley et al., 1990, 1993) are the main mediator of this VCS-induced reflex inhibition, for transection of the pelvic nerves almost completely abolishes the ability of VCS to block withdrawal reflexes (Cunningham et al., 1991). Vasoactive intestinal peptide (VIP) is a possible mediator of this effect of VCS, for pelvic nerve contains VIP (Basbaum and Glazer, 1983), VCS releases VIP into spinal cord superfusates (Komisaruk et al., 1989), and VIP and certain of its fragments administered directly to the spinal cord, intrathecally, mimic the analgesia-producing effects of VCS (Komisaruk and Jordan, 1995). And in a different context, VIP exerts neurotrophic effects (Brenneman and Eiden, 1986; Gozes et al., 1991; Gressens et al., 1993; Brenneman et al., 1999).

These findings combined led us to hypothesize that VCS could promote recovery of spinal cord-mediated behavioral function. To test this hypothesis, we first reduced the inhibitory effect of VCS on the tail flick latency (TFL) to radiant heat test by transecting the pelvic nerve unilaterally. In order to maximize the unilateral genital neurectomy effect, we also ipsilaterally transected the other genital sensory nerves, i.e., the pudendal and hypogastric (Komisaruk et al., 1972; Peters et al., 1987; Berkley et al., 1990, 1993). Then we applied repeated VCS three times daily for 1 week. We reasoned that the daily repeated VCS would stimulate sprouting of the remaining intact pelvic nerve terminals, and thereby increase the effectiveness of the VCS. Thus, in the present study, we ascertained whether: (a) unilateral genital neurectomy would reduce the magnitude of VCS-induced inhibition of the TFL, (b) daily repeated VCS would overcome this attenuation, and (c) the daily repeated VCS would induce sprouting of the remaining intact pelvic nerve terminals.

MATERIALS AND METHODS

EXPERIMENT 1. DOES DAILY REPEATED VCS IN GENITAL UNILATERAL-NEURECTOMIZED RATS INCREASE ITS INHIBITORY EFFECT ON TFL?

Subjects

Sprague Dawley female rats at least 90 days of age and weighing 300–400 g, were purchased from Charles River Breeding Laboratories. All animals were maintained on a reversed-light cycle

(lights on 7:00 p.m. to 10:00 a.m.) with food and water *ad libitum*. Rats were housed two per cage in tubs with absorbent material flooring. All rats were ovariectomized at least 1 month prior to nerve transections to eliminate variability in response to VCS due to fluctuating levels of ovarian hormones (Crowley et al., 1976).

Groups

Animals were randomly assigned to the following groups: C: sham-operated controls, $n = 10$; GenX: unilateral pelvic-hypogastric-pudendal neurectomy, $n = 11$; GenX + VCS: unilateral pelvic-hypogastric-pudendal neurectomy receiving three times daily VCS, $n = 11$; C + VCS: intact controls receiving three times daily VCS, $n = 12$.

Surgery

All experimental procedures used in the present study received approval from the Rutgers University Institutional Animal Care and Use Committee. Nerve transections: rats were anesthetized with a solution of 0.5 ml Rompun/10 ml ketamine; dose: 0.07 ml/100 g body weight. A ventral abdominal incision, approximately 6 cm length, exposed the abdominal–pelvic region and facilitated the identification of the genitospinal nerves.

Unilateral hypogastric neurectomy. We identified the left ureter by its vigorous peristaltic activity, then visualized the left hypogastric nerve between the colon and the ureter as it courses parallel to the ureter, and removed a 4 mm segment of the nerve.

Unilateral pelvic neurectomy. The bifurcation of the vena cava into the common iliac veins was identified, the left common iliac vein was followed caudad, approximately 1 cm, to the origin of the left internal iliac vein. Approximately 5 mm dorsal to the origin of the left internal iliac vein, perpendicular to its axis, we identified the left pelvic nerve as it courses rostrocaudad, lying across the vein, and removed a 4 mm segment.

Unilateral pudendal neurectomy. The left pudendal nerve was located dorsal and parallel to the left pelvic nerve as it, also, lies across the internal iliac vein. The pudendal nerve is well-myelinated and glistens, relative to the pelvic nerve. A 4 mm segment was removed.

Sham neurectomy. The hypogastric, pelvic, and pudendal nerves were located unilaterally, and then each nerve was gently lifted with a microsurgical hook and returned to its original site.

Behavioral testing

Baseline TFL responses to VCS were obtained 1 week prior to neurectomy or control procedures. Each rat was placed in a hemi-cylindrical Plexiglas restrainer for approximately 5 min prior to testing. TFL responses before and during VCS (100 and 300 *g* force) were established prior to neurectomy or sham surgery.

TFL test. This measures the latency (in s) to flick the tail away from a focused regulated radiant heat source. The radiant heat source (IITC Inc., Model 33) was located 8 cm above and 4 cm proximal to the tip of the tail. The temperature of the heat lamp of the tail flick apparatus was adjusted to elicit a group mean approximate TFL of 3 s. The TFL reading displayed automatically when the rat withdrew the tip of its tail away from the radiant heat source, breaking a photocell circuit. To prevent tissue damage, the heat source was stopped at 9 s if the rat did not withdraw its tail. Each test consisted of three trials 15 s apart. The score for each test was calculated as the mean latency of three successive trials.

Acute VCS. VCS (100 and 300 *g* force) was applied to the cervix using a calibrated "Force Dial Hand Held Dynamometer" (Model FD 500, Wagner Instruments, Greenwich, CT, USA). The tip of the metal plunger of the force-calibrated dynamometer was shaped with a dental sanding disk in order to match the tip of a 1 cc plastic syringe plunger. To provide cushioning during VCS, we placed the rubber tip of a 1 ml plastic syringe onto this modified tip.

Daily repeated VCS began 18 h after unilateral neurectomies and continued for 1 week. VCS (100 *g* force, 10 s on, 10 s off × 5 min) was applied three times daily, 2 h apart. The effect of acute VCS on TFL (100 and 300 *g* force) was ascertained 1 week after the daily VCS treatment ended, using a blind procedure.

Statistical analysis

The data are expressed as mean ± SEM. Comparison among groups was made using repeated measures, Two-way analysis of variance (ANOVA), and subsequent Fisher's protected *t*-tests.

EXPERIMENT 2. DOES DAILY REPEATED VCS INDUCE SPROUTING IN GENITAL PRIMARY AFFERENT NERVE TERMINALS?

Subjects

Eight Sprague Dawley female rats were purchased from Charles River Breeding Laboratories, at least 90 days of age and weighing 250–400 g at the time of surgery. They were ovariectomized at least 2 weeks before surgery and maintained on a reversed-light cycle (lights on 7:00 p.m. to 10:00 a.m.) with food and water *ad libitum*.

Groups

Animals were randomly assigned to the following groups: Control group: the genitospinal nerves (hypogastric, pelvic, and pudendal nerves) were transected unilaterally (right side) as described above, $n = 5$; VCS-treated group: the genitospinal nerves were transected unilaterally (right side). Then, beginning 18 h after unilateral neurectomies VCS (100 *g* force) was applied for 5 min (10 s on/10 s off), 2 h apart, three times a day for 1 week, $n = 3$. Two weeks post-surgery, in both the control and VCS-treated groups, the remaining intact contralateral nerve (pelvic nerve) was transected and the proximal end was dipped into horseradish peroxidase (HRP) (see details of method below). Two to three days after the nerve dip, the animals were sacrificed and the density of HRP particles in the terminal field of the nerve in the spinal cord was determined.

Pelvic nerve transection and pelvic nerve dip

Fourteen days post-lesion, the rats were anesthetized as above and the left pelvic nerve was exposed at the pelvic ganglion level and transected with microscissors. Approximately 5 μl HRP dissolved in saline (50 U/μl) was used to fill a small cup made by cutting PE-10 tubing to fit the nerve and closing one end with petroleum

jelly. The cut end of the proximal pelvic nerve was placed into the cup and exposed to the tracer for 1–2 h. At the end of this period, the cup was gently removed from the pelvic nerve end and the incision sutured. The animals were then allowed to survive for 2–3 days.

Perfusion and tissue preparation

Two to three days after the nerve dip, the animals were deeply anesthetized as described above, and perfused, via the ascending aorta, with physiological saline at room temperature (50 ml/rat), followed by a solution of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer at room temperature (500 ml/rat). After perfusion, the L6–S1 spinal cord segments were identified and removed, placed in a sucrose-buffer solution at 4°C for 12–24 h and cut into 30 μm sections using a cryostat.

Horseradish peroxidase histochemistry

The mounted sections were processed for HRP histochemistry by using tetramethylbenzidine as the chromogen and stabilized with sodium nitroprusside according to the method of Mesulam and Brushart (1979). The sections were then air-dried overnight, cleared in hemoD, coverslipped with Permount mounting medium and examined under a Nikon light microscope using darkfield illumination. In all cases, the investigator making the histological observations was blind as to the treatments that corresponded to the slides. All sections L6–S1 were examined for the presence of HRP particles. HRP particles (reaction product granules) were identified and counted manually using the software program, "Neurolucida," under darkfield illumination. Spinal cord regions were identified and delineated according to Rexed's laminae, and the area of each region determined with the software program, "Neuroexplora." The selection of these sections was based on the presence of maximal HRP label intensity at the sacral parasympathetic nucleus (SPN) as observed under darkfield microscopy. The average of three sections (L6–S1) per animal was used to measure the density of HRP particles for each spinal cord region. HRP particle density was calculated by counting the total number of HRP reaction product granules and then dividing this value by the area of the corresponding spinal cord region. The images of the selected sections were analyzed using a Kodalith threshold that was adjusted for each image after manually identifying HRP particles and excluding background levels and artifacts. The average particle size was set at (0.01–5 μm^2) and the average size of 3 μm was used to separate and thereby identify individual particles.

Statistical analysis

The data are expressed as the medians and interquartile ranges. The groups were compared using Mann-Whitney *U*-tests.

RESULTS

EXPERIMENT 1. EVIDENCE THAT DAILY REPEATED VCS INCREASES THE INHIBITORY EFFECT OF VCS ON TAIL FLICK

As summarized in **Figure 1**, all groups showed equivalent baseline (pre-VCS; "0 *g* force") TFLs. As anticipated, the TFL in response to acute VCS (100 or 300 *g* force) was significantly lower in the unilateral genital neurectomy (GenX) group than in the intact control (C) group ($p < 0.01$, Fisher's protected *t*-tests). Thus, the unilateral neurectomy significantly reduced the ability of VCS to inhibit the tail flick response. The TFL elevation to 300 *g* (although not 100 *g*) VCS was significantly higher in the unilateral neurectomy + daily VCS group (GenX + VCS) than in the unilateral neurectomy group that did not receive daily VCS (GenX). However, in the intact group, daily repeated VCS (C + VCS) did not increase the ability of acute VCS to increase TFL any more than in the intact group that did not receive daily repeated VCS (C). Thus, the higher VCS force revealed the effect of daily repeated VCS to increase the ability of VCS to inhibit the tail flick response in the genital-neurectomized rats.

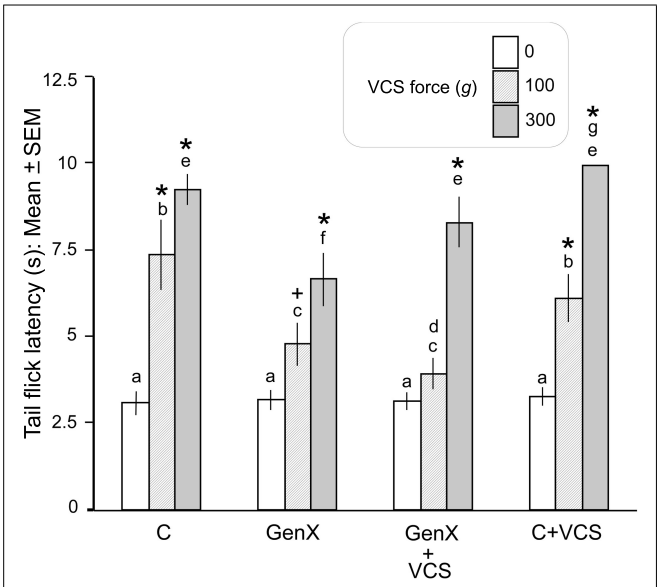

FIGURE 1 | Unilateral genital neurectomy significantly reduced the ability of VCS to elevate TFL. Daily repeated VCS in unilateral genital-neurectomized rats increased the ability of acute VCS to elevate TFL compared to their controls that did not receive daily repeated VCS (see text for details). C, intact controls; GenX, unilateral genital neurectomy; GenX + VCS, unilateral genital neurectomy plus daily repeated VCS; C + VCS, intact rats receiving daily repeated VCS. $^{*}p < 0.01$, $^{+}p < 0.05$; significantly greater, by Fisher's protected *t*-test, than within-group baseline (0 *g* force of acute VCS). Bars that do not share the same letters are significantly different from each other, within- or between-groups (ANOVA plus subsequent paired comparisons; $p < 0.05$)

EXPERIMENT 2. EFFECT OF UNILATERAL NERVE TRANSECTION AND DAILY REPEATED VCS ON HRP LABELING OF PELVIC NERVE TERMINALS

HRP reaction product was located in the L6–S1 segments of the spinal cord. The primary afferent fibers enter this region through Lissauer's tract, diverging into a medial and a lateral band surrounding the superficial border of the dorsal horn. The collaterals of some primary afferents that course along the lateral band approach, and form connections with, SPNs and dendrites. Other collaterals course among SPNs and dendrites to form a terminal field in the upper dorsal gray commissure with fewer fibers crossing to the contralateral side of the spinal cord. The primary afferent collaterals emerging from the medial band form

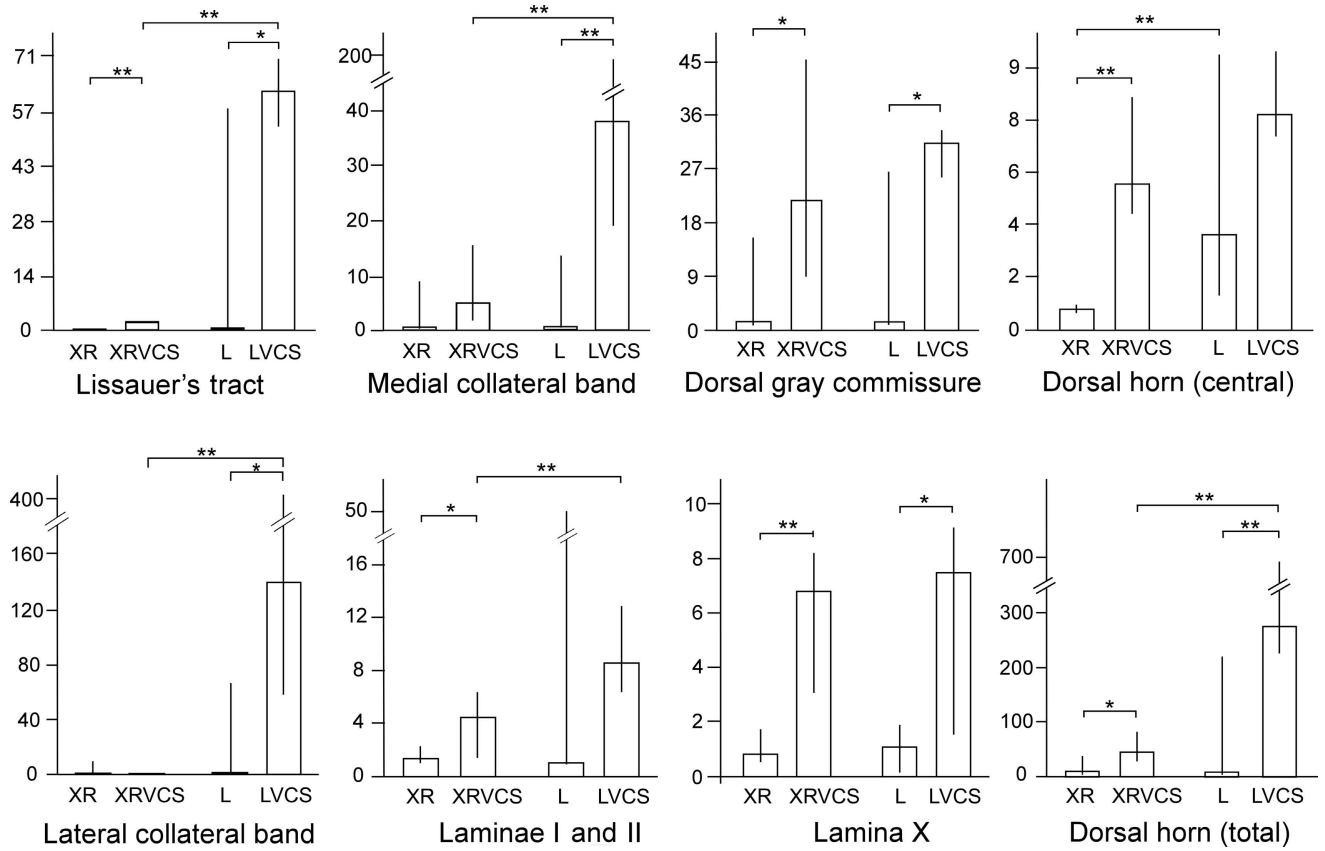

FIGURE 2 | In rats that received daily repeated VCS, there was increased HRP labeling in the terminal fields of the pelvic nerve at L6–S1 compared to rats that did not receive such VCS, suggesting that the daily repeated VCS induced sprouting (details in the text). The *y*-axes depict the number of HRP particles/1×10^4 μm^2. XR, HRP counts on the right side of the spinal cord; XRVCS, HRP counts on the right side of the spinal cord after 1 week of daily repeated VCS; L, HRP counts on the left side of the spinal cord; LVCS, HRP counts on the left side of the spinal cord after 1 week of daily repeated VCS. In general, daily repeated VCS increased the number of HRP granules compared to rats that did not receive such VCS. $^{**}p < 0.02$ (Mann–Whitney *U*-tests), $^{*}p < 0.05$ (Mann–Whitney *U*-tests). Notes: In all four groups, the right pelvic nerve was first transected 2 weeks earlier. Scales for HRP granule counts are different in the different groups.

a terminal field in the upper dorsal gray commissure. Primary afferent collaterals/terminals are also located in Laminae I, II, and X (**Figure 3**).

EVIDENCE THAT DAILY REPEATED VCS INCREASES HRP LABELING

The purpose of transecting the genitospinal nerves unilaterally (on the right side) was to clear a field for the ipsilateral and contralateral terminals of the remaining contralateral pelvic nerve to potentially proliferate. As can be seen in **Figure 2**, in the non-VCS group (XR and L), there was minimal HRP labeling in either the ipsilateral or the contralateral side of the spinal cord in the regions specified. By contrast, in the VCS group (XRVCS and LVCS), there was significantly more labeling than in the non-VCS group in six of the eight ipsilateral (i.e., left side) intact regions. And in six of the eight neurectomized (i.e., right side) regions, there was significantly more labeling in the XRVCS group than in the non-VCS (XR) group. Thus, whereas relatively minimal HRP labeling occurred in the terminals in the absence of VCS, there was a marked increase in HRP labeling on both the ipsilateral and the contralateral sides in the group that received daily repeated VCS. **Table 1** summarizes the median number of HRP particles observed in each region of the spinal cord.

DISCUSSION

In the present study, unilateral denervation of the reproductive tract significantly reduced the magnitude of VCS-induced inhibition of the tail flick response to radiant heat. Daily repeated VCS for 1 week then significantly increased (restored) its analgesic effectiveness in these unilateral genital-denervated rats, whereas in the intact control rats, the same type of VCS did not increase further the analgesia magnitude. We tested a possible morphological basis for this effect by first transecting the pelvic nerve on the right side in order to vacate its terminal projection field. Subsequently, we applied HRP to the acutely cut proximal end of the remaining intact pelvic nerve on the left side, and then counted HRP reaction product in the pelvic nerve terminal projection zone at spinal cord level L6–S1 (Nadelhaft and Booth, 1984). The present findings provide support for the interpretation that daily repeated VCS increased the terminal proliferation, on the basis that the daily repeated VCS significantly and markedly increased the counts of

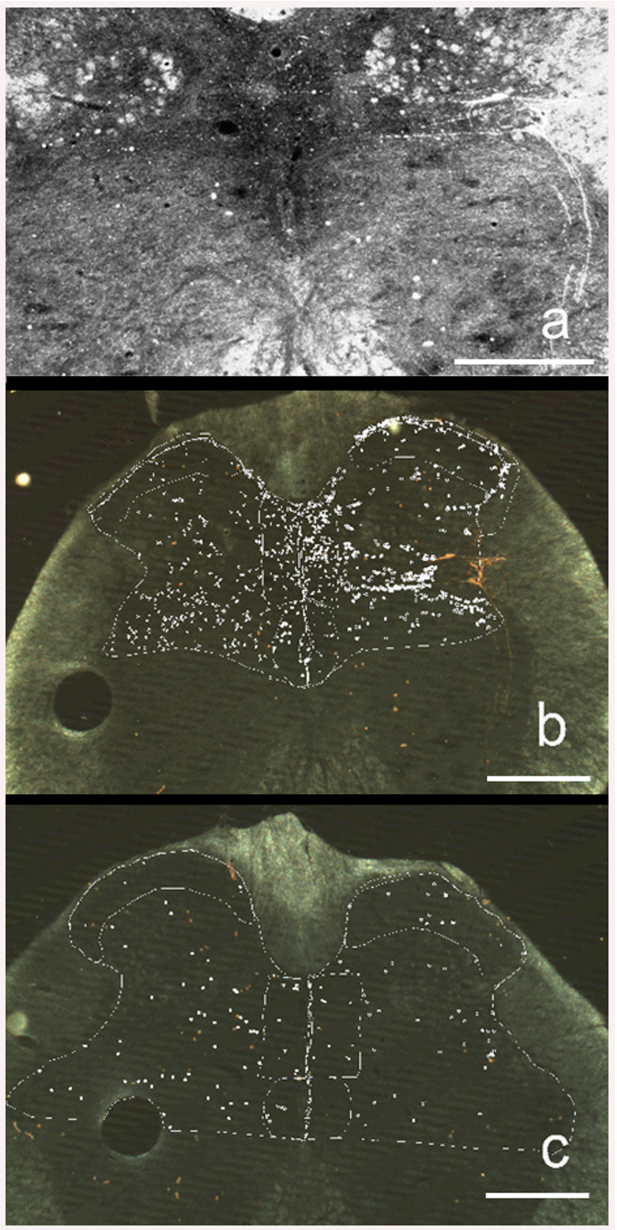

FIGURE 3 | Darkfield illumination photomicrograph (Neurolucida) of transverse 30-μm sections of the sacral 1 (S1) segment of the spinal cord. The right side of the figures is the left side of the spinal cord. (a) HRP labeling in a rat that received daily repeated VCS. Brightened image to emphasize appearance and location of HRP particles (white dots). (b) Same section as above showing how HRP counting was made. The white marker dots indicate the locations of HRP particles. Different types of marker dots were used to differentiate the selected regions of the spinal cord. (c) HRP labeling in a control rat that did not receive daily repeated VCS. Calibration bar = 200 μ.

HRP reaction product in the pelvic nerve terminal field in six of the eight ipsilateral terminal fields measured, as well as in six of the eight contralateral terminal fields measured (The pelvic nerve distributes its terminals ipsi- and contra-laterally; Basbaum and Glazer, 1983; Chung et al., 1993).

Table 1 | Regional distribution of median number of HRP particles/1 × 10^4 μm^2.

Spinal cord region	XR	XRVCS	L	LVCS
Lissauer's tract	0	1.88	0	61.33
Medical collateral band	0	4.47	0	37.76
Dorsal gray commissure	1.15	21.38	0.90	30.88
Dorsal horn (central)	0.70	5.19	3.39	7.73
Lateral collateral band	0	0	0	138.09
Laminae I and II	1.20	4.33	0.90	8.55
Lamina X	0.86	6.64	1.04	7.43
Dorsal horn (total)	4.04	44.01	5.12	273.47

This is a numerical summary of the data shown in Figure 2. In the control group (n = 5), the right genitospinal (hypogastric, pelvic, pudendal) nerves were transected (XR) 2 weeks before HRP administration to the intact contralateral pelvic nerve [left side of the spinal cord (L)]. In the experimental group (n = 3) rats were treated with a daily regimen of VCS following unilateral right side genitospinal nerve transection (XRVCS). Following daily VCS treatment HRP was administered to the intact contralateral pelvic nerve (LVCS). HRP counts were done in both sides of the spinal cord (see text for details).

The validity of the quantitative HRP method used in the present study is supported by our finding of a distribution of primary afferent fiber terminals consistent with the distribution described by others (Nadelhaft and Booth, 1984). According to those authors, pelvic nerve primary afferent fibers enter Lissauer's Tract in the spinal cord in the same segments (L6–S2) as those in which the preganglionic neurons are located that enter the pelvic nerve efferents. This supports our criterion for selecting, for quantitative HRP analysis, sections with SPN neurons showing maximal HRP label, since these sections also contain primary afferent fibers and terminals of the pelvic nerve.

We speculate that the marked quantitative increase in HRP reaction product in the pelvic nerve terminal fields in the daily repeated VCS group represents VCS-induced proliferation, i.e., sprouting, of pelvic nerve primary afferent terminals in the spinal cord, thereby mediating the ability of repeated daily VCS to increase its tail flick response-inhibiting potency.

A mechanism for this effect may be the release of neurotrophic substances into the dorsal horn of the spinal cord, inducing sprouting of intact pelvic nerve fibers into the denervated region. Sprouting occurs spontaneously within 2 weeks of trauma (Woolf et al., 1995); the present findings suggest that daily repeated VCS can markedly intensify this process. Several other mechanisms could play a role in functional recovery of the CNS in response to injury, e.g., a rearrangement of spared neuronal circuits, an upregulation of neuropeptides with neurotrophic abilities (Muir and Steeves, 1997), and/or an acceleration of transport of neurotrophic substances to the terminal fields, perhaps represented by HRP accelerated transport of HRP particles. VCS, which activates the pelvic nerve, releases VIP into the spinal cord (Komisaruk et al., 1989). Evidence that VIP is a neurotrophic factor is that it induces neurite growth *in vitro* and protects neurons from cell death (Brenneman and Eiden, 1986; Pincus et al., 1990; White and Mansfield, 1996). VIP increases neuronal survival indirectly, via the release of several neurotrophic substances from glial cells

(Brenneman et al., 1987, 1990). VIP exerts neurotrophic effects on developing neurons in the CNS (Brenneman and Eiden, 1986); dissociated cell cultures and whole embryo cultures showed a marked increase in growth markers in response to VIP treatment (Gressens et al., 1993); and VIP increases the survival of electrically blocked spinal neurons (Brenneman and Eiden, 1986; Brenneman et al., 1999). Also, blocking the action of VIP with neutralizing VIP antisera or with VIP receptor antagonists resulted in neuronal cell death for a subpopulation of CNS neurons in spinal cord, hippocampus, and cerebral cortex (Brenneman and Eiden, 1986; Gozes et al., 1991).

These findings led us to speculate that VCS, by releasing VIP into the spinal cord, could promote the recovery of spinal cord-mediated behavioral function for which evidence is presented in the present study.

Because the TFL test measures a motor response, from this test one cannot distinguish whether the inhibitory effect of VCS on the tail flick response is on the motor response only, nociception only, or both. However, there is substantial evidence that VCS does at least produce analgesia. That is, in rats VCS inhibits nociceptive responses in thalamic sensory neurons (Komisaruk and Wallman, 1977), and in women vaginal/cervical self-stimulation produces a marked elevation in pain thresholds (Komisaruk and Whipple, 1984; Whipple and Komisaruk, 1985). Those effects are analgesic, rather than anesthetic, because in both cases, responses to innocuous tactile stimulation were not affected by the VCS.

The present study focuses on the proliferation of primary afferent pelvic nerve terminals in the spinal cord in response to daily repeated VCS and the resulting increase in the analgesic effect of VCS. While this process involves an active inhibitory process within the spinal cord, there is evidence that a parallel supraspinal process is also activated by VCS. That is, in women with complete spinal cord injury at T10 or above, we previously showed that vaginal or cervical self-stimulation produces analgesia, evidenced by significant increases in pain thresholds to calibrated compressive force applied to the fingers (Komisaruk et al., 1997). Evidence that this analgesia is mediated by the vagus nerves is that by using functional MRI in these women, we found that the vaginal or cervical self-stimulation activated the terminal projection zone of the vagus nerves in the medulla oblongata, i.e., the nucleus of the solitary tract (Komisaruk et al., 2004). Thus, the vagus nerves (cranial nerve 10) provide a previously unrecognized genital sensory pathway directly to the brain, bypassing the spinal cord. And stimulation of this nerve via the vagina or cervix can produce analgesia.

On the basis of the present findings that daily repeated VCS can increase the analgesic action of VCS by proliferating pelvic nerve terminals in the spinal cord, it is tempting to speculate that the same stimulus could stimulate the proliferation of vagus nerve terminals above complete spinal cord injury at any level, for the terminal fields of genital afferents ascending to the brain via the spinal cord would also be vacated by the de-afferentation. An intriguing possibility is whether, via the vagus nerve afferents, in women with "complete" spinal cord injury at or above T10, vaginal or cervical self-stimulation could attenuate pain that is perceived above – and, via the vagus nerve pathway even *below* – the level of complete spinal cord injury, and whether repeated VCS in women could augment the attenuation of such pain.

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Plasticity in ascending long propriospinal and descending supraspinal pathways in chronic cervical spinal cord injured rats

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The high clinical relevance of models of incomplete cervical spinal cord injury (SCI) creates a need to address the spontaneous neuroplasticity that underlies changes in functional activity that occur over time after SCI. There is accumulating evidence supporting long projecting propriospinal neurons as suitable targets for therapeutic intervention after SCI, but focus has remained primarily oriented toward study of descending pathways. Long ascending axons from propriospinal neurons at lower thoracic and lumbar levels that form inter-enlargement pathways are involved in forelimb-hindlimb coordination during locomotion and are capable of modulating cervical motor output. We used non-invasive magnetic stimulation to assess how a unilateral cervical (C5) spinal contusion might affect transmission in intact, long ascending propriospinal pathways, and influence spinal cord plasticity. Our results show that transmission is facilitated in this pathway on the ipsilesional side as early as 1 week post-SCI. We also probed for descending magnetic motor evoked potentials (MMEPs) and found them absent or greatly reduced on the ipsilesional side as expected. The frequency-dependent depression (FDD) of the H-reflex recorded from the forelimb triceps brachii was bilaterally decreased although H_{max}/M_{max} was increased only on the ipsilesional side. Behaviorally, stepping recovered, but there were deficits in forelimb–hindlimb coordination as detected by BBB and CatWalk measures. Importantly, epicenter sparing correlated to the amplitude of the MMEPs and locomotor recovery but it was not significantly associated with the inter-enlargement or segmental H-reflex. In summary, our results indicate that complex plasticity occurs after a C5 hemicontusion injury, leading to differential changes in ascending vs. descending pathways, ipsi- vs. contralesional sides even though the lesion was unilateral as well as cervical vs. lumbar local spinal networks.

Keywords: spinal cord injury, propriospinal, supraspinal, inter-enlargement, motor-evoked potentials, magnetic stimulation, H-reflex

INTRODUCTION

As the majority of human injuries are incomplete and occur at the cervical level (www.sci-info-pages.com), experimental models of cervical SCI are highly clinically relevant. Functional deficits include impaired fore- and hindlimb function due to damage to the white matter that affects both descending and ascending systems, and to the gray matter containing the segmental circuitry for processing sensory input and generating motor output.

Long ascending propriospinal axons are likely candidates to be involved in coupling and coordination between the cervical and lumbar central pattern generators (CPGs; Miller et al., 1973a,b; English et al., 1985) as the rhythmicity in cervical segments can be driven by the lumbar CPG (Juvin et al., 2005). A subgroup of long propriospinal axons ascend in the ventrolateral funiculus (VLF) and projects to cervical lateral motoneuronal pools, giving them a direct influence on forelimb control (Sterling and Kuypers, 1968; Giovanelli and Kuypers, 1969). Propriospinal neurons have recently emerged as attractive targets for treatment after spinal cord injury (SCI) as regeneration over long distances is very limited. They have the potential to form new intraspinal circuits (Bareyre et al., 2004; Courtine et al., 2008) and regenerate (Fenrich and Rose, 2009) after SCI, giving these pathways a high potential of success if integrated in a comprehensive combinatory treatment such as cellular transplant and neurotrophin delivery (Jordan and Schmidt, 2002; Conta and Stelzner, 2004; Fouad et al., 2005).

Because the lesion characteristics evolve over an extended period of time after the initial spinal injury, non-invasive non-noxious methods are warranted to address the potential effect of injury/treatments but not many electrophysiological outcome measures of this nature are available or clinically relevant. Previous studies showed that magnetic inter-enlargement responses (MIER) and magnetic motor-evoked potentials (MMEPs) are reproducible, non-invasive and represent an objective assessment of axonal conduction and functional integrity in the ascending intersegmental lateral funiculus and descending VLF, respectively (Loy et al., 2002; Beaumont et al., 2006). This study characterizes plasticity occurring in the intact ascending propriospinal pathways after an incomplete cervical injury and describes concomitant changes in the function of

descending supraspinal pathways as well as cervical and lumbar spinal local circuits.

MATERIALS AND METHODS

All procedures were performed in accordance with protocols approved by Drexel University College of Medicine Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines for the care and use of laboratory animals.

SURGICAL PROCEDURES AND POST-OPERATIVE CARE

A total of 38 adult female Sprague-Dawley rats (225–250 g, Charles River) were used for this study. **Table 1** summarizes the condition and testing for each group of animals. A cervical hemicontusion injury was performed on thirty-one animals. Animals were anesthetized with a mixture of ketamine (60 mg/kg), xylazine (6 mg/kg), and acepromazine (6 mg/kg) and spinal cord injuries performed as previously described (Sandrow et al., 2008; Sandrow-Feinberg et al., 2009). Briefly, a unilateral cervical laminectomy (C5) was performed and a moderate unilateral contusion injury was created by an impact force of 200 Kdyne with tissue displacement to a depth of 1600–1800 μm using the Infinite Horizon Impact Device (Precision Systems and Instrumentation, Lexington, KY). Upon completion of surgery, overlying muscles were sutured and the skin incision closed with wound clips. Animals were given dextrose in saline (5 mL, s.c.), buprenorphine (0.05 mg/kg, i.m.) for 3 days as an analgesic, and ampicillin (100 mg/kg, s.c.) for 7 days to prevent infection. Bladders were expressed manually as needed.

BEHAVIORAL ASSESSMENTS

After acclimation to the testing apparatus over a 2 week period, baseline scores were established for each animal. Due to the unilateral nature of our injury model, we evaluated the ipsilesional (right) and contralesional (left) limbs separately. Right and left hindlimb and forelimb scores were recorded preoperatively and weekly thereafter for 6 weeks.

Open field locomotion

Forelimb and hindlimb function was evaluated using the Forelimb Locomotor Scale (FLS; Sandrow et al., 2008) and the Basso, Beattie, Bresnahan Locomotor Rating Scale (BBB; Basso et al., 1995), respectively. The FLS is an 18-point scale that ranks ipsilesional forelimb locomotion after unilateral cervical SCI based on range of motion, degree of weight support, and paw placement. The FLS scale is not sensitive enough to identify compensation on the contralesional forelimb, thus, only data for the ipsilesional forelimb is included. The BBB is a 22-point scale which ranks hindlimb locomotion after SCI from complete paralysis (0) to normal and coordinated movement (21).

CatWalk locomotion

Interlimb coordination was assessed preoperatively and at 6 weeks after SCI on the CatWalk using methods described previously by Kloos et al. (2005). Six passes of runway locomotion for each testing session were processed and analyzed for footfall patterns. Three aspects of coordination were quantified: the patterns of limb placement, the consistency by which the rat was able to implement this pattern and the ratio of limb placements by calculating the mean phase dispersion, the mean standard deviation of the phase dispersion, and the incidence of mismatches, respectively, across trials for diagonal (Ipsilesional Forelimb-Contralesional Hindlimb (IF-CH; CF-IH), girdle (CF-IF; CH-IH), and ipsilateral (IF-IH; CF-CH) pairings. To quantify the pattern of interlimb coordination, we measured the synchrony or phase dispersion of initial contact between different limbs. Phase dispersion is defined as the degree of synchrony between two limbs during locomotion. For limb pairings that are "in phase" (i.e., RF-LH or LF-RH), initial contact of stance occurs at the same time for the limb pairing and yield a phase dispersion of 0%. For limb pairs that alternate (i.e., ipsilateral RF-RH or girdle LF-RF), typically yield a phase dispersion of 50% and appear out of phase, as one limb of the pairing is in stance while the other limb is in swing. Importantly, when the initial contact of one limb follows the diagonal limb, the phase dispersion value is expressed as positive. Conversely, when the initial contact of one limb precedes that of the diagonal limb, the phase dispersion value is reported as negative. During locomotion of SCI animals, the degree of synchrony between limb pairings is compromised. When the phase dispersion or timing of limb pairings drifts beyond 75%, a mismatch occurs. The number of mismatches of diagonal pairs is summed for each pass and the percentage of mismatches to the total number of step cycles was determined.

ELECTROPHYSIOLOGICAL ASSESSMENTS USING MAGNETIC STIMULATION: INTER-ENLARGEMENT RESPONSES AND MOTOR-EVOKED POTENTIALS

Magnetic inter-enlargement responses (MIERs) were measured to assess conductivity in propriospinal ascending pathways between the lumbar and cervical enlargement (Beaumont et al., 2006; Cao et al., 2010). Magnetic motor-evoked potentials (MMEPs) were measured to evaluate impairment of transmission in descending

Table 1 | Number of animals in behavioral, electrophysiological, and histological section of the study.

Group	*n* =	Behavior	White matter sparing	MMEP	MIER (magnetic)	MIER (electric)	H-reflex
Hemicontusion	11	X	X	X	X		
	15	X	X	X	X		X
	5*				X	X	
Intact	5	X	X				X
	2	X			X	X	

** This group had a survival time of 2 weeks.*

motor pathways using methods described elsewhere (Magnuson et al., 1999; Loy et al., 2002). Magnetic stimulation was performed in awake, non-sedated animals positioned on a wooden board. Animals were restrained using a piece of stockinet pinned to the board around the animal. The four limbs remained accessible for electrode placement. EMGs were recorded via 27-gauge stainless steel needle electrodes (Cadwell Laboratories, Kennewick, WA) inserted into either the forelimb triceps brachii (MIER) or hindlimb gastrocnemius muscle (MMEPs). The active electrode was placed in the muscle belly whereas the reference electrode was placed near the distal tendon of the muscle. The ground electrode was placed subcutaneously in the neck area (MIER) or at the base of the tail (MMEP). Magnetic stimulation was administered using a Magstim 200^2 stimulator (The Magstim Company, Wales, UK) delivering monophasic stimuli (100 μs approximate rise time, 1 ms duration). **Figure 1** illustrates the stimulus and recording configuration. MIERs were elicited via a double 25 mm coil (figure of eight) positioned at the hip to stimulate the sciatic nerve. MMEPs were evoked by transcranial magnetic stimulation delivered via a 50 mm circular coil held over the rat cranium to elicit EMGs in distal musculature. The 25 mm coil generates 4.6 T magnetic field whereas the circular 50 mm coil generates 3.6 T (www.magstim.com). The stimulation intensity is expressed as the percent of absolute maximal output of the stimulator. In each case, stimulation was elicited with a series of increasing intensities (40, 60, 80, 100%). To determine the motor threshold (MT), the stimulus intensity was decreased by intervals until no response was evoked. The MT was defined as the stimulation intensity at which a response was evoked at least 50% of the time. Each assessment included the determination of the MT followed by recordings at 60 and 80%. A minimum of five responses were recorded at each of two intensity levels and averaged.

To confirm the MIER results, electrical stimulation of the sciatic was performed in five contused animals 2 weeks after SCI and also in three normal animals. This time point was chosen because our results showed that MIER responses were already modulated at 1 week post-SCI. A cuff electrode was surgically implanted on the sciatic nerve and the animal recovered from isoflurane anesthesia. Recordings similar to those described above were performed in the awake animal "suspended" on a vinyl cloth platform with the hindlimbs hanging free beneath the animal. Single bipolar pulses of 500 μs total duration were applied with intensities of 1 mA.

EMG signal was amplified (×100–1000, A-M Systems), bandpass filtered (10–300 to 5000 Hz), digitized and fed to a custom software (Labview, National Instruments). Averages were computed and peak-to-peak amplitude and onset latency (initial deflection from baseline) were measured for each animal, at each time point and intensity of stimulation.

H-REFLEX RECORDINGS AND ANALYSIS

Eight weeks after SCI, a terminal experiment was performed to assess the frequency-dependent depression (FDD) of the H-reflex in all 4 limbs of 14 of the contusion injured animals and 5 normal

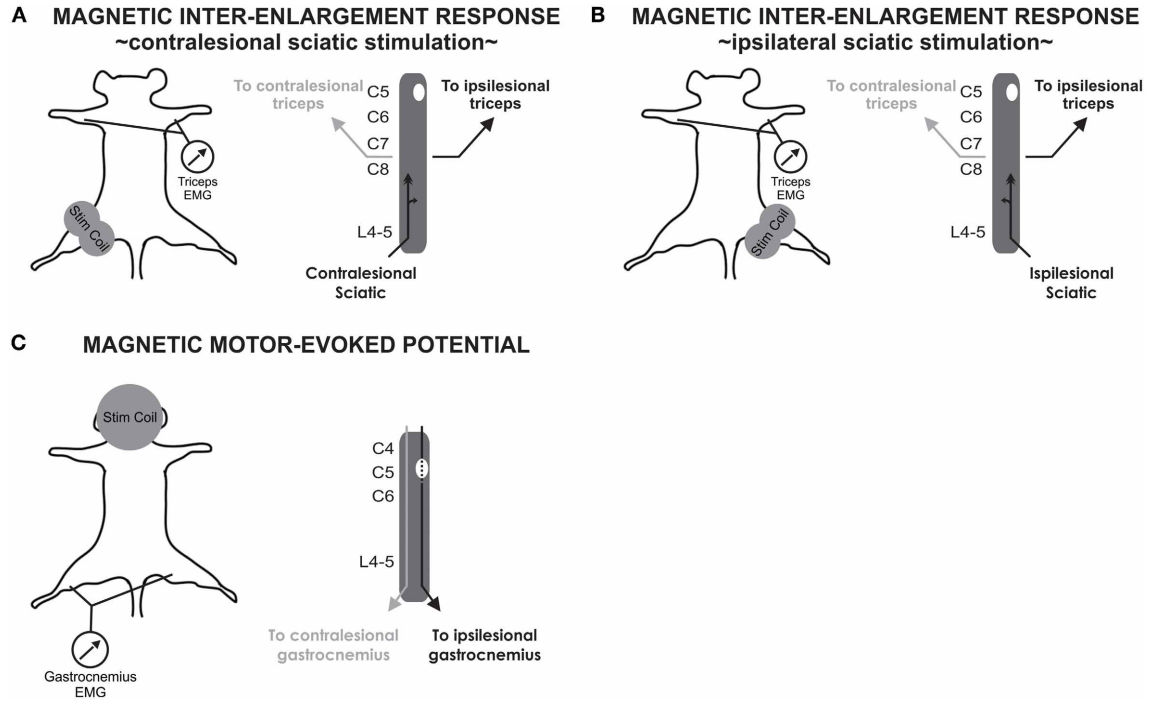

FIGURE 1 | Experimental procedure for stimulation and recordings of MIER and MMEP. (A) MIERs are evoked with a figure of eight coil positioned at the hip to stimulate the contralesional sciatic nerve. Recordings are performed in the triceps brachii bilaterally. **(B)** MIER recordings were additionally performed with the coil positioned on the ipsilesional hip. **(C)** MMEPs are evoked with a circular coil positioned over the cranium while recording in the gastrocnemius muscle bilaterally.

animals. Rats were anaesthetized with a mixture of ketamine (60 mg/kg), xylazine (10 mg/kg) and acepromazine (6 mg/kg). H-reflex was recorded as previously reported for the hindlimbs (Côté et al., 2011) and forelimbs (Hosoido et al., 2009). Briefly, the tibial (hindlimbs) or ulnar nerve (forelimbs) was isolated, dissected free and mounted on a bipolar hook electrode for stimulation. Skin flaps were used to form a pool of mineral oil to prevent dessication of the nerves throughout the recording period. EMG was recorded using bipolar wire electrodes (Cooner Wire, Chatsworth, CA) inserted in the hindlimb or forelimb interosseus muscles. H-reflexes were evoked via an isolated pulse stimulator (A-M Systems, Carlsborg, WA) delivering single bipolar pulses (100 μs each phase) to the tibial or ulnar nerve. Stimuli of increasing intensities were used to determine the MT, the H-reflex threshold and to determine the maximal response amplitude for both M and H-wave (M_{max} and H_{max}). The stimulation intensity which elicited H_{max} response (∼1.2 MT) was then used for 3 series of 17 consecutive stimulation pulses delivered at 0.3 Hz, 5 Hz or 10 Hz. The 0.3 Hz series was then replicated. The trial was discarded if the M-wave amplitude was not within 95% of the initial 0.3 Hz control series. EMG recordings were amplified (×1000, A-M Systems) and bandpass filtered (10-5k Hz). Signal was digitized (10 kHz) and fed to custom software.

Response latency (onset of response) and peak-to-peak amplitude were measured for the H and M responses evoked by single pulses. The recruitment curve was plotted by expressing the amplitude of the H and M responses as a function of stimulus intensity. The H_{max}/M_{max} ratio, which is believed to give an estimate of motoneuronal excitability, was calculated to assess the relative proportion of motoneurons recruited through the monosynaptic reflex loop as compared to the activation of the entire motor pool. The H_{max}/M ratio was also calculated to estimate the relative activation of the motor pool required to reach maximal reflex amplitude. For the analysis of FDD, the first five responses to a train of stimulation were discarded to allow reflex stabilization and the last twelve responses were averaged for every stimulation frequency (0.3, 5, and 10 Hz). H-reflex amplitude was normalized to M_{max} and the change in H-reflex response at 5 Hz and 10 Hz was calculated as a percentage of the response measured at 0.3 Hz (control).

LESION ANALYSIS

Rats were given an overdose of Euthasol (390 mg/kg sodium pentobarbital, 50 mg/kg phenytoin, ip.) and perfused transcardially with 4% paraformaldehyde. Cervical spinal cord between C4 and C6 was removed, post-fixed in paraformaldehyde at 4°C overnight and submersed in 30% sucrose for cryoprotection. Transverse serial sections throughout the rostral to caudal extent of the lesion 250 μm apart were mounted on glass slides, air-dried and stained for cresyl violet (Sigma) for Nissl and euriochrome cyanine (Sigma) for myelin. Sections were cover slipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

To determine the amount of spared tissue, the contralesional gray and white matter of the spinal cord and spared gray and white matter on the ipsilesional side were measured separately in 10 sections (250 mm apart) spanning the rostrocaudal extent of the lesion using the Cavalieri estimator method (Stereo Investigator, MicroBrightfield, Burlington, VT). To quantify the degree of spared tissue at the lesion epicenter, we figured the proportion of the spared tissue area on the ipsilesional hemisphere to the spared tissue area on the contralesional (uninjured) spinal cord (Sandrow-Feinberg et al., 2009). To further delineate regions within the white matter that are thought to be responsible for eliciting MMEP or MIER responses (Magnuson et al., 1999; Loy et al., 2002), we used a pie-shaped overlay that had twelve equal wedges. The center of the overlay was aligned with the central canal (see schematic in **Figure 6**. To determine the effects of sparing on MIER, the proportion of spared tissue for wedges 3 and 4 was calculated and the data were partitioned into groups based on the amplitude of their MIER (high amplitude >9.7 mV or low amplitude. The effect of sparing on MMEP was determined by calculating the proportion of spared tissue for wedge 5 only. For these analyses, animals were grouped based on the presence or absence of an ipsilesional MMEP. Correlational analysis determined whether a relationship exists between the amplitude of the MIER or MMEP response to the proportion of spared tissue in these specified regions of the lesion epicenter.

STATISTICAL ANALYSIS

One-Way ANOVA followed by Holm–Sidak *post-hoc* test were used to determine significant differences across groups for all data unless stated below. If the sample variables did not fit a normal distribution or were not equally variant, a One-Way ANOVA on ranks followed by Dunn's *post-hoc* test was performed. All data are reported as mean ± SEM. Statistical analysis was performed using Sigma Plot software 11.0 and PASW Statistics 18. For all statistical tests, the significance level was set to $p < 0.05$. A Two-Way ANOVA followed by Holm–Sidak *post-hoc* test was used to assess whether stimulation frequency and treatment group had a significant effect on the amplitude of the H-reflex and to evaluate if the interaction of these factors affected the variable. Correlation analysis using Spearman's Rank Correlation was used to determine a relationship between the amplitude of the MMEP or MIER responses to spared tissue at the lesion epicenter.

RESULTS

ANATOMICAL AND BEHAVIORAL MEASURES

Nissl-myelin staining revealed that contusive SCI produced a core lesion limited to one side of the spinal cord (mean lesion area of $1.52 \pm 0.09\ mm^2$) that nearly eliminated all gray matter and spared a thin rim of myelinated tissue in the lateral and ventral funiculi ($1.09 \pm 0.10\ mm^2$; **Figure 2A**). Contiguous myelin staining and nuclear labeling in the contralesional spinal cord indicated that the ascending and descending spinal cord tracts and gray matter remain normal ($3.44 \pm 0.13\ mm^2$). On average less than one-third of the ipsilesional spinal cord was spared at the lesion epicenter of contused rats.

The ability of SCI animals to utilize their ipsilesional forelimb in the open field was assessed using the FLS (**Figure 2B**). Two days after SCI, animals exhibited extensive movement of one forelimb joint or slight movement of two forelimb joints (FLS score <3; $p < 0.01$ vs. baseline). By four weeks after SCI, animals exhibited continuous plantar stepping of the ipsilesional forepaw

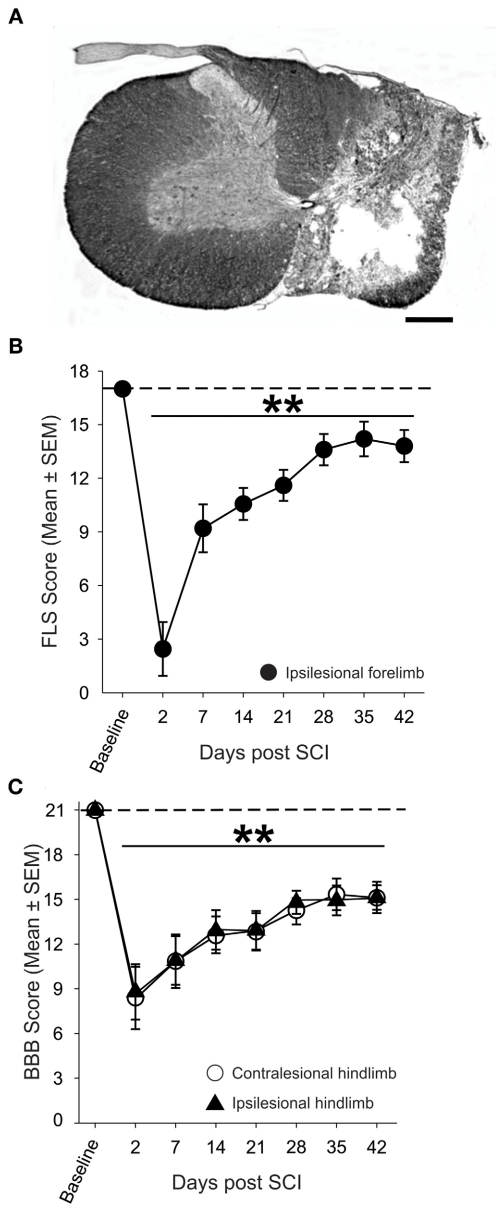

FIGURE 2 | Anatomical and behavioral evaluation of unilateral spinal cord contusion. (A) Representative transverse section of the lesion epicenter stained for Nissl and myelin. 200 k dyne impact produced a unilateral lesion with a spared rim of lateral white matter on the right side of the spinal cord, while the gray and white matter of the contralesional spinal cord appears normal (scale bar = 0.5 mm). **(B)** Assessment of the ipsilesional forelimb with the Forelimb Locomotor Scale (FLS) revealed significant deficits in ipsilesional forelimb function acutely after SCI that partially recovers over time. **(C)** Unilateral SCI produced significant, bilateral deficits in hindlimb locomotor function in the open field as assessed by the Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale.

with parallel paw position and occasional toe clearance (FLS score =14; $p < 0.01$ vs. baseline). Assessment of the hindlimbs using the BBB scale revealed that unilateral cervical contusion caused bilateral deficits in hindlimb locomotion that persisted for the duration of the study (**Figure 2C**). At one week after SCI, all animals were able to frequently or consistently plantar step with both hindlimbs, but they were unable to coordinate forelimb and hindlimb movements (BBB score = 11) at this early post-injury interval. Spontaneous locomotor recovery reached a plateau by four weeks after SCI but deficits in trunk stability, paw placement and toe clearance persisted in all animals. Importantly, just over 80% of animals demonstrated consistent FL-HL coordination at 4 weeks after injury.

TRANSMISSION IN ASCENDING INTER-ENLARGEMENT PATHWAYS IS ENHANCED ON THE IPSILESIONAL SIDE AFTER INCOMPLETE SCI

Conductivity and connectivity of ascending spared/reorganized fibers after cervical hemicontusion injury were estimated using MIER. The MIER was evoked by stimulating magnetically the ipsi- or contralesional sciatic nerve and recording from the triceps brachii bilaterally in awake animals. We found out that maximal response was reached at 60% of the maximum output of the stimulation unit. To avoid possible occlusion of the response, all results below were obtained at 60% of the stimulator output. **Figure 3A** displays representative recordings of MIER at baseline, 1 and 6 weeks after contusion injury. The latency, the time it takes for the magnetic impulse to travel from the stimulation site to the recording electrode, i.e., from the stimulus onset to the onset of evoked muscle potential, was modulated after SCI. Before injury, the latency of the response in the triceps was significantly shorter on the ipsilesional side if the ipsilesional sciatic was stimulated. Similarly, the response was shorter on the contralesional triceps when the contralesional sciatic was stimulated (**Table 2**). After hemicontusion, the MIER latency on the contralesional side was delayed compared to the ipsilesional side whether the stimulation was evoked from either the ipsilesional or contralesional sciatic (**Table 2**). In addition, MIER amplitude was significantly larger in the ipsilesional triceps when compared to contralesional triceps at 1 and 6 weeks and also was larger than baseline (**Figure 3B**). We confirmed that signals could not be transmitted through the injury by attempting to record EMGs from the masseter muscle (*data not shown*). The MT for activation of the ipsilesional and contralesional triceps was ∼38% of the stimulator output before injury. Six weeks after injury, the threshold to evoke a response in the contralesional triceps was increased to 48% when the ipsilesional sciatic was stimulated and to 43% when the contralesional sciatic was stimulated. The MT for the ipsilesional triceps was not significantly changed after SCI (**Table 2**).

The ascending signal between the lumbar and cervical enlargement appeared to be enhanced after injury on the side of the lesion. To rule out any unwanted effect of magnetic stimulation, we further investigated transmission in this pathway using electrical stimulation in a subset of animals. Cuff electrodes were implanted bilaterally on the sciatic nerve for stimulation and recordings were performed as described using needle electrodes. Magnetic and electrical stimulation yielded similar results, i.e., the amplitude of the response was much larger in the ipsilesional as compared to the contralesional triceps no matter if the contralesional or ipsilesional sciatic was stimulated (**Figure 3C**). Before injury, the MIER amplitude response in either triceps was comparable. The amplitude of the MIER in the ipsilesional triceps

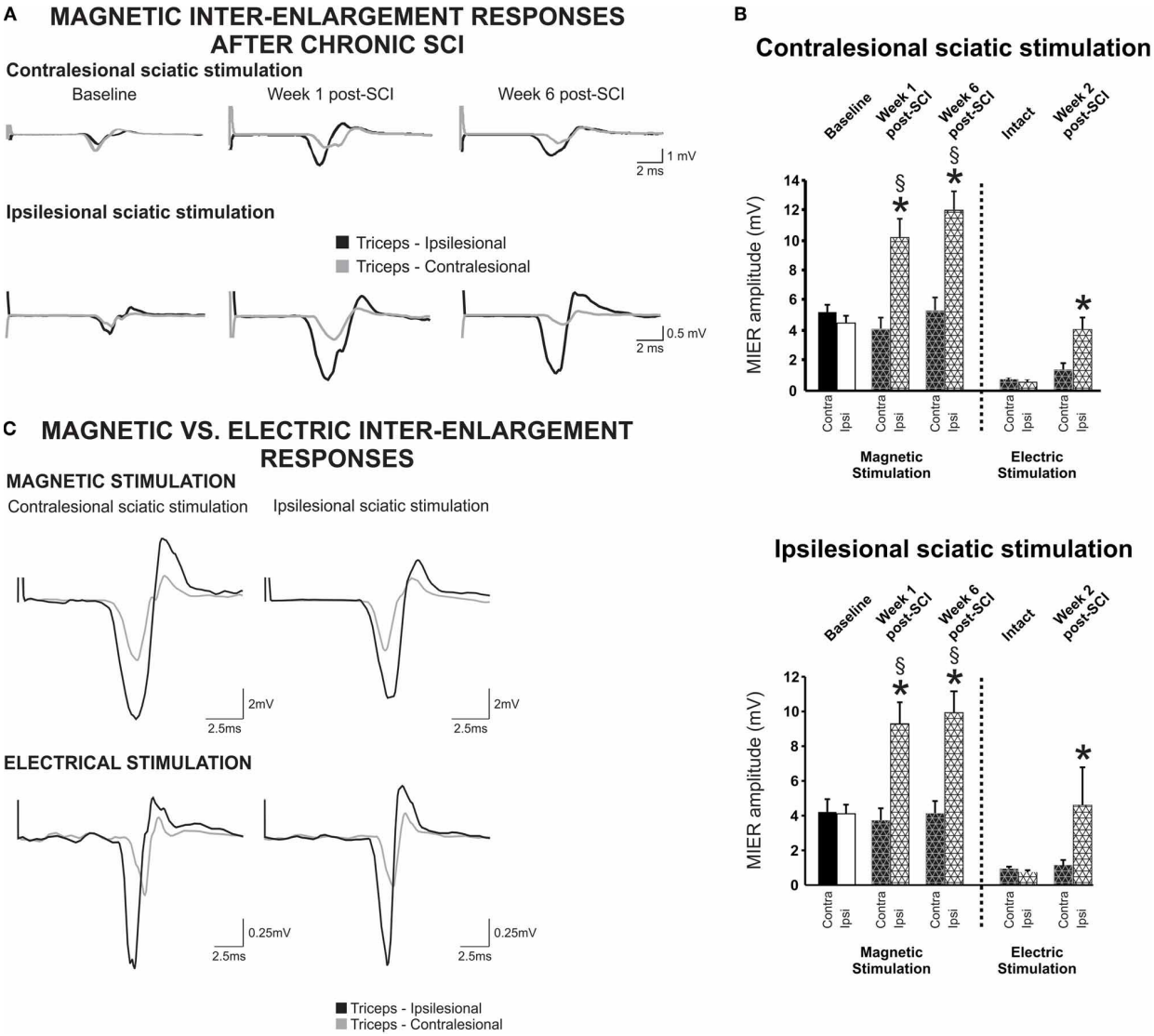

FIGURE 3 | Inter-enlargement responses are enhanced on the ipsilateral side of the lesion after a cervical hemicontusion injury. **(A)** Inter-enlargement responses are recorded in the ipsilesional and contralesional triceps muscles following magnetic stimulation of the ipsilesional or contralesional sciatic. Representative traces recorded from a single animal illustrate that the response in the ipsilesional triceps (black) is larger than in the contralesional triceps (gray) after SCI. The MIER is also delayed, i.e., the latency in the contralesional triceps is significantly longer than in the ispilesional side whether the stimulation is evoked from the ispilesional or contralesional sciatic. **(B)** Regardless of ipsi- or contralesional stimulation, the amplitude of the magnetically and electrically-evoked inter-enlargement response in the ipsilesional triceps is significantly increased as compared to baseline and as compared to the contralesional triceps. **(C)** Inter-enlargement responses recorded from the same animal with magnetic (top panel) or electrical (bottom panel) stimulation of the contralesional or ipsilesional sciatic nerve. Note that the magnetic and electric stimulation elicit similar responses in the ipsilesional and contralesional triceps. EMG responses are larger in the ipsilesional triceps than in the contralesional triceps. In addition, the response in the contralesional triceps is delayed as compared to baseline or ispilesional triceps.

was increased after SCI by ∼2.5 fold whether we used magnetic or electrical stimulation (**Figure 3B**). The amplitude of MIER did not correlate with recovery of locomotion.

TRANSMISSION IN DESCENDING PATHWAYS IS IMPAIRED ON THE IPSILESIONAL SIDE AFTER INCOMPLETE SCI

We assessed conductivity and connectivity of descending spared fibers after hemicontusion injury over 6 weeks post injury in awake animals using transcranial magnetic stimulation to elicit MMEPs in the right and left gastrocnemius (GS) muscles. **Figure 4A** illustrates averaged traces recorded with a stimulus delivered at 60% of the maximal output of the stimulator for both hemicontused animals. We found no difference in response latency in either GS before injury or between the contralesional GS at 1 or 6 weeks vs. baseline (**Table 2**). However, MMEP amplitude was significantly decreased in the contralesional GS at 1 week post-SCI and 6 weeks post-SCI as compared to baseline (**Figure 4B**). Also, the response in the ipsilesional

Table 2 | Magnetic inter-enlargement responses and motor-evoked potentials latency and motor threshold 1 week and 6 weeks after SCI.

			Baseline		Hemicontusion			
					Week 1		Week 6	
			Contra	Ipsi	Contra	Ipsi	Contra	Ipsi
Stimulation	**MIER contralesional sciatic stimulation**	**Latency (ms)**						
		Mean	5.83	**6.17***	6.12	**5.53*§**	6.04	**5.57*§**
		SEM	0.09	0.12	0.20	0.15	0.19	0.13
		Motor threshold						
		Mean	38%	38%	**42%***	33%	**43%***	35%
		SEM	2%	2%	4%	2%	2%	2%
	MIER ipsilesional sciatic stimulation	**Latency (ms)**						
		Mean	6.50	**6.24***	5.82	**5.53*§**	6.07	**5.61*§**
		SEM	0.14	0.13	0.16	0.15	0.15	0.14
		Motor threshold						
		Mean	37%	38%	**42%*§**	37%	**48%*§**	37%
		SEM	3%	2%	3%	4%	3%	2%
	MMEP	**Latency (ms)**						
		Mean	6.04	6.04	6.27	**6.73§**	6.09	**7.23§**
		SEM	0.06	0.07	0.10	0.38	0.11	0.30
		Motor threshold						
		Mean	31%	31%	31%	35%	31%	**43%*§**
		SEM	1%	1%	2%	4%	2%	4%

Contra, contralesional; Ipsi, ipsilesional. NA indicates no response. § *indicates a significant difference vs. baseline and *a significant difference vs. contralesional.*

GS remained delayed (∼1.2 ms) and of significantly smaller amplitude compared to both baseline and contralesional GS at matched times at least up to 6 weeks post-SCI. To validate these results, we confirmed that animals with C5 lateral hemisection and complete disruption of descending pathways on the ipsilesional side exhibited no MMEP in the ipsilesional GS (*data not shown*).

The MT, i.e., the lowest stimulation intensity that evoked a response at least 50% of the time, was also measured at baseline and after SCI. As reported by others, all animals exhibited responses at 40% stimulation level, a majority at 30%, and a few at 20% (Linden et al., 1999). Six weeks after injury, 10 animals failed to display MMEPs in the ipsilesional GS even at 100% of the stimulator output. For those animals who presented MMEPs, they arose with stimulus intensity between 50 and 60%. Six weeks after hemicontusion injury, activation threshold was significantly increased by 12% in the ipsilesional GS but was not changed at 1 week post-SCI (**Table 2**). Importantly, the amplitude of the ipsilesional MMEP response positively correlated to the ipsilesional hindlimb BBB score ($\rho = 0.462$; $p = 0.03$). Animals with MMEPs in the ipsilesional GS exhibited better recovery of locomotion in the open field compared to those with no ipsilesional MMEP (**Figure 4C**).

THE FREQUENCY-DEPENDENT DEPRESSION OF THE H-REFLEX AFTER UNILATERAL SCI IS IMPAIRED IN BOTH FORELIMBS BUT NOT IN THE HINDLIMBS

Stimulation of the tibial nerve evoked two consecutive muscle responses, the M and the H waves (**Figure 5A**). H-reflex gain and threshold were determined from recruitment curves generated with stimuli of increasing amplitude. No significant difference was observed in the electrical threshold to evoke a motor response (M-wave) after injury (*data not shown*). Similarly, the threshold for H-reflex initiation (H-wave) was unchanged in the hindlimbs but it was increased in the ipsilesional side (2.62 ± 1.19MT) as compared to the contralesional side (1.16 ± 0.25MT) or normal forelimbs (1.16 ± 0.14MT). Eight weeks after injury, there was an increase in H_{max}/M_{max} ratio in the ipsilesional forelimb (0.67 ± 0.13) but not in the contralesional forelimb (0.40 ± 0.09) as compared to non injured animals (0.26 ± 0.09, **Figures 5B**,**C**). The same ratio was not significantly different in the hindlimbs 8 weeks SCI.

Averaged H-reflex recordings evoked by train of stimulation at 0.3 Hz (black), 5 Hz (gray) and 10 Hz (dotted) in the ispi- and contralesional forelimb and hindlimbs from hemicontused animals are illustrated in **Figure 5A**. These recordings demonstrate that increasing the frequency of stimulation leads to a substantial decrease in H-reflex amplitude in the forelimbs and hindlimbs of normal animals. As a group, hemicontused animals displayed a significant modulation of the H-reflex in the hindlimbs with increasing stimulus frequency with depression values similar to normal animals. The greater the depression, the lower the value is on the *y axis*. Although FDD of the H-reflex was not impaired in the hindlimbs, it was significantly impaired in both the ipsilesional and contralesional forelimbs at 5 Hz and 10 Hz as compared to the normal group. A Two-Way ANOVA revealed statistically significant differences across stimulation frequency, across experimental groups, and

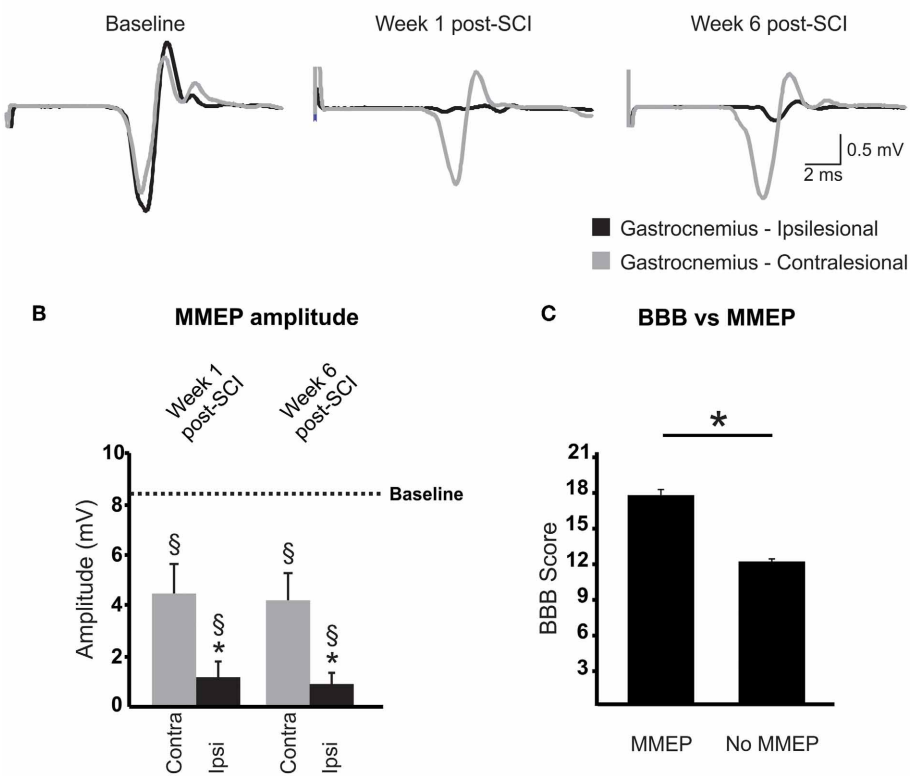

FIGURE 4 | Magnetic motor-evoked potential (MMEP) partly recovers after a cervical hemicontusion injury. (A) MMEPs recorded from the contralesional (gray) and ipsilesional (black) gastrocnemius (GS) muscles disappear on the ipsilesional side after SCI and partly recovers over time. MMEPs are delayed and of smaller amplitude **(B)** in the ipsilesional GS (black) as compared to baseline (dotted line) or the contralesional GS at matched times (gray). **(C)** Animals with no MMEP in the ipsilesional GS exhibited a lower extent of locomotor recovery in the open field.

an interaction between frequency and groups. *Post-hoc* comparisons showed that 5 Hz and 10 Hz depression values were different than 0.3 Hz in all limbs and groups and that hemicontused forelimb values were different than normal animals both at 5 Hz and 10 Hz. At 10 Hz, there was 34% less depression in the contralesional and 26% in the ipsilesional forelimb. These results suggest that the excitability of cervical motor pools, that are located several spinal segments below the lesion, is affected in a bilateral manner.

CONTRIBUTIONS OF SPARED WHITE MATTER TO MMEP AND MIER RESPONSES

A previous report by Loy et al. (2002) determined that the ventrolateral white matter of the spinal cord is necessary to elicit an MMEP response in the GS muscle. In order to limit the analysis of white matter sparing to the ventrolateral spinal cord, we determined the amount of sparing in the gray "ventrolateral" wedge depicted in **Figure 6A**. This analysis of the lesion site revealed that the presence of an ipsilesional MMEP was directly related to the amount of sparing in the ventrolateral white matter of the spinal cord (**Figure 6A**). Regression and correlational analysis using non-parametric Spearman's rank correlation test showed that animals with no ipsilesional MMEP had significantly less spared tissue within the ventrolateral white matter than those demonstrating bilateral MMEPs (**Figure 6A**; Spearman's $\rho = 0.544$; $p < 0.009$; $r^2 = 0.35$).

We examined the dorsolateral and lateral spinal cord responsible for eliciting a MIER response in a similar manner (Beaumont et al., 2006; **Figure 6B**). By partitioning the animals into two groups with low or high amplitude responses and comparing the proportion of spared tissue in the dorsolateral and lateral wedge (**Figure 6B**) we saw no effect of sparing on the amplitude of the MIER response (**Figure 6B**). Likewise, regression and correlational analysis using Spearman's rank test showed no significant relationship between MIER amplitude and the amount of tissue sparing at the epicenter, indicating that the fibers responsible for transmitting the MIER signal are located in the lateral-most edge of the dorsolateral white matter (**Figure 6B**; Spearman's $\rho = 0.0.007$; $p < 0.097$; $r^2 = 0.009$).

Interlimb coordination is a complex skill based on the ability to consistently implement a predominant, rhythmic pattern with little variability. Animals with no MMEP response in the

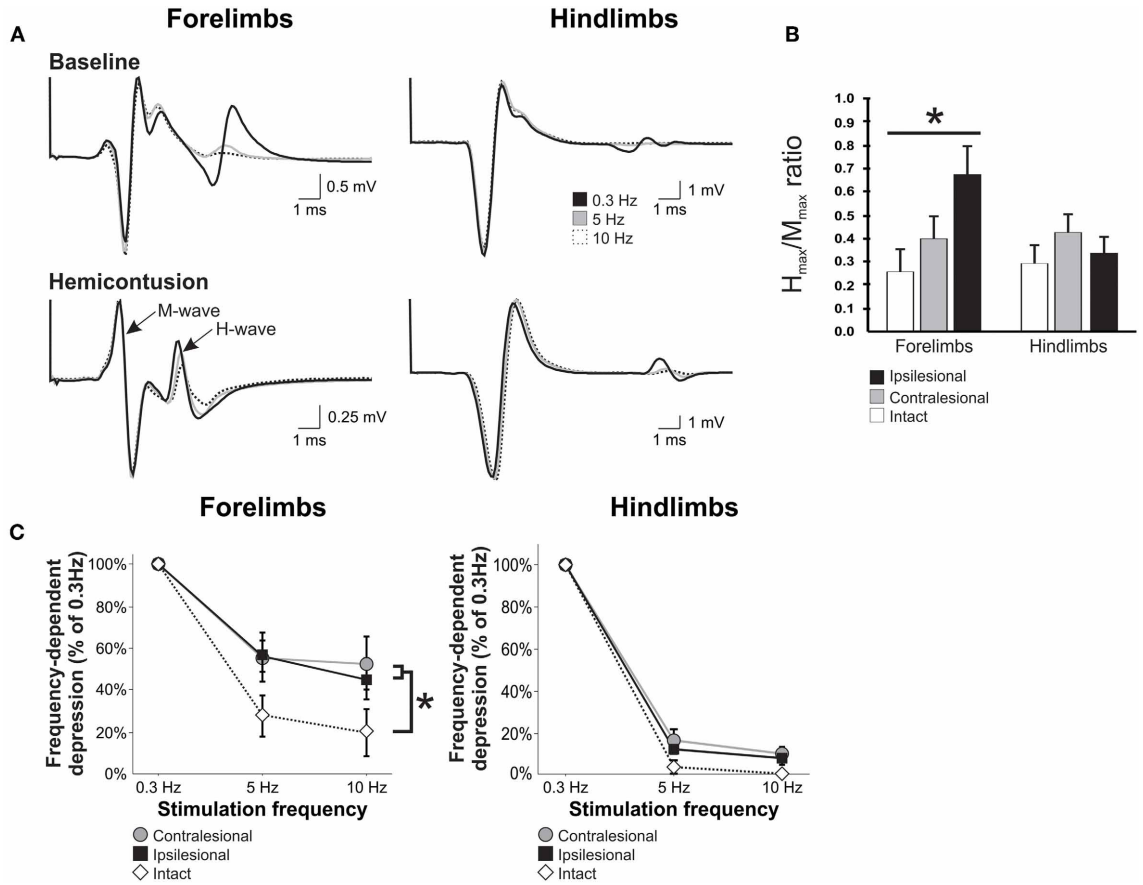

FIGURE 5 | Unilateral cervical SCI affects H-reflex modulation bilaterally in the forelimbs, but not in the hindlimbs. H-reflex traces were evoked by the stimulation of the tibial or ulnar nerve and recorded from the interosseus muscle of ipsilesional and contralesional hindlimb and forelimb. **(A)** Representative average of H-reflex recordings following a train of stimulation at 0.3 Hz (black), 5 Hz (gray), and 10 Hz (white) in normal and SCI animals. There was a decrease in the average amplitude of the H-reflex with increasing stimulus frequency in the hindlimbs and forelimbs of a normal animal and also in the hindlimbs of an SCI animal. However, this decrease was modest in the impaired forelimb of the hemicontused animal. **(B)** H_{max}/M_{max} ratio is increased in the ipsilesional forelimb after SCI but not in the contralesional forelimb or the hindlimbs. **(C)** Overall, the frequency-dependent depression of the H-reflex displayed in the hindlimbs of SCI animals is similar to normal animals 8 weeks after injury. However, the depression is impaired in both forelimbs as compared to normal.

ipsilesional GS muscle exhibited deficits in forelimb–hindlimb (FL–HL) coordination. In the open field, all animals with bilateral MMEPs were able to execute FL–HL coordination more than 95% of the time (BBB = 14), while animals with no ipsilesional MMEP exhibited FL–HL coordination <50% of the time. In order to examine FL–HL coordination in greater detail, we created footfall diagrams of CatWalk passes to allow us to determine how all four limbs move relative to each other (**Figures 7A–C**). Solid lines represent the time a limb is in stance and open spaces represent time when the limb is in the swing phase of locomotion. In normal animals, the ipsilesional forelimb and contralesional hindlimb move through stance and swing phase in concert (**Figure 7A**). The footfall pattern changes with SCI (**Figures 7B,C**). The ability to abruptly transition from one diagonal pairing to the other is not retained with injury, as depicted by instances of three or more limbs in stance at one time. Furthermore, quantification of these changes in the transition between diagonal pairings by determining the mean phase dispersion 6 weeks after SCI revealed that animals without ipsilesional MMEPs exhibited a significant deficit in their ability to implement a single coordinative strategy during bouts of runway locomotion. The deficits in coordination were associated with FL–HL limb pairing rather than those across the shoulder or pelvic girdles.

Figure 7C shows a histogram of the phase dispersion for both diagonal limb pairings in normal animals. For each diagonal pairing (IF-CH or CF-IH) the FL and HL are in phase with each other (i.e., the FL and HL initiate initial contact at the same time), with a mean phase dispersion at 4.36%. Normally, phase dispersion values for diagonal limb pairings are tightly distributed between –20% and 20%, and the standard deviation is small (7.65 + 0.62%) (**Figure 7D**). Regardless of the presence of ipsilesional MMEP, SCI animals demonstrated better synchrony of the IF-CL compared to the CF-IH pairing. Animals with bilateral MMEPs demonstrated tight synchrony between the IF and CH (**Figure 7E**, mean phase dispersion: 0.72 + 1.26%) that was not different than baseline. Animals with no ipsilesional MMEP produced a positive

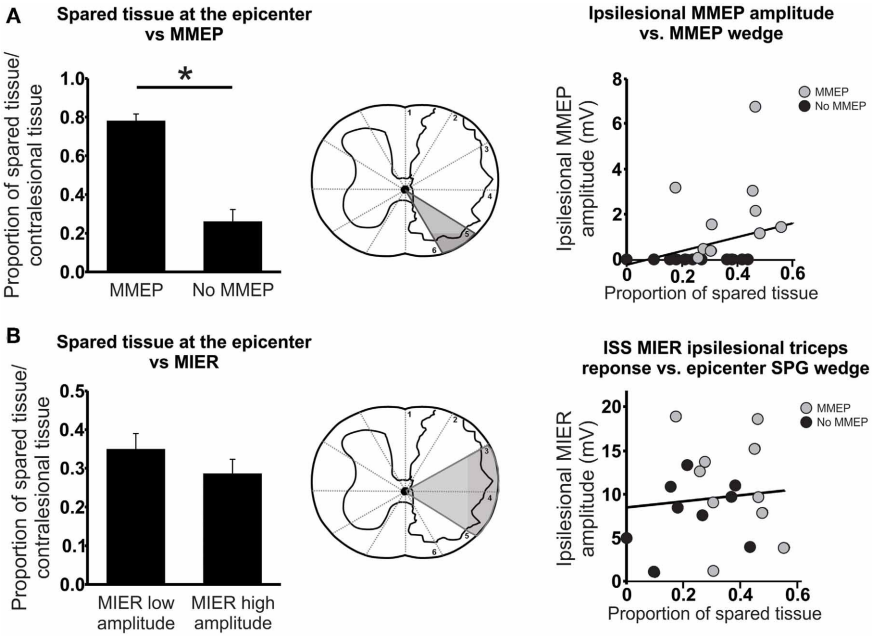

FIGURE 6 | Anatomical evaluation of tissue sparing related to the MMEP or the MIER responses. (A) Magnetic motor evoked potentials (MMEP) are transmitted in the ventrolateral white matter of the spinal cord. Animals that failed to exhibit ipsilesional MMEPs had significantly less spared tissue within this ventrolateral portion of the spinal cord at the lesion epicenter. Correlational analysis revealed a significant relationship between the amplitude of the MMEP response and the proportion of tissue sparing in the ventrolateral white matter responsible for MMEP transmission (gray wedge of schematic). **(B)** Magnetic Inter-Enlargement Responses (MIER) travel through the lateral white matter of the spinal cord. The amount of tissue sparing within these regions at the lesion epicenter did not affect the amplitude of the MIER. A scatter plot and correlational analysis failed to show a relationship between the amplitude of the ipsilesional MIER and the proportion of tissue sparing in the lateral white matter (gray wedge of schematic).

shift in the coincidence (mean phase dispersion: 18.64 + 7.26%, **Figure 7F**) suggesting that the coordination for the IF-CH is approximately 20% out of phase compared to normal. Standard deviation of the phase dispersion remained small, indicating that animals with MMEPs were able to consistently implement a single coordinative strategy for this limb pairing (Baseline: 7.26 + 2.5%; MMEP: 8.31 + 0.1.3%) with the coincidence ranging from −20% to +20%. Animals with no ipsilesional MMEP exhibited significantly greater variability in phase dispersion compared to normal and animals with MMEPs (No MMEP: 14.38 + 1.43%) that extended over a range of –20% to +60%.

Deficits in coordination were most visible in the CF-IH pairing. While the distribution of the phase dispersion appeared similar to normal, animals with MMEP exhibited a positive shift in mean phase dispersion and increased variability compared to normal (**Figure 7G**; mean phase dispersion: baseline: 4.36%, MMEP:16.38 + 6.21%; standard deviation: baseline 7.65 + 0.62%, MMEP: 17.12 + 1.34%). Animals with no ipsilesional MMEP were unable to execute synchronous placement of the CF and IH during runway locomotion (**Figure 7H**). Their mean phase dispersion was shifted 19.29 + 15.22% and the variability of this limb pairing was 26.5 + 2.64%. For the CF-IH relationship, phase dispersion values were evenly distributed across the range −20% to +70%. All animals, regardless of MMEP response, showed an increase in the incidence of mismatched limb pairings compared to normal.

DISCUSSION

It is now recognized that long distance regeneration might not be necessary to contribute to functional recovery after SCI; a short distance regeneration and/or formation of new relays appears to be sufficient (Jordan and Schmidt, 2002; Bareyre et al., 2004; Courtine et al., 2008). Long descending propriospinal neurons have been shown to be involved in new circuits that are reorganized after SCI and act as a relay between the cortex and their spinal target of origin (Bareyre et al., 2004). Propriospinal neurons thus are well suited to accomplish this role and the localization of their axons in the lateral and ventral white matter make them easily identified targets for therapeutic strategies after SCI (Conta and Stelzner, 2004). The results presented here extend these findings by characterizing plasticity occurring in ascending propriospinal networks after SCI. We show enhanced transmission in ascending propriospinal pathways and a concomitant increase in H_{max}/M_{max} ratio in the ipsilesional triceps. Surprisingly, while MMEPs correlated with tissue sparing and locomotor recovery, MIER did not show any relationship with these outcomes and did not predict better interlimb coordination.

INCREASED TRANSMISSION IN ASCENDING LONG PROPRIOSPINAL FIBERS

Long ascending propriospinal neurons that originate in the lumbar enlargement and terminate in the cervical enlargement have been anatomically identified, shown to ascend in the ipsilateral

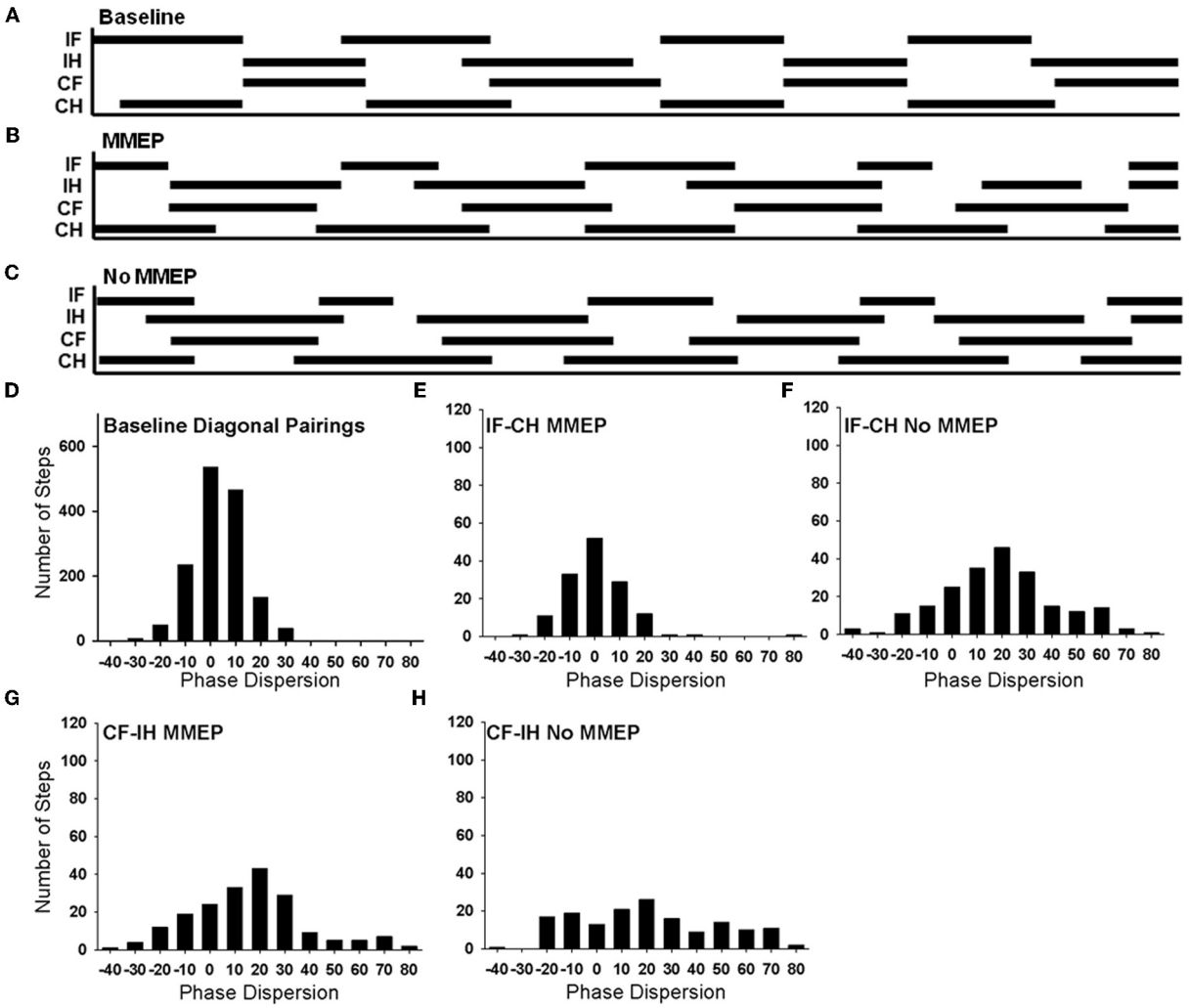

FIGURE 7 | Analysis of forelimb–hindlimb coordination in animals with and without ipsilesional MMEPs 6 weeks after SCI. Footfall diagrams during CatWalk locomotion for normal **(A)**, SCI animals with **(B)**, and without **(C)** ipsilesional MMEP responses allow the comparison of movements of all four limbs (IF, ipsilesional forelimb; IH, ipsilesional hindlimb; CF, contralesional forelimb; CH, contralesional hindlimb). The stance phase of each limb is indicated by black bars and the swing phase is indicated by the open spaces. Overlap of the solid lines for the MMEP and No MMEP groups indicate times when the limbs are moving synchronously. Comparison of the correspondence of stance and swing phase reveals a distribution pattern or phase dispersion for normal animals that centers at 0%, indicating that the initial contact of both diagonal limb pairings are tightly coupled **(D)**. **(E)** Animals with ipsilesional MMEP recovered a synchronous coordinative relationship that was similar to baseline values for the ipsilesional FL-contralesional HL limb pairing, yet for the other diagonal limb pairing (contralesional FL-ipsilesional HL; **(G)**, animals with ipsilesional MMEPs adopted a more lax strategy to coordinate the forelimbs and the hindlimbs as seen by a positive shift of the mean phase dispersion compared to baseline values and greater variability in the histogram. Animals with no ipsilesional MMEP demonstrated a lower degree of recovery of FL–HL coordination **(F,H)**. For the ipsilesional FL-contralesional HL limb pairing for this group of animals showed a positive shift in the mean phase dispersion compared to baseline values. The variability covered a broad range of –20% to +60%. There was no evidence of a predominant coordinative pattern for the contralesional FL-ipsilesional HL in animals with no ipsilesional MMEP **(H)**. A significant shift of mean phase dispersion (~20%) and the values of phase dispersion were evenly distributed across the range –20% to +70%.

VLF and to send collaterals to the contralateral side of the spinal cord (Giovanelli and Kuypers, 1969; Reed et al., 2006, 2009). Also, the stimulation of the peroneal nerve in humans can elicit responses in arm muscles (Zehr et al., 2001) that are modulated by leg movements during walking in a phase-dependent manner (Zehr and Haridas, 2003; Zehr et al., 2007). We used a recently described electrophysiological measure of the circuitry between the lumbar and cervical enlargements (Beaumont et al., 2006) to address the possible contribution of this pathway to functional recovery after SCI. We confirmed that signals could not be transmitted through the injury by attempting to record EMGs from the masseter muscle which is known to receive inputs from group II afferents of the sciatic nerve (Deriu et al., 2001, see also Beaumont et al., 2006). While we recorded no signals in the masseter muscle, we found that transmission between the lumbar and cervical enlargement was increased ipsilaterally after a hemicontusion

injury, with no effect on the contralateral side. Volume-conducted transmission from the site of stimulation or direct stimulation of cervical muscles that would then conduct the stimulus caudally to the recording site is unlikely since the response was absent in some animals or in any of the masseter muscles we recorded from. In addition, a similar artifact was never observed with the MMEP which was not enhanced in any group or experimental condition.

The mean latencies of the MIER at baseline ranged between 5.83 ms to 6.5 ms (see **Table 2**). With a distance between the sciatic at the iliac crest and the cervical enlargement of ∼105 mm, the conduction velocity including synaptic delay would be ∼16−18 m/s similar to what has previously been reported (Beaumont et al., 2006). Assuming a conduction velocity of 30 m/s (Carp et al., 2003), the synaptic delay would range from 2.33 to 3 ms, and the pathway potentially involve 3−5 synapses. Before injury, we observed that the response latency was significantly longer in the triceps contralateral to the hip stimulated. Whether this difference reflects an extra synapse on a commissural interneuron or added distance to travel across the cord remains to be determined. After injury, the latency was shorter on the ipsilesional side no matter which sciatic (contra or ipsi) was stimulated. Conduction velocity has been shown to be influenced by firing/depolarization threshold (Carp et al., 2001, 2003) which, itself, is modulated by activity and neuromodulators, two factors that are considerably affected by SCI. The inverse relationship between firing threshold and conduction velocity, suggest that the decrease in MIER latency that we observe after SCI on the ipsilesional side could rely on the increased excitability of the forelimbs motoneuronal pools as evidenced by the increased H_{max}/M_{max} ratio. More experiments would be necessary to address this question.

Ascending propriospinal neurons are involved in the coupling of lumbar and cervical CPGs and also interlimb coordination (Miller et al., 1973a,b), allowing the lumbar circuitry to drive or entrain the cervical enlargement (Juvin et al., 2005) and imposing a left-right alternation on the cervical networks. Behaviorally, we showed that our animals had not fully recovered appropriate interlimb coordination. Proper activation of the triceps brachii, a forelimb extensor, is crucial during locomotion and postural tasks which require coordination between the hindlimbs and forelimbs (Jordan and Schmidt, 2002). We did not observe a significant relationship between white matter sparing in the LF/VLF, BBB scores, and the amplitude of the MIER as previously reported in a thoracic contusion animal model (Beaumont et al., 2006). This is not surprising since our cervical unilateral injury was located rostral the triceps motor pool and did not directly affect the fibers that transmits the MIER whereas a thoracic contusion injury directly damage the MIER pathway. The increased transmission between the sciatic nerve and triceps brachii we observed with the MIER could be due to facilitation and/or disinhibition, both of which have been shown to occur after SCI. Disruption of supraspinal inhibitory pathways was confirmed by the analysis of the lesion site. Our experimental design did not allow us to determine if facilitation due to sprouting or increased synaptic strength also contributed to enhance the MIER in the ipsilesional triceps, although an increase in H_{max}/M_{max} suggests an increase in excitability of the ipsilesional motoneuron pool. Further study of how enhancement of intersegmental transmission to ipsilesional forelimb extensor muscles contributed to spontaneous functional recovery would require a detailed EMG and kinematics analysis.

DECREASED TRANSMISSION IN DESCENDING MOTOR PATHWAYS

Use of MMEPs as a functional measurement of motor pathway damage after SCI (Magnuson et al., 1999) depends on the activation of subcortical structures (Kamida et al., 1998) and the transmission of generated signals through axons that travel in the VLF of the spinal cord (Linden et al., 1999; Loy et al., 2002). It has been suggested that transcranial magnetic stimulation excites fast pathways (∼50 m/s) which reflect rodent motor function more accurately than the dorsal corticospinal tract that is activated by electrical motor-evoked potentials (MEP, Kamida et al., 1998; Luft et al., 2001) but not by MMEPs (Ryder et al., 1991). Therefore, MMEPs have great advantages over MEPs to assess motor recovery after SCI since the corticospinal tract is not crucially involved in locomotion whereas the ventral spinal cord is the main carrier of locomotion and postural control (Basso et al., 1996).

There is still debate as to whether the VLF carries descending fibers that are necessary (Steeves and Jordan, 1980; Noga et al., 1991) or not for the locomotor pattern to be expressed (Vilensky et al., 1992; Brustein and Rossignol, 1998) because of the redundancy of the pathway in the ventral column (Loy et al., 2002). Here, all our animals spontaneously recovered some degree of coordinated locomotion. The partial preservation of the ventral white matter tracts may account for the spontaneous locomotor recovery we observed. Sparing of 10−15% of ventral/lateral white matter is sufficient for locomotor movements after SCI (Schucht et al., 2002), while complete lesions of ventral white matter prevent the recovery of stepping. This suggests that sparing of at least part of the reticulospinal tract is necessary to initiate stepping and the recovery of rat locomotion (Loy et al., 2002). In our experiment, animals that did not present MMEPs had lower BBB scores than animals which recovered MMEP on the ipsilesional side. Analyzing specific regions of the ventral and lateral funiculi that contain the reticulospinal fiber tract that carries MMEP signals was a better predictor of locomotor recovery than overall white matter sparing at the lesion site (Schucht et al., 2002). MMEP latency remained delayed in the ipsilesional side 6 weeks after injury. We feel that this delay could arise from demyelination of spared fibers, formation of a new relay with propriospinal interneurons or unmasking of a previously "silent" polysynaptic pathway. It is likely that the return of MMEP signal over time represents the return of function in spared fibers rather than reorganization through the contralateral side since signal remained absent in hemisected animals even 6 weeks after the injury and the amplitude of the MMEP in the contralesional GS was ∼50% of pre-injury values. This decrease was not the result of damage to the contralesional cervical spinal cord since the white matter on the contralesional side appeared contiguous, did not have vacuoles and did not show anatomical evidence of demyelination.

Interlimb coordination during locomotion is a multifaceted skill that requires the ability to sustain a predominant rhythmic pattern with limited variability during locomotion that

results in one-to-one limb movements. Interlimb coordination has been most often studied in laboratory animals using the BBB Locomotor Rating Scale. The evaluation of interlimb coordination using the BBB relies on visual assessment of a one-to-one step ratio between the FLs and HLs, and due to the live nature of BBB testing, the consistency of the patterned movements cannot be accurately determined (Kloos et al., 2005). Phase dispersion is a highly sensitive method to measure interlimb coordination. It quantifies three components of interlimb coordination: the pattern, consistency, and step ratio. Kloos et al. (2005) used these three values to identify two degrees of coordination in mild SCI that were indistinguishable using the BBB. They termed these two groups a synchronous group where animals consistently and repeatedly displayed a one-to-one coordinative relationship between limb pairs, and modified concordance where animals implemented a more lax coordination strategy. Here, our examination of interlimb coordination using BBB showed significant differences between animals with and without ipsilesional MMEPs, and phase dispersion was able to further identify differences in the coordination of diagonal pairings after unilateral cervical SCI. That phase dispersion is a precise indicator of the pattern and underlying variability of limb placement during locomotion suggests that it may be a predictor of spared ascending and descending fibers necessary to mediate FL–HL coordination.

EXCITABILITY OF LOCAL CERVICAL SPINAL NETWORKS

The H-reflex is a measure of both peripheral sensory and motor nerve conductivity as well as segmental integrity of function (Thompson et al., 1998, 2001). After SCI, physiological and biomolecular changes such as ischemia, inflammation, and modification of neurotransmitter expression at the epicenter and beyond can affect the M- and H-wave of the H-reflex. Injury leads to increased reflex excitability (Thompson et al., 1992, 1998, 2001; Hiersemenzel et al., 2000), disruption of serotonergic descending pathways (Schmidt and Jordan, 2000) and enhanced monosynaptic transmission of group Ia fibers (Cope et al., 1988). The functional and anatomical changes that occurred in the cervical spinal cord are reflected by the increase in the H_{max}/M_{max} ratio in the ipsilesional forelimb in our experiments.

Although a change in H_{max}/M_{max} ratio is typically thought to reflect altered motoneuron excitability, this one-dimensional assumption is widely contested in the literature as many other factors can contribute to the modulation of the amplitude of the H- and/or M-wave. Amplitude of the H- and M-wave varies significantly with stimulus intensity and a variety of factors contribute to set the input-output gain across a motoneuronal pool (reviewed in Hultborn et al., 2004). Among those, the slope of the ascending part of the recruitment curve can provide information about the input–output relationship (Mazzocchio et al., 2001). The reflex gain can also be estimated as the slope between motoneuron activation (measured as background activity) and H-reflex amplitude. Although it is essential to determine background EMG activity for studies carried out during movement, its effect is minimal under anesthesia and our baseline recordings were silent. We found no difference in steepness of the slopes of the H-reflex or M-wave recruitment curve for stimulation on either the contra- or ipsilesional side (*data not shown*). Conversion of muscle fibers toward faster phenotypes has been shown to occur after SCI and/or unloading and could have a marked effect on the amplitude of the M-wave (Roy et al., 1991; Talmadge, 2000). However, this increase in FF motor units is transitional after contusion injury, gradually returns to normal level and correlates with weight-bearing recovery (Hutchinson et al., 2001; Roy et al., 2011). Therefore, a switch in muscle unit properties is unlikely to have contributed to changing the H_{max}/M_{max} ratio as our animals have recovered weight-bearing in the forepaws by 3 weeks post-SCI and the amplitude of the M-wave was not significantly different between intact animals and the ipsi- or contralesional forepaw 8 weeks after hemicontusion injury. The amplitude of H_{max} depends on pre and post synaptic events and concomitant antidromic activity elicited in motor nerves (Misiaszek, 2003; Knikou, 2008). The advantage of using the H-reflex by stimulating the nerve (vs. stretch reflex) is that it bypasses the muscle spindles and fusimotor activity that could influence the sensitivity of Ia afferents. Also, when reflexes are recorded at rest as it is the case in this study, the influence from supraspinal pathways are anticipated to be minimal and Renshaw cells and Ia/Ib interneurons less active. Although other mechanisms cannot fully be ruled out, the model we used here tended to limit the effect of other confounding factors and we believe the change in H_{max}/M_{max} ratio truly represents a change in motor pool activity in this context.

Our injury altered local cervical reflex excitability in the ipsilesional forelimb only, but decreased FDD bilaterally suggesting that the injury induced changes in motoneuronal properties and motor pool excitability only on the ipsilesional side. The contralesional cervical neurons did not seem to be affected by the injury in the same manner with a H_{max}/M_{max} ratio similar to the normal group. The ipsilesional increase in H_{max}/M_{max} ratio may be due to increased sprouting of Ia afferents onto motoneuronal dendrites with reduced supraspinal inputs; similar openings onto the dendritic arbor would not be observed contralesionally as the supraspinal inputs are for the most part preserved on that side. A possible explanation for the contralesional decrease in FDD would be the lack of presynaptic inhibition from ipsilesional collaterals onto Ia afferents of the contralesional triceps. Changes in the H-reflex pathway after SCI suggest both spinal reorganization and an associated decrease in presynaptic inhibition (Schindler-Ivens and Shields, 2000) with some of the effects being unilateral and others bilateral. Of supraspinal neurons, only reticulospinal neurons appear to contribute to presynaptic inhibition of group Ia afferents in any major way; the axons of these neurons run in the medial longitudinal fascicle and the ventral quadrant of the spinal cord (Carpenter et al., 1963, 1966; Reed et al., 2008) which are greatly compromised in our model.

SUMMARY

Two months after a unilateral contusive injury to the C5 spinal cord, animals demonstrated a persistent disruption of signal transmission in ascending and descending pathways despite partial functional recovery. Our results indicate that complex plasticity occurs, leading to differential changes in both the ipsi- and contralesional spinal networks even though the lesion was

unilateral. Animals that did not recover MMEPs on the ipsilesional side had significantly less spared tissue and presented inconsistent interlimb coordination as compared to those who recovered MMEPs. The lack of relationship of the MIER to functional outcomes prevents us to establish a clear role of ascending propriospinal pathways in either partial recovery or lasting deficits we observed. Nonetheless, that this pathway is intact and excitable with stimulation suggests that with the right type of stimulation (exercise, epidural stimulation) it may represent a potential avenue to further drive and improve locomotor recovery.

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The transcriptional response of neurotrophins and their tyrosine kinase receptors in lumbar sensorimotor circuits to spinal cord contusion is affected by injury severity and survival time

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Traumatic spinal cord injury (SCI) results in changes to the anatomical, neurochemical, and physiological properties of cells in the central and peripheral nervous system. Neurotrophins, acting by binding to their cognate Trk receptors on target cell membranes, contribute to modulation of anatomical, neurochemical, and physiological properties of neurons in sensorimotor circuits in both the intact and injured spinal cord. Neurotrophin signaling is associated with many post-SCI changes including maladaptive plasticity leading to pain and autonomic dysreflexia, but also therapeutic approaches such as training-induced locomotor improvement. Here we characterize expression of mRNA for neurotrophins and Trk receptors in lumbar dorsal root ganglia (DRG) and spinal cord after two different severities of mid-thoracic injury and at 6 and 12 weeks post-SCI. There was complex regulation that differed with tissue, injury severity, and survival time, including reversals of regulation between 6 and 12 weeks, and the data suggest that natural regulation of neurotrophins in the spinal cord may continue for months after birth. Our assessments determined that a coordination of gene expression emerged at the 12-week post-SCI time point and bioinformatic analyses address possible mechanisms. These data can inform studies meant to determine the role of the neurotrophin signaling system in post-SCI function and plasticity, and studies using this signaling system as a therapeutic approach.

Keywords: spinal cord injury, neurotrophins, neurotrophin receptors, contusions, transcription, injury mechanisms, sensory neurons, genetic regulation

INTRODUCTION

Traumatic injury to the spinal cord (SC) results in a variety of changes to sensorimotor circuits. Sensory neurons of the dorsal root ganglia (DRG) rapidly undergo long-lasting changes in their electrophysiological properties and growth capacity (e.g., Bedi et al., 2010, 2012; Walters, 2012). Locomotor circuitry in the SC caudal to an injury site undergoes plasticity at the cellular, synaptic, and connectivity levels in an activity-dependent manner after injury in humans and experimental models (e.g., Edgerton et al., 2004; Rossignol, 2006; Petruska et al., 2007). One strategy to restore function after spinal cord injury (SCI) is physical therapy and/or locomotor rehabilitation training (e.g., Wernig et al., 1995). The neurotrophins Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), and Neurotrophin 3 (NT3) are secreted growth factors that were first characterized for their important role in the survival of subpopulations of sensory neurons and in formation of SC sensorimotor circuits during development (e.g., Barbacid, 1995; Lindsay, 1996; Huang and Reichardt, 2001). In addition to these essential roles in establishing the physiological patterns of developing neural circuitry, neurotrophins are implicated as having a role in activity-dependent changes associated with restoration of function after SCI (described below).

Neurotrophins have key roles in modulating the anatomical, neurochemical, and physiological properties of cells in the central and peripheral nervous system. The effects of neurotrophins on responses to stimuli in both the intact and injured nervous system have been extensively investigated and studies have demonstrated an important role in modulation of sensorimotor physiology (for reviews, see Huang and Reichardt, 2001, 2003; Reichardt, 2006; Skaper, 2008, 2012). The neurotrophins have therefore become a frequent target for manipulation after injury. Delivery of exogenous BDNF and NT3 to the transected SC improves recovery of hindlimb function (Blits et al., 2003; in rats) and results in a level of function similar to that seen in animals receiving locomotor training after spinal transection (Boyce et al., 2007; in cats). Such demonstrations of enhanced post-SCI function in response to exogenous neurotrophins suggests a role for neurotrophin signaling in models of activity-dependent plasticity after injury, possibly

including physical therapy. For example, in the lumbar SC of rats, post-SCI locomotor training causes an increase in both BDNF and NT3 above levels of non-trained animals (Hutchinson et al., 2004; Côté et al., 2011). In light of the demonstrated and suggested roles in modulating sensorimotor physiology, characterizing the endogenous regulation of neurotrophins and their receptors after injury is particularly relevant.

Neurotrophins influence cellular processes by binding to membrane-bound receptors which transduce the extracellular signal into intracellular effect – their high affinity tyrosine kinase receptors. In general, NGF binds TrkA, BDNF bindsTrkB, and NT3 binds TrkC (e.g., Barbacid, 1995; Patapoutian and Reichardt, 2001; Huang and Reichardt, 2003), although cross-talk is recognized and there is a low-affinity receptor, p75, which we do not consider here. To determine the role of neurotrophins in any process or condition one must examine not only the neurotrophins, but also the receptors.

Prior characterizations of changes in neurotrophins and Trk receptors in lumbar neural circuitry have been instrumental in elucidating the complex regulation of these important molecules after injury (**Table 1**). However, these have largely focused on time points of less than 6 weeks (Hayashi et al., 2000; Liebl et al., 2001; Nakamura and Bregman, 2001; Widenfalk et al., 2001; Qiao and Vizzard, 2002, 2005; Gulino et al., 2004; Zvarova et al., 2004; Qin et al., 2006; Li et al., 2007; Hajebrahimi et al., 2008; Qian et al., 2011; Keeler et al., 2012). Although valuable for elucidating the role of neurotrophin signaling in the first 6 weeks after SCI, these data are of uncertain value for relating to longer-term post-SCI function. Given the many demonstrations of continued changing conditions after SCI (e.g., Beattie et al., 2002; Profyris et al., 2004; Ung et al., 2008; Beck et al., 2010), it is important to recognize that the temporal character of experiments has a significant influence on the outcome.

The impact of SCI also varies depending on the location of the injury itself and the spatial relation of the investigated tissue to the SCI. Clearly, the relative composition of types of tissues innervated changes throughout the course of the neuraxis as does the specific function of local circuitry. For example, in rat, the spinal components of bladder control are focused on the T13/L1 and L6/S1 segments, colon function is focused in L6/S1, and the locomotor central pattern generator appears focused in (though not limited to) the L1/2 segments, spinal sympathetic circuitry regulating outflow exists roughly from T1-L2, and spinal parasympathetic circuitry exists in the sacral-caudal SC. Thus it follows that the effect on spared function and/or recovery is influenced by the level of the injury (e.g., Magnuson et al., 1999, 2005; Garcia-Alias et al., 2006), but this also extends to less direct functions (Campagnolo et al., 2000; Lucin et al., 2007). It is also very important to consider that both neural and non-neural tissues remote from the SCI can be affected (e.g., Collazos-Castro et al., 2005; Massey et al., 2006; Gris et al., 2008).

Sensory input to the SC plays a role in establishing natural and therapy-induced recovery and regulating spinal function in the absence of descending control. For example, urinary bladder function after SCI is highly reliant on sensory input and plasticity of sensory afferents (e.g., Tai et al., 2006; de Groat and Yoshimura, 2009), and SCI affects the trk receptor profile of neurons in DRG segments innervating bladder differently than for DRG innervating hindlimb (Qiao and Vizzard, 2002, 2005), a finding that extends to spinal trk receptors as well (Zvarova et al., 2004). Additionally, the type and amount of sensory input can influence spontaneous recovery after SCI (e.g., Grau et al., 2004, 2012; Ollivier-Lanvin et al., 2010; Caudle et al., 2011; Ferguson et al., 2012a,b) and also influence the effectiveness of physical therapy (e.g., Bouyer and Rossignol, 1998, 2003; Edgerton et al., 2004, 2008; Gomez-Pinilla et al., 2004; Frigon and Rossignol, 2009; Ollivier-Lanvin et al., 2010), all of which may involve neurotrophin signaling (e.g., Gomez-Pinilla et al., 2004; Hutchinson et al., 2004; Boyce et al., 2007, 2012; de Leon, 2007; Côté et al., 2011). Further, autonomic dysreflexia (AD), a life-threatening condition that is common for those living long-term with cervical or high thoracic SCI, is triggered most frequently by nociceptive sensory input (Maiorov et al., 1998; Krassioukov and Fehlings, 1999; Garstang and Miller-Smith, 2007), and sprouting of central terminals of nociceptive neurons, purportedly modulated by NGF, is proposed as a mechanism contributing to AD (Weaver et al., 1997; Krenz et al., 1999; Marsh et al., 2002; Cameron et al., 2006; Ackery et al., 2007). It is important, therefore, to examine not only the SC, but also the sensory neurons providing information to the SC, and to consider that the effects of SCI on these neurons may differ with their spatial relation to the SCI, and/or to the different tissues they innervate (e.g., Qiao and Vizzard, 2002; Zvarova et al., 2004; Bedi et al., 2010, 2012; Keeler et al., 2012). The spatial character of experiments, in terms both of the level of SCI and the relation to the SCI of the tissue investigated, has a significant influence on the outcome.

Injury severity, or more precisely the degree and nature of the tissue spared after injury, is one of the key factors determining the functional capabilities of the SC caudal to the SCI. The literature is replete with examples of this when reports are considered together (e.g., Rossignol and Frigon, 2011). Far fewer single studies examine multiple injury severities (e.g., Magnuson et al., 2005; Smith et al., 2006), although the injury severity character of experiments has a significant influence on the outcome.

We sought to characterize the natural regulation of neurotrophin and trk receptor genes in tissues and conditions that were applicable to experimental studies of long-term function and recovery after SCI and to the human condition. We therefore characterized the transcriptional response of neurotrophins and their cognate Trk receptors to SC contusion temporally (6 and 12 weeks post injury), spatially (in lumbar SC and DRG), and relative to injury severity (12.5 and 25 g cm NYU contusions).

MATERIALS AND METHODS

All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville, Louisville, KY, USA. Experimental animals were 7 week old female Sprague–Dawley rats (Taconic Labs, Hudson, NY, USA). Animals were housed in pairs throughout the course of our experiments.

SURGICAL SPINAL CORD INJURY

Rats ($n = 47$) were anesthetized with 50 mg/kg sodium pentobarbital (Sigma, St Louis, MO, USA). Once sedated, Lacquer Lube was applied to the eyes to prevent drying. After skin incision,

Table 1 | Summary of recent experiments assessing expression levels of neurotrophins and neurotrophin receptors after SCI.

PMID	Reference	Molecule(s)	Injury model	Injury site	Sampling site	Experimental methods	Post injury time course	Findings	Notes
10757326	Hayashi et al. (2000)	NGF, BDNF, NT3, TrkA, TrkB, TrkC	Spinal cord crush (60 g, 1 s)	Under T10 vertebra	Five segments centered on epicenter	ISH	Six times; up to 3 days	Increase in BDNF and NT3, weaker increase for NGF; TrkA and TrkC not detected; TrkB detected in non-neurons and motoneurons, and increased in both with SCI	Functional status of animals was not assessed, but reference was given to Guth et al., 1994 (model shows mild motor deficit after awakening from anesthesia with apparent full recovery at 72 h); BDNF observed in non-neurons after SCI; qualitative data only, no statistics
						qPCR (NGF only)		NGF increased weakly	
11161589	Liebl et al. (2001)	TrkA, TrkB, TrkC	12.5 g cm NYU contusion	Under T9, T10 vertebra	Entire SC	ISH	1 day	No difference in TrkA, TrkB, or TrkC expression rostral or caudal to injury	Absent Trk expression around injury site and reduced in penumbra, no statistics
11331375	Widenfalk et al. (2001)	NGF, BDNF, NT3, TrkA, TrkB, TrkC	25 g cm NYU contusion transection	Under T9 vertebra	Cross-sections taken from regions throughout length of spinal cord injury epicenter and up to 1 cm caudal	ISH	Six times; up to 6 weeks	No change in TrkA, TrkB, TrkC; increase in NGF and BDNF up to 1 day, but no change vs. intact at 6 week; NT3 not detected in either intact or injured spinal cord	Functional status of animals with contusion was not assessed; reports on multiple injury types; statistical analysis uses optical density measures for ISH, and radioactivity for RPA
						RPA (NGF, BDNF, NT3)	6 weeks after contusion	No change in NGF and BDNF; NT3 not detected	
							1 day after transection	Increase in NGF and BDNF; NT3 not detected	

(Continued)

Table 1 | Continued

PMID	Reference	Molecule(s)	Injury model	Injury site	Sampling site	Experimental methods	Post injury time course	Findings	Notes
11358454	Nakamura and Bregman (2001)	NGF, BDNF, NT3, NT4	Lateral over hemisection	Under T6 vertebra	Entire SC	RPA	Five times; up to 2 weeks	Increase in NGF and BDNF up to 4 days, NT3 and NT4 not detected	Used whole SC mRNA, no assessment of injury or post-SCI function, expression data represented as % GAPDH
12115676	Qiao and Vizzard (2002)	TrkA, TrkB	Transection	Under T8-T10 vertebrae	L1-S1 DRG	IHC	5–6 weeks	Increase in # of TrkA and TrkB positive cells in L1, L6/S1, no change at L4/5	No assessment of post-SCI function, data expressed as # of Trk-IR positive cells
15193526	Gulino et al. (2004)	BDNF, NT4	Lateral hemisection	Under T9 vertebra	L4/5 SC	IHC	Four times; up to 2 weeks	Decrease in BDNF, NT4 starting at 30 min, lasting up to 2 weeks	Coronal sections; no assessment of post-SCI function; data expressed as relative optical density of IR positive cells in ipsilateral vs. contralateral hemisected cord
15236239	Zvarova et al. (2004)	NGF, BDNF	Transection	Under T7–T9 vertebrae	T7-S1	ELISA	<1, 6 weeks	Increase in NGF T7–T8 (rostral), and T13-L1, L6-S1 (caudal) 6 weeks post injury; Increase in NGF T9-T10 (caudal), and T13-L1, L6-S1 (caudal) < 1 week post injury; increase in BDNF T7-T10, T13-L1, L6-S1 6 weeks post injury; Increase in BDNF T7-L1, L3-S1 < 1 week post injury	No assessment of post-SCI function; neurotrophin concentration expressed as proportion of total protein

(Continued)

Table 1 | Continued

PMID	Reference	Molecule(s)	Injury model	Injury site	Sampling site	Experimental methods	Post injury time course	Findings	Notes
15611995	Qiao and Vizzard (2005)	TrkA, TrkB	Transection	Under T8-T10 vertebrae	L1-S1 DRG	IHC	2 days and 2 weeks	Increase in # of TrkA and TrkB positive cells in L1, L6/S1, no change at L4/5	No assessment of post-SCI function; data expressed as # of Trk-IR positive cells
17055159	Qin et al. (2006)	NGF, BDNF, NT3	Lateral hemisection	Under T10 vertebra	Ventral horn caudal to T10 injury site	IHC	Three times; up to 3 weeks	Increase in # of BDNF, NT3, NGF positive cells in ventral horn	Characterized injuries by spared function (BBB locomotor score); data expressed as optical density of IR positive cells in hemisected cords relative to control cords
17459471	Li et al. (2007)	NGF, BDNF, NT3	Transection	Under T9–T10 vertebra	Laminae I-IX, ∼1.5 cm caudal to injury site	IHC	Four times; up to 3 weeks	Increase in # of NGF IR cells and relative IR in laminae I-IX up to 3 weeks, # of NT3 IR cells and relative IR in laminae VIII and IX up to 3 weeks and laminae I–VII at 2 weeks, # of BDNF IR cells and relative IR in laminae I-IX up to 7 days	Characterized injuries by spared function (BBB locomotor score); data expressed as relative optical density of IR positive cells in hemisected vs. control cords
18585435	Hajebrahimi et al. (2008)	NGF, BDNF, NT3, TrkA, TrkB, TrkC	25 g cm NYU contusion	Under T9-T10 vertebra	∼1 cm block of SC	EthBr staining intensity	Eight times; up to 3 weeks	Decrease in NGF after 6 h that increases until 3 week where greater than control, BDNF and NT3 decrease after 6 h up to 3 weeks; TrkA, TrkB, TrkC decrease up to 3 weeks	B2m used as internal control; no assessment of injury or post-SCI function; data expressed as levels of mRNA relative to B2m

(Continued)

Table 1 | Continued

PMID	Reference	Molecule(s)	Injury model	Injury site	Sampling site	Experimental methods	Post injury time course	Findings	Notes
21441969	Qian et al. (2011)	TrkC	Transection/ resection	Under T8–T9 vertebrae	Motor cortex; SC: adjacent to injury, rostral/caudal to injury	mRNA, protein	Four times; up to 2 weeks	Decrease at and around injury site from 1 to 7 days, then rapid increase until 14 days	mRNA more highly expressed at injury site than neighboring segments; protein shows same pattern; no assessment of post-SCI function
22244304	Keeler et al. (2012)	BDNF, NT4, NT3, TrkB, TrkC	Transection	Under T9 vertebra	L4–L6 SC (laser-captured motoneurons, select laminae); L4–6 DRG (large neurons)	Laser-capture, qPCR, WB	up to 31 days	Increase in NT4 and TrkB mRNA at 10 days, NT4 mRNA at 31 days; increase in NT3, NT4, BDNF protein at 31 days; increases in NT4 at 10 days, TrkB at 31 days in motoneurons; no change in expression in intermediate gray matter or large DRG neurons	More robust increase in expression after exercise; no assessment of post-SCI function; data expressed as mRNA or protein relative to control
	Hougland et al. (this article)	NGF, BDNF, NT3, TrkA, TrkB, TrkC	12.5 and 25 g cm NYU contusion	Under T9 vertebra	L4/5 spinal cord and DRG	qPCR	6 and 12 weeks	Gene regulation differed by injury severity and by post-SCI time; correlated expression of genes at 12 weeks in DRG	Characterized injuries by spared function (BBB locomotor score) and by histology (white matter sparing); examined co-regulation of genes

Abbreviations: n.c, no change; IHC, immunohistochemistry; ISH, in situ hybridization; WB, western blot; RPA, ribonuclease protection assay; EthBr, ethidium bromide; LCM, laser-capture microdissection; qPCR, quantitative polymerase chain reaction; SC, spinal cord; DRG, dorsal root ganglion.

laminectomy was performed at vertebral level T9, to expose the T10 SC. Contusion injuries were produced using the New York University (NYU) Impactor. Either "Moderate" or "Moderately severe" injuries were produced by releasing a 10 g, 2 mm rod from 12.5 or 25 mm height, respectively, onto the exposed dura mater of the SC. These will subsequently be referred to as 12.5 and 25 g cm injuries. After producing the contusion the wound was closed in layers and the skin incision was stapled. Rats received fluids (10cc 0.9% saline subcutaneously), and antibiotic treatment (0.1cc Gentamicin (50 mg/mL) intramuscularly, and Bacitracin was topically applied on the incision site). Animals were housed overnight in a recovery room with a heating pad under their cage, and were taken to the animal facilities in the morning.

Assessment of mRNA expression in SCI animals was compared to control animals. These consisted of naïve rats (two per time point group) and rats receiving laminectomy-only (three rats per time point group), for a total of five controls per time point. There were four additional laminectomy-only control rats included with the animals used for the 6-week post-SCI DRG assessment. All surgical procedures (except for the SCI), were as described above for the laminectomy-only control rats.

INJURY CHARACTERIZATION

Behavior

Experiments were performed on rats separated into groups based on injury severity, survival time, and the tissue to be analyzed for mRNA expression. Rats were familiarized with the testing procedures and personnel by handling for 1 week before injury. Pre-surgical behavioral assessments were done to ensure no pre-existing conditions were present that would subsequently affect our locomotor outcome measures. Seventeen rats received 12.5 g cm NYU (moderate) and 16 rats received 25 g cm NYU (moderately severe) injuries. Hindlimb locomotor function was assessed with the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (Basso et al., 1996). BBB testing was carried out prior to injury and 7, 14, 21, 28, 35, 42 for the 6-week SC group, and 7, 14, 21, 28, 35, 42, 49, 56, 63, 72, 79, and 84 days post injury for the 12-week SC groups, 12 week DRG group, and at 7, 14, 28, and 42 days post injury for the 6-week DRG group. For testing, rats were placed in an open field (a plastic tank that was 105 cm in diameter with 30 cm high walls) for 4 min. BBB testing was done after animal care in the morning. Hindlimb movement and locomotion were scored simultaneously by two observers who were blind to the treatment groups. We include the BBB measures as a means to characterize the injuries with commonly used assessments so that the mRNA measures can be placed in context.

Histology

At the end of the testing period, rats were anesthetized with sodium pentobarbital and euthanized via transcardial perfusion with 30% RNA Later (Qiagen) in 0.1 M Phosphate Buffered Saline (PBS). An approximately 10 mm long block of SC containing the injury epicenter was removed from each animal and immersed in 4% paraformaldehyde. After 1 week cords were immersed in PBS containing 30% sucrose for cryoprotection until further processing. For sectioning, tissue was embedded in TissueTek®(VWR) and frozen. The blocks were cut 50 μm thick in the transverse plane on a cryostat and were sampled every 250 μm. A series of sections spanning the rostrocaudal extent of the lesion was stained with eriochrome cyanine (EC) to assess amounts of spared myelin as described (Rabchevsky et al., 2007). Light microscopy was used to determine spared white matter (SWM). Images were captured using a SPOT digital camera (Diagnostic Instruments) mounted on a Zeiss Axioskop. From these, the area of spared tissue was manually designated (Intuos drawing tablet; Wacom, Otone, Japan). Areas of white matter sparing were calculated using the ImageJ program and expressed as a proportion of control (defined as group mean of the smallest white matter area from an analogous section of SC from all control animals). For each injured animal, the SCI epicenter was defined quantitatively as the section containing the least amount of intact tissue. Percent white matter sparing is reported as mean (±SD). As with the BBB, we include the WMS measures as a means to characterize the injuries with commonly used assessments so that the mRNA measures can be placed in context.

mRNA EXPRESSION

Isolation and cDNA conversion

Animals were euthanized after final behavioral assessments and exsanguinated by transcardial perfusion using 30% RNA later (Qiagen) in PBS. Lumbar SCs (L4/5) and DRG were removed and immersed in 100% RNA later and stored at −20°C until further processing. SCs were homogenized on ice in 1 mL Trizol and RNA was isolated using Trizol/chloroform extraction method. Briefly, homogenate was transferred to a 1.5 mL tube and spun at 12,000 *g* for 10 min at 2°C. The supernatant was transferred to a new tube and 200 μL chloroform added. This mixture was spun for 15 min at 2°C to separate into aqueous and organic phases. The aqueous phase was transferred to a new tube and alcohol precipitation was performed with 100% isopropanol, then 70% ethanol. After removal and drying of excess ethanol, the pellet was resuspended in 30 μL nuclease free H_20, solubilized in 600 μL Buffer RLT with beta-Mercaptoethanol (BME), and processed through RNeasy MiniKit (Qiagen) per manufacturers protocol. DRGs were homogenized directly in Buffer RLT + BME and processed through RNeasy MiniKit. RNA was analyzed by Nanodrop (ThermoScientific, Waltham, MA, USA) to obtain concentration and 500 ng of RNA from each sample was reverse transcribed into cDNA using Quanta Biosciences qScript cDNA SuperMix. All RNA was converted to cDNA using the same lot of reverse transcriptase. Performing the reverse-transcription for all samples with the same reagents is a methodological procedure meant to reduce the cross-sample variability which in turn can enhance the reliability of statistical assessments.

qRT-PCR

mRNA expression levels were quantified by qRT-PCR on Corbett Research 6000 (Qiagen) using FastStart Universal SYBR Green Master Mix(Roche). Duplicate reactions were run for each sample for both the gene of interest and the normalizer [Beta-3 Tubulin – demonstrated as a suitable normalizer gene for SCI work (Strube et al., 2008)]. Relative expression levels were calculated as ΔΔCT of gene of interest vs. normalizer. Primer sequences for the genes

analyzed are provided in **Table 2**, along with their relationship to the known gene structure and transcript species.

STATISTICS

Statistical analyses were performed using SPSS (IBM, North Castle, NY, USA) or SigmaPlot/SigmaStat (Systat Software, San Jose, CA, USA). A Student's *t*-test was performed to determine if expression levels differed between control groups. In cases where gene expression did not differ between control groups the 6- and 12-week control groups were combined and the expression values for the experimental groups are reported as a fold-change of the unified control group. One-way analysis of variance (ANOVA) was performed on these values with *post hoc* Tukey's test for all pairwise comparisons. All groups with $p < 0.05$ difference are reported as significant. Pearson Product Moment was calculated to determine the relationships between the expression levels of the different transcripts, and to determine the relationships between BBB/WMS vs. expression levels. Differences between BBB scores were assessed using a mixed model repeated measures ANOVA with a *post hoc* Bonferroni *t*-test.

RESULTS

INJURY CHARACTERIZATION

To assess the degree of injury severity, we characterized SC injuries based on two parameters; behavior as measured by BBB, and the amount of SWM at the epicenter after staining with eriochrome cyanin (Rabchevsky et al., 2007). BBB scores were significantly greater in the 12.5 g cm injury groups than the 25 g cm groups beginning at week 5 (**Figure 1A**). These differences in behavior were reflected in the amount of SWM, as the 25 g cm groups had 8.5% (±1.8%) and the 12.5 g cm groups had 13.9% (±3.6%) SWM at the epicenter. In accord with prior literature (Basso et al., 1996; Schucht et al., 2002; Magnuson et al., 2005), a significant correlation ($r = 0.88$, $p < 0.001$) was observed between white matter sparing at epicenter and BBB scores (**Figure 1B**). BBB scores of the 12.5 g cm group showed a high degree of variability and continued to increase between 6 and 12 weeks instead of reaching a plateau. Within this group, two animals had BBB scores consistent with the range observed in previous literature (Basso et al., 1996; Agrawal et al., 2010; 12 and 13) and four animals that had higher BBB scores than expected for this injury severity (mean 17.9) at 12 weeks post injury. We considered that these results may be due to both greater amount of SWM and/or asymmetry of the lesion (**Figures 1C,D**). Indeed, of the four animals whose BBB scores continued to increase, all had a greater amount of SWM (mean 16.1% for four animals with higher BBB scores, 10.1% for two animals with lower BBB scores), and all had asymmetrical injuries (arbitrarily defined as more than 4% greater SWM on one side vs. the other). Animals with the lower BBB scores in the 12.5 g cm group did not represent statistical outliers (Grubbs outlier test). Separate statistical analyses of gene expression were performed with the exclusion of the two animals whose BBB scores did not continue to increase and the results generally did not differ from those found when all six animals were considered together. The lone exception was the results for expression of one Trk receptor in the SC, which is noted below. We thus consider all six animals

Table 2 | Primer sequences for qPCRand relationship to gene/transcripts.

Gene	Forward primer	Location of forward primer	Reverse primer	Location of reverse primer	Gene intervals probed	Isoforms probed
TrkC	GATTCAGGGAACAGCAATGG	Within exon 1	TTGATGGTCAGCTTCTGGAG	Spans exons 2–3	Bp 231–382 of NM_001270656	All isoforms
NT3	CGATCTTACAGGTGAACAAGG	Spans exons 1–2	CTGGCAAACTCCTTTGATCC	Spans exons 2–3	Bp 177–318 of NM_031073	Full length NT3
TrkB	CATTGACCCAGAGAACATCAC	Within exon 2	TCGAGTGAAATTGATGTGCC	Spans exons 4–5	Bp 846-1032 of NM_012831.2	Full length TrkB
BDNF	CGAGACCAAGTGTAATCCCA	Within exon 2	TCTATCCTTATGAACCGCCA	Within exon 2	Bp 920–1075 of NM_001270630.1	All isoforms
TrkA	TTCAGTGATACCTGTGTCCAC	Spans exons 12–13	GACGAGCATTCTCAGATGTC	Spans exons 13–14	Bp 1558–1732 of NM_021589.1	All isoforms
NGF	CTTCAACAGGACTCACAGGA	Within exon 3	TTGTTAATGTTCACCTCGCC	Within exon 3	Bp 517-681 of XM_003749364.1	All isoforms
TubB3	CGAGTGAAGTCAGCATGAG	Within exon 1	ACATACTTGTGAGAGGAGGC	Spans exons 2–3	Bp 36–228 of NM_139254.2	All isoforms

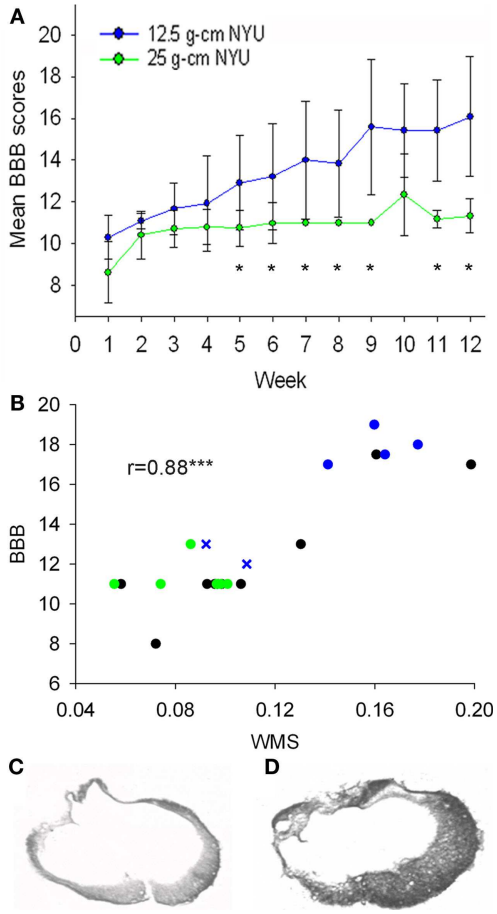

FIGURE 1 | Injuries were characterized using BBB scores to assess hindlimb locomotor function and white matter sparing (WMS) at epicenter using EC stain. (A) BBB scores of groups that received 12.5 g cm (blue) or 25 g cm (green) NYU injuries. Starting at week 5, a significant difference was observed between the two injury severities. Both groups were significantly different ($p < 0.05$) from controls (not shown) at all time points. **(B)** White matter sparing at epicenter (*x* axis) plotted vs. BBB score. Black dots represent 6 week values. Green dots represent 12 week, 25 g cm injured animals. Blue x represent the two animals from the 12-week, 12.5 g cm group with the lowest BBB scores within the group. Blue dots represent the four animals from the 12-week, 12.5 g cm group with the highest BBB scores within the group. **(C)** Image taken from a 12.5 g cm contused animal showing a laterally-symmetrical injury pattern at the epicenter. Note the difference from **(D)**, which was taken from an animal that also received a 12.5 g cm spinal cord contusion but which yielded an asymmetrical injury at epicenter. $*p < 0.05$, $***p < 0.001$.

together in the group in all subsequent figures and analyses of mRNA expression.

EXPRESSION OF Trk RECEPTORS IN THE DRG

One purpose of this study was to determine whether these different contusion severities result in a differential transcriptional response of neurotrophins and their Trk receptors in lumbar sensorimotor circuits. Hence, we sought to determine the expression level of Trk receptors in the DRG 6 and 12 weeks after our two severities of contusion injury. Expression of TrkA, TrkB, and TrkC each differed significantly between the 6- and 12-week groups, with the magnitude and direction of difference depending on receptor type and injury severity. Expression of TrkA mRNA in DRG from the 12-week group at both injury severities was significantly greater than that in DRG from the corresponding 6 week group. Expression of TrkA in DRG from the 12-week group that received 12.5 g cm injury was also elevated relative to the control groups. We also observed a difference in TrkA expression between injury severities at the 12-week time point. Similar to TrkA, expression of TrkC mRNA in DRG from the 12-week group was greater than that in DRG from the corresponding 6 week group at both injury severities, but the difference only reached significance in the 12.5 g cm animals. Unlike the findings for TrkA, we detected no significant difference in TrkC expression between DRG from the 12.5 g cm group and from the 25 g cm group at the 12 week time point. Expression of mRNA for TrkB in DRG at 12 weeks after 25 g cm injury was significantly lower than in DRG from both the 6-week SCI and control groups. No significant difference in TrkB expression was observed between injury severities at 6 or 12 week time points in the 12.5 g cm injury group (**Figure 2**).

EXPRESSION OF NEUROTROPHINS IN THE DRG

As with TrkA, NGF mRNA expression in DRG from the 12.5 g cm injury severity group was significantly greater in the 12-week group than in both the 6-week and control groups. However, no significant changes in NGF expression were observed between survival time groups in the 25 g cm injury severity group. As with TrkB, BDNF expression in the 12-week 25 g cm group was significantly less than in the 6-week 25 g cm group, but did not differ from the control group (**Figure 3**). No other differences were observed in BDNF expression levels. There was a large increase in the mean expression of NT3 in DRG from the 12-week, 12.5 g cm injury group, however due to high variance no significant differences were observed from 6 to 12 weeks.

EXPRESSION OF Trk RECEPTORS IN THE SPINAL CORD

Expression levels of mRNA for neurotrophin receptors TrkA, TrkB, and TrkC were assessed from samples of lumbar SC (L4/5). In the groups that received a 12.5 g cm injury, the level of TrkA in SC from the 12-week group was significantly greater than that from the 6-week group, whereas there was no significant difference between the two post-SCI times in the 25 g cm injury group. Like TrkA, the level of TrkC in SC from the 12-week 12.5 g cm group was significantly greater than that from the 6-week group, with no significant difference between the two post-SCI times in the 25 g cm injury group. No significant changes in TrkB expression levels were detected between any groups (**Figure 4**).

EXPRESSION OF NEUROTROPHINS IN THE SPINAL CORD

The results for neurotrophins in the SC are displayed differently from the data regarding expression levels of neurotrophins and Trk receptors in the DRG, and Trk receptors in the SC. In the latter assessments, the expression of neurotrophins and trks did not differ between the 6- and 12-week control animals. Thus, those data were analyzed and presented relative to the mean and variation of a single unified control group. This allowed us to simultaneously assess the effect of both injury severity and survival time on gene

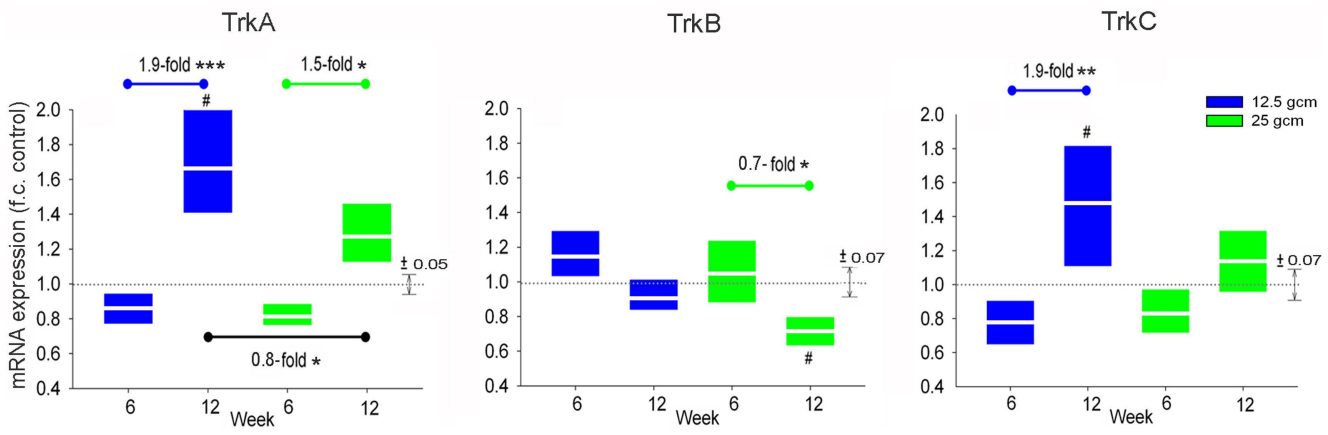

FIGURE 2 | mRNA expression of Trk receptors is altered in L4/5 DRG at 6 and 12 weeks after receiving either 12.5 g cm (blue) or 25 g cm (green) NYU contusion injury to spinal cord at vertebral level T9. Fold-change (FC) is reported as change of 12 week relative to 6 week time points in all figures. Black bar on TrkA reports fold-change (fc) of 25 g cm at 12 weeks relative to 12.5 g cm at 12 weeks. X axis denotes weeks post injury. White lines in box-plots indicate group mean. Dotted gray lines indicate expression level of controls (normalized to 1), with ±SEM indicated by the vertical arrows at right end of the control line. #$p < 0.05$ vs. control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

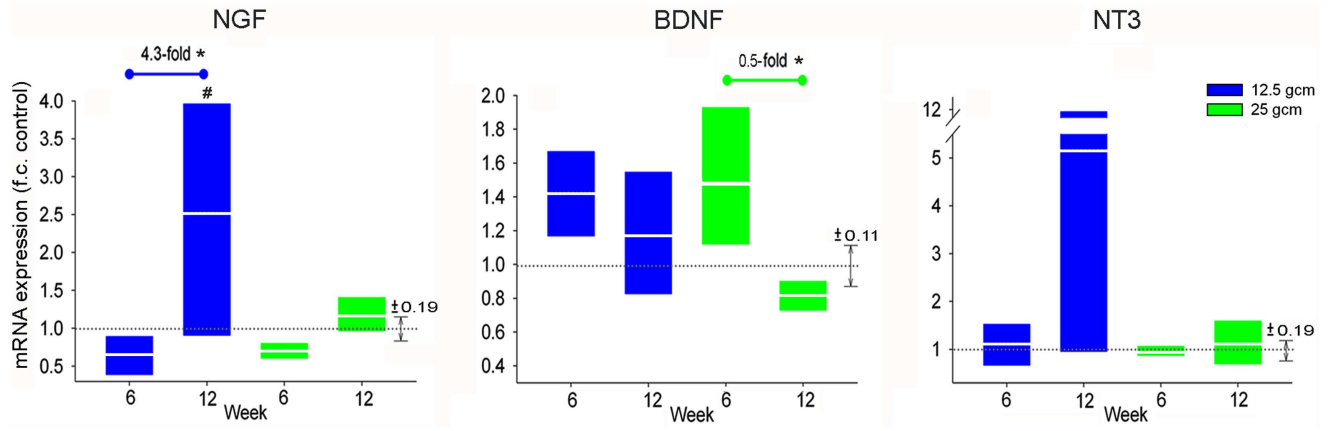

FIGURE 3 | mRNA expression of Neurotrophins is altered in L4/5 DRG at 6 and 12 weeks after receiving either 12.5 g cm (blue) or 25 g cm (green) NYU contusion injury to spinal cord at vertebral level T9. Fold-change (fc) is reported as change of 12 week relative to 6 week time points in all figures. X axis denotes weeks post injury. White lines in box-plots indicate group mean. Dotted gray lines indicate expression level of controls (normalized to 1), with ±SEM indicated by the vertical arrows at right end of the control line. #$p < 0.05$ from control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

expression. For the neurotrophin genes in SC, however, expression differed significantly between the 6- and 12-week control groups (**Figure 5A**). We first analyzed these gene expression data exactly as was done for the other tissues – comparing each injury severity and survival time to the mean and variation of a single unified control group – but for the sake of clarity we have presented the data from the individual animals in each group. Caution must be exercised when considering the expression data for the experimental groups in this analysis (**Figure 5A**) because of the use of a unified control group – i.e., these data were generated exactly as were the other expression values, but are relative to a unified control group that, in this case, is not a suitable control group. We found decreases between our 6 and 12 week control groups in expression levels of NGF, BDNF, and NT3 in the SC in the absence of SCI. It is worth noting that our quality control measures were repeated for these samples, but the assessments remained the same. In ruling out technical issues and variability due to the necessity of using animals from different litters, a single factor appears to account for the altered expression levels in the control groups, that being age.

Because the gene expression differed between the 6- and 12-week control groups, we cannot incorporate the temporal characteristic of the experimental design in our assessment of neurotrophin expression in SC. We are limited to analyzing the effect of injury severity on gene expression within each separate survival time group, where the data from experimental groups is expressed relative to the time-matched control group only (**Figure 5B**). Considered in this way, SCI itself did not significantly influence expression of any neurotrophin at any time considered, with the

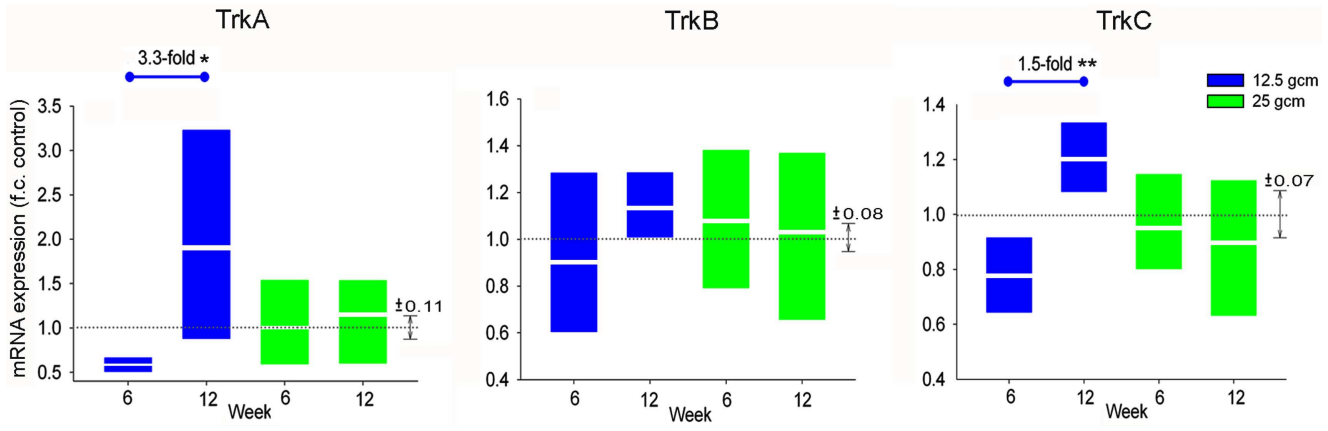

FIGURE 4 | mRNA expression of Trk receptors is altered in L4/5 spinal cord at 6 and 12 weeks after receiving either 12.5 g cm (blue) or 25 g cm (green) NYU contusion injury to spinal cord at vertebral level T9. Fold-change (fc) is reported as change of 12 week relative to 6 week time points in all figures. *X* axis denotes weeks post injury. White lines in box-plots indicate group mean. Dotted gray lines indicate expression level of controls (normalized to 1), with ±SEM indicated by the vertical arrows at right end of the control line. #$p < 0.05$ from control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

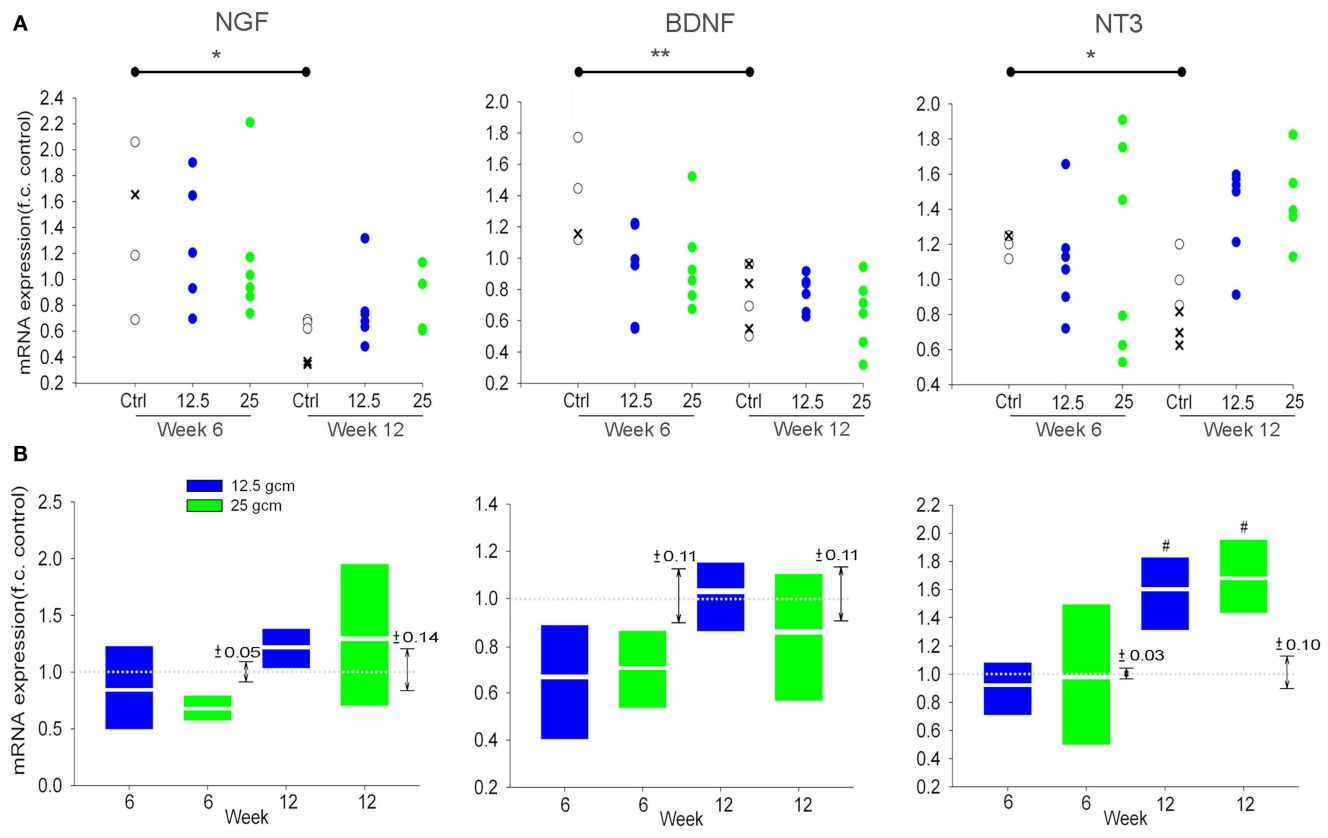

FIGURE 5 | (A) Scatterplots of Neurotrophin mRNA expression in L4/5 SC at 6 and 12 weeks after receiving either 12.5 g cm (blue) or 25 g cm (green) NYU contusion injury to spinal cord at vertebral level T9, *relative to the unified control group as was done for the other data. x*'s represent mRNA expression of age-matched naive animals. Open circles represent expression of age-matched laminectomy control animals. *$p < 0.05$, **$p < 0.01$ **(B)** mRNA expression of Neurotrophins in L4/5 SC at 6 and 12 weeks after receiving either 12.5 g cm (blue) or 25 g cm (green) NYU contusion injury to spinal cord at vertebral level T9, *relative to their respective age-matched control groups. X* axis denotes weeks post injury. White lines in box-plots indicate group mean. Dotted gray lines indicate expression level of controls (normalized to 1), with ±SEM indicated by the vertical arrows to the right of each time point pair. #$p < 0.05$ from control.

exception of NT3 at 12 weeks post-SCI. At this time, NT3 was elevated relative to the time-matched control group, with no effect of injury severity.

RELATIONSHIP OF TRANSCRIPTIONAL ASSESSMENTS TO FUNCTIONAL AND ANATOMICAL ASSESSMENTS

Our experimental design was intended to embrace the variability that exists with models of contusive SCI in that we also examined whether a statistical correlation existed between expression levels of each transcript and BBB or white matter sparing on an animal by animal basis. We observed no statistically significant correlation between the expression levels of the transcripts and BBB score or white matter sparing.

COORDINATED EXPRESSION OF NEUROTROPHINS AND Trk RECEPTORS IN DRG AND SPINAL CORD 12 WEEKS POST INJURY

To further characterize the relationship between the neurotrophins and their receptors in lumbar DRG and SC, we analyzed the expression levels of neurotrophins and Trk receptors relative to each other, and without respect for injury severity. In the SC, the only significant relationship was that of TrkB and TrkC in the control and 6 week groups. No relationship was found between any other expression levels at any time points in the SC (**Table 3**). In the DRG, there was a relationship between NGF and NT3 in all groups. In the 6-week groups the only other significant correlation observed was between BDNF and TrkB. After 12 weeks there was a significant correlation in the expression levels of all neurotrophins in the DRG, a relationship that existed for the Trk receptors as well (**Table 4**). Additionally, a significant correlation was observed between expression levels of neurotrophins and their cognate Trk receptors at 12 week time points (**Table 4**). This coordinated expression pattern occurred in all animals independent of injury severity (**Figures 6** and **7**). The reliability of this statistical assessment is enhanced by our performing the reverse-transcription for all samples with the same reagents, a procedure which reduces the cross-sample variability.

BIOINFORMATIC ANALYSIS OF NEUROTROPHIN AND Trk RECEPTOR GENE REGULATION

In light of the apparent coordinated expression of neurotrophins and trk receptors in DRG at 12 weeks after SCI, we used bioinformatic analyses to examine some possible mechanisms that may be at play. In order to assess possible coordination of regulation via gene promoters, we retrieved from the TransFac database (Wingender et al., 2000; Wingender, 2008; gene-regulation.com) all transcription factors (TFs) known/predicted to bind to (1) the annotated promoter region or (2) the sequence 1 kb upstream of the annotated translation start-site if the annotated promoter was less than 1 kb, of all six genes examined here. For this procedure the RGSC 5.0/rn5 (March 2012; genome.ucsc.edu) rat genome assembly was used and all sequences and locations are relative to this assembly (**Table 5**). These broad results were filtered for those TFs with annotations indicating expression in nervous tissue, and results for different transcript entries for the same gene were pooled. TrkC was the only gene to lose all TFs in this filtering process, reflecting the fact that the assembled sequence upstream of the TrkC gene has numerous stretches of undefined bases, and that the annotated promoter is very short. In

Table 3 | Correlations between expression of mRNA for trk receptors in spinal cord.

	Control		6 week		12 week	
	***r*-Value**	***p*-Value**	***r*-Value**	***p*-Value**	***r*-Value**	***p*-Value**
TrkA vs. TrkB	0.47	0.2	0.25	0.46	0.19	0.56
TrkA vs. TrkC	0.42	0.26	0.21	0.53	0.4	0.19
TrkB vs. TrkC	0.75	**0.01**	0.76	**0.004**	0.44	0.16

Data in bold are statistically significant.

Table 4 | Correlations between expression of mRNA for neurotrophins, Trk receptors, and cognate pairs in DRG.

	Control		6 week		12 week	
	***r*-Value**	***p*-Value**	***r*-Value**	***p*-Value**	***r*-Value**	***p*-Value**
NGF vs. BDNF	0.20	0.61	0.15	0.69	0.84	**0.001**
NGF vs. NT3	0.72	**0.03**	0.87	**0.003**	0.92	**0.00006**
BDNF vs. NT3	0.10	0.80	0.33	0.38	0.88	**0.0004**
TrkA vs. TrkB	0.04	0.90	0.005	0.989	0.89	**0.0007**
TrkA vs. TrkC	0.56	0.11	0.61	0.08	0.79	**0.006**
TrkB vs. TrkC	0.40	0.28	0.38	0.31	0.78	**0.004**
NGF vs. TrkA	0.65	0.06	0.55	0.13	0.88	**0.0004**
BDNF vs. TrkB	0.45	0.22	0.69	**0.04**	0.77	**0.005**
NT3 vs. TrkC	0.57	0.11	0.61	0.08	0.77	**0.006**

Data in bold are statistically significant.

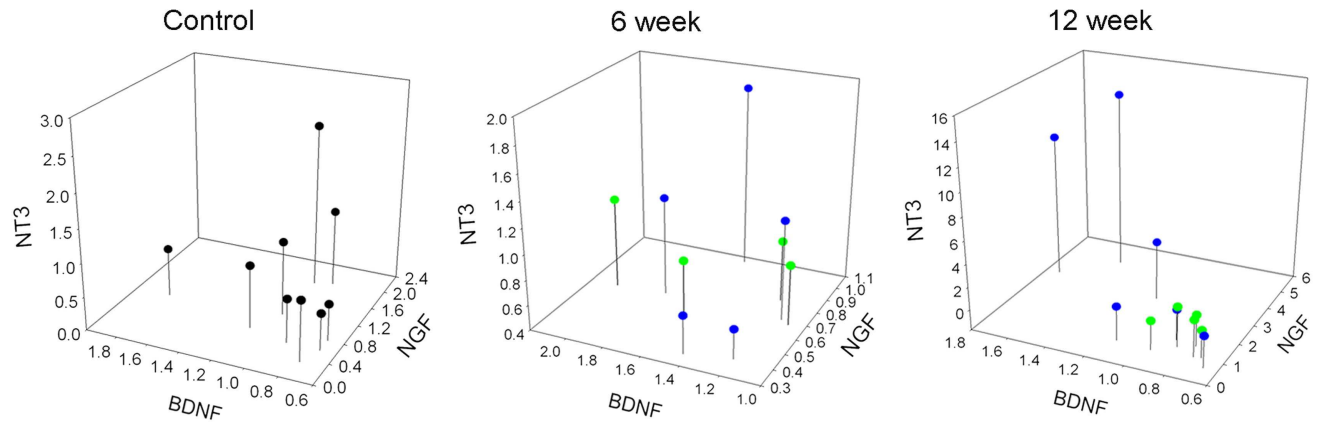

FIGURE 6 | Correlated expression of neurotrophins in DRG emerges at chronic time points. Values represent fold-change of the individual animals vs. mean of control group. Blue dots represent animals with 12.5 g cm injuries. Green dots represent animals with 25 g cm injuries.

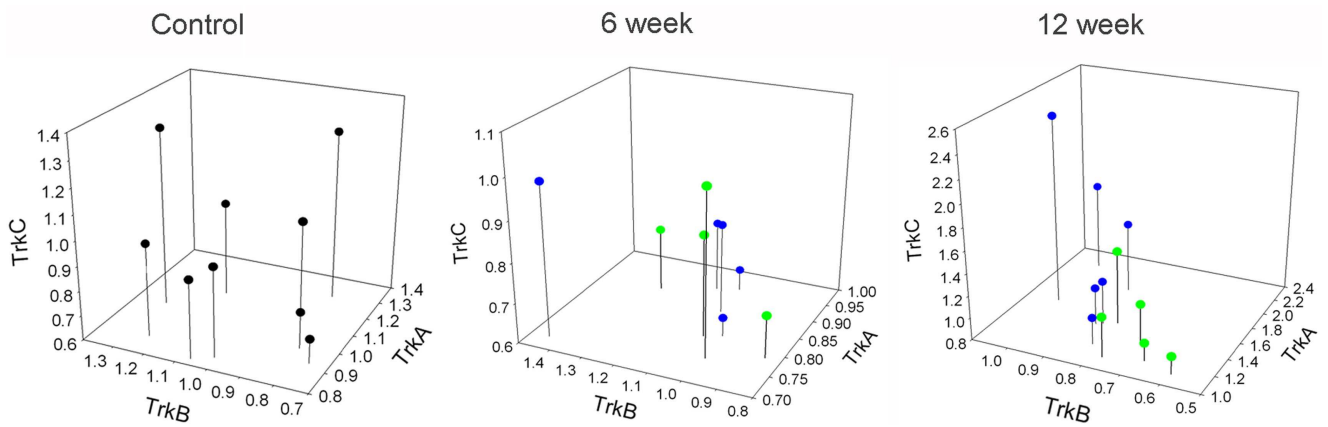

FIGURE 7 | Correlated expression of Trk receptors in DRG emerges at chronic time points. Values represent fold-change of the individual animals vs. mean of control group. Blue dots represent animals with 12.5 g cm injuries. Green dots represent animals with 25 g cm injuries.

spite of this, numerous TFs remained for three or more genes, and four TFs remained for all genes (except trkC) – cyclic AMP response element binding protein (CREB), MafB, NeuroD, and Pax3 (**Table 6**).

Another possible means of regulating the levels of mRNA is by micro-RNA (miRNA), which can influence the stability and/or turn-over rate of transcripts, among other effects (e.g., Kosik and Krichevsky, 2005). In order to assess possible coordination of regulation via miRNA, we retrieved from the TargetScan database those miRNA-binding sites that are conserved between human and rat neurotrophin and trk genes (TargetScanHuman release 6.2, e.g., Grimson et al., 2007; targetscan.org; **Table 7**). Although numerous miRNA species were retrieved, none were shared across any of the neurotrophin and trk genes.

DISCUSSION

EXPRESSION AND FUNCTION OF NEUROTROPHINS AND Trks

Spinal cord injury engenders a host of changes to both the central and peripheral nervous system, indeed for the entire organism, with residual functional capacity that is largely dependent on the location and severity of the injury. A variety of different approaches have been used in efforts to re-establish function, including enhancement of regeneration across the injury site (e.g., Bregman et al., 2002; Moon and Bunge, 2005; Sharma et al., 2012; Smith et al., 2012) and plasticity of intact circuits below the level of the lesion (e.g., Edgerton et al., 2004; Boulenguez and Vinay, 2009; Rossignol and Frigon, 2011). One means for achieving plasticity of intact circuits is through activity-dependent reorganization of inputs (e.g., Edgerton et al., 2004). This phenomenon has been described in studies of both animal (reviewed in Edgerton et al., 2008) and human (reviewed in Harkema, 2008) of SCI. Neurotrophins have been implicated as having a role in such changes (Hutchinson et al., 2004; Boyce et al., 2007, 2012; Côté et al., 2011). However, activity-dependent changes in the capacity for locomotion often manifest at times later than those examined in studies of post-SCI expression of neurotrophins and trk receptors (De Leon et al., 1998, 1999; **Table 3**). Indeed, the dynamic period of spontaneous locomotor recovery generally lasts for approximately

Table 5 | Genomic coordinates used for Bioinformatic analyses.

Gene	RefSeq	Chromosome	CDS Beg	CDS End	Strand	5′UTR Beg	5′UTR End	3′UTR Beg	3′UTR End
Ntrk1 (TrkA)	NM_021589	chr2	206548727	206565310	–	206565311	206570310	206543727	206548726
Ntrk2 (TrkB)	NM_012731.2	chr17	8158054	8463473	–	8463474	8468473	8153054	8158053
Ntrk2 (TrkB)	NM_001163168.1	chr17	8340214	8463473	–	8463474	8468473	8335214	8340213
Ntrk2 (TrkB)	NM_001163169	chr17	8389944	8463473	–	8463474	8468473	8384944	8389943
Ntrk3 (TrkC)	NM_001270655.1	chr1	140868438	141239903	–	141239904	141244903	140863438	140868437
Ntrk3 (TrkC)	NM_001270656.1	chr1	140868438	141239903	–	141239904	141244903	140863438	140868437
Ntrk3 (TrkC)	NM_019248.1	chr1	140868438	141239903	–	141239904	141244903	140863438	140868437
NGF	NM_001112698.1	chr2	224368770	224369496	+	224363770	224368769	224369497	224374496
NGF	NM_013609.2	chr2	224362515	224369496	+	224357515	224362514	224369497	224374496
BDNF	NM_001270631	chr3	107418271	107419021	+	107413271	107418270	107419022	107424021
BDNF	NM_001270632	chr3	107418271	107419021	+	107413271	107418270	107419022	107424021
BDNF	NM_001270633	chr3	107418271	107419021	+	107413271	107418270	107419022	107424021
BDNF	NM_001270634	chr3	107418271	107419021	+	107413271	107418270	107419022	107424021
BDNF	NM_001270635	chr3	107418271	107419021	+	107413271	107418270	107419022	107424021
BDNF	NM_001270636	chr3	107418271	107419021	+	107413271	107418270	107419022	107424021
BDNF	NM_001270637	chr3	107418271	107419021	+	107413271	107418270	107419022	107424021
BDNF	NM_001270638	chr3	107418271	107419021	+	107413271	107418270	107419022	107424021
BDNF	NM_001270630	chr3	107390677	107419021	+	107385677	107390676	107419022	107424021
BDNF	NM_012513	chr3	107371964	107419021	+	107366964	107371963	107419022	107424021
Ntf3 (NT3)	NM_031073	chr4	225639116	225705803	–	225705804	225710803	225634116	225639115
Ntf3 (NT3)	NM_001270869	chr4	225639116	225705803	–	225705804	225710803	225634116	225639115
Ntf3 (NT3)	NM_001270868	chr4	225639116	225675123	–	225675124	225680123	225634116	225639115
Ntf3 (NT3)	NM_001270870	chr4	225639116	225639893	–	225639894	225644893	225634116	225639115

6 weeks after SCI, a time well beyond most prior studies (**Table 3**). In addition to a likely role in locomotor function, neurotrophin signaling is implicated in pathologic outcomes of plasticity such as post-SCI pain and autonomic dysreflexia (e.g., Brown and Weaver, 2012). The role of neurotrophin signaling has principally been examined in terms of initiation of these conditions in the near-term after SCI in animal models (Krenz et al., 1999; Marsh et al., 2002; Cameron et al., 2006), as opposed to later-phase initiation or maintenance. The regulation we have demonstrated at extended time points may provide new rationale for examining the role of neurotrophin signaling in later stages of these conditions.

Neurotrophins exert modulatory effects on cellular physiology through activation of their cognate Trk receptors (Lindsay, 1996; Patapoutian and Reichardt, 2001; Huang and Reichardt, 2003). In the DRG, expression of neurotrophin receptors is restricted to specific populations of cells. Generally, TrkA is expressed in neurons with small soma size, TrkB in neurons with intermediate size, and TrkC in neurons with large soma size; populations of TrkA and TrkC expressing neurons remain largely separate, whereas TrkB is co-expressed in overlapping populations of TrkA and TrkC positive cells (Mu et al., 1993; McMahon et al., 1994; Wright De, 1995; McMahon, 1996; Phillips and Armanini, 1996). Trk receptors are not ubiquitous, however, as there is a large subpopulation of small diameter DRG neurons which do not express any of the Trk receptors or the low-affinity neurotrophin receptor p75 in the adult (McMahon et al., 1994; Molliver and Snider, 1997; Bennett et al., 1998). In the mammalian SC, TrkA is expressed in second order nociceptors of the dorsal horn, TrkB has a broad pattern of expression which overlaps with both TrkA and TrkC expression, and TrkC is expressed in neurons of the intermediate and ventral horn (e.g., Duberley et al., 1997; Curtis et al., 1998; Schober et al., 1999; Copray and Kernell, 2000; Liebl et al., 2001; Allen Institute for Brain Science, 2009).

As long as 6 weeks after SC transection injury, the number of cells expressing TrkA and TrkB protein in L1 and L6/S1 DRG (containing bladder afferents) increases over controls, though the numbers of cells expressing these genes does not significantly change in L4/5 DRG (Qiao and Vizzard, 2002, 2005). Our analysis of Trk expression, which was also performed in L4/5 DRG and included a 6-week post-SCI time point, found no significant change in the trkA or trkB mRNA levels for either severity of contusion injury, in agreement with the prior work. In intact sensory and sympathetic ganglia of the adult rat, NGF and NT3 (as well as TrkA, full length TrkB, and TrkC), localize exclusively to neurons; BDNF and the truncated isoform of TrkB are expressed more extensively, however, localizing to neuronal cells and some glial and satellite cells (Wetmore and Olson, 1995). These observations are consistent with the notion that full length Trk expression predominantly occurs in neurons, though since the latter study was performed with intact animals, we cannot exclude the possibility that our injuries potentially resulted in expression in other cell types. Indeed, there are numerous reports of trk receptor expression by non-neuronal cells. In particular Schwann cells can express trks, as can cancer cells (e.g., Funakoshi et al., 1993; Tacconelli et al., 2005; Hess et al., 2007; Jin et al., 2011). Further, neurotrophins are

Table 6 | Transcription factor binding sites for neurotrophin and trk receptor genes.

TF Binding-site name	HGNC symbol	TrkA	NGF	TrkB	BDNF	TrkC	NTF3
AhR	AHR	x		x	x		
AhR: Arnt					x		
AP-1	FOS; FOSB; JUN; JUND		x		x		
AP-2	TFAP2A	x	x	x	x		
AR	AR			x	x		
Arnt	ARNT			x			
ATF	ATF	x		x			
ATF2	ATF2	x	x	x	x		
ATF2: c-Jun			x				
Brn-2	POU3F2				x		
C/EBP	CEBPA, B, D, E, G, Z	x			x		
CAR	NR1I3	x					
c-Ets-2	ETS2		x				
c-Jun	JUN	x		x			
c-Myc: Max		x					
COUP-TF1	NR2F1		x		x		x
CREB	CREB1	x	x	x	x		x
CREB, ATF					x		
CREM	CREM	x		x			
DEC	BHLHE40	x	x		x		
E2A	TCF3			x			
Ebox	TCF3; MYOD1; MYOG			x			
ER-alpha	ESR1				x		x
Ets	ETS1, 2; ETV1, 2, 3, 4, 5, 6, 7		x		x		x
Foxj1	FOXJ1						x
FOXO1	FOXO1						x
GATA-3	GATA-3				x		x
GR	NR3C1		x	x	x		
HES1	HES1			x			x
HOXA5	HOXA5		x				
HOXB8	HOXB8	x					
KROX	EGR1, 2; ZNF22; ZBTB7B				x		
MAF	MAF		x	x	x		x
MAFB	MAFB	x	x	x	x		x
Max	MAX	x			x		x
MEF-2	MEF-2A						x
MEF-2C	MEF-2C				x		
Myc	MYC	x			x		
Neuro D	NEUROD1	x	x	x	x		x
NFAT1	NFATC2	x	x		x		x
NF-AT4	NFATC3	x	x		x		x
NF-kappaB	NFKB1		x				
NKX2B	NKX2-2		x				
NRSF	REST		x				
NURR1	NR4A2	x	x		x		
Oct-1	POU2F1		x		x		
Octamer	POU family of proteins		x		x		
Oct-x	STAT1		x				
p53	TP53						x
Pax3	PAX3	x	x	x	x		x
Pax6	PAX6						x

(Continued)

Table 6 | Continued

TF Binding-site name	HGNC symbol	TrkA	NGF	TrkB	BDNF	TrkC	NTF3
Pax8	PAX8		x	x	x		
Pbx1	PBX1		x		x		x
POU6F1	POU2F1				x		
POUF2F1	POU6F1		x				
PPARgamma	PPARG			x			
PPARgamma: RXR-alpha				x			
PXR	NR1I2	x					
RXR-alpha	RXRA			x	x		
SF1	SF1						x
SMAD	MADH family of proteins				x		x
Smad3	SMAD3				x		x
Sox1	SOX1				x		x
Sox2	SOX2		x				
Sp1	SP1	x	x		x		x
SRF	SRF		x				
Sry	SRY		x		x		x
STAT	SOAT1		x		x		x
STAT1	STAT1		x				
STAT3	STAT3		x		x		
Tax	CNTN2			x			
Tax/CREB					x		x
Tbp	TBP			x	x		x
TCF4	TCF4	x					
Tcfap2a	TFAP2A				x		
Tcfap2b	TFAP2B				x		
Tst-1	CCDC6		x				
USF	USF1				x		
USF2	USF2				x		
VDR	VDR	x					
VDR, CAR, PXR					x		x

Entries with transcription factors separated with a ":" have binding sites situated such that they act in a cooperative fashion, rather than independent from each other. Entries with transcription factors separated by a "," share binding sites or have binding sites situated near each other such that the factors act in competition with each other.

often expressed in non-neuronal cells, most notably by cells outside the nervous system where they influence both developmental and adult processes (e.g., Lewin, 1996; Petruska and Mendell, 2004).

Previous assessments of changes in neurotrophin/Trk receptor expression levels after SCI have typically focused at time points of less than 6 weeks. BDNF expression increases up to 2 weeks after injury in the SC after thoracic transection and crush injury (Hayashi et al., 2000; Li et al., 2007), though both increases and decreases in expression have been reported after hemisection during a similar time period post injury (Gulino et al., 2004; Qin et al., 2006). Expression levels of NGF and NT3 in the cord increase for up to 3 weeks after SCI (Hayashi et al., 2000; Li et al., 2007). In another study, NGF and BDNF transcripts were found to increase up to 4 days following injury in the adult cord, however, by 2 weeks post injury all neurotrophins were expressed at levels similar to that of control (Nakamura and Bregman, 2001; Widenfalk et al., 2001), suggesting expression decreases after an early increase, though these studies used different injury models. Trk mRNA expression is downregulated acutely in the SC at and around the injury site after contusion (Liebl et al., 2001; Hajebrahimi et al., 2008), however by 6 weeks expression levels are not different from control (Liebl et al., 2001). However, after SC transection TrkC has been shown to increase after 2 weeks (Qian et al., 2011). Similarly, in a recent study assessing mRNA and protein changes after transection at 10 and 31 days post injury, whole SC TrkB mRNA was elevated at 10 days post injury, and whole SC NT3 and TrkB protein was elevated at 31 days post injury, with expression differences also observed depending on the location within the parenchyma of the SC (Keeler et al., 2012). **Table 1** summarizes the findings of recent experiments to facilitate comparison of these results.

We found TrkA expression increases in both the DRG and SC of animals after contusion in a manner that was dependent on injury severity. This finding is of particular interest with regards to the functions of NGF and TrkA. NGF plays a well-defined role in sensitization of nociceptive afferent neurons (e.g., Shu

Table 7 | Micro-RNA binding sites for neurotrophin and trk receptor genes.

Gene symbol	miRNA symbol
Ntrk1 (TrkA)	n/a
Ntrk2 (TrkB)	rno-miR-325p
Ntrk2 (TrkB)	rno-mir-211
Ntrk2 (TrkB)	rno-miR-204
Ntrk3 (TrkC)	rno-mir-128
Ntrk3 (TrkC)	rno-miR-466b
Ntrk3 (TrkC)	rno-miR-297
Ntrk3 (TrkC)	rno-miR-3592
NGF	rno-let-7e
NGF	rno-let-7d
NGF	rno-let-7b
NGF	rno-let-7c
NGF	rno-let-7a
NGF	rno-miR-98
NGF	rno-let-7f
NGF	rno-let-7i
BDNF	rno-miR-10a-5p
Ntf3 (NT3)	rno-miR-222
Ntf3 (NT3)	rno-miR-221

and Mendell, 1999, 2001; Galoyan et al., 2003; Zhu et al., 2004b). Nociceptive DRG neurons undergo changes after SCI, including development of spontaneous activity (Bedi et al., 2010) and an enhanced intrinsic growth promoting state (Bedi et al., 2012). Such changes in anatomical and physiological properties of nociceptors may contribute to development of conditions such as autonomic dysreflexia (e.g., Marsh et al., 2002). TrkA antagonists prevent the sensitization (thermal and mechanical hyperalgesia) normally induced by partial nerve injury (Ma et al., 2010), and antagonism of TrkA signaling has been effective for controlling human pain (Mantyh et al., 2011). Hence, elevation in the levels of TrkA and NGF in response to contusive injury could play a role in some of the maladaptive processes after incomplete SCI.

TrkB activation has also been implicated in hypersensitivity to nociceptive input and sensitization of nociceptors (Kerr et al., 1999; Shu et al., 1999; Garraway et al., 2003). However, after either SC transection or contusion injury, BDNF induced facilitation of afferent responses in lamina II of the dorsal horn is significantly reduced (Garraway et al., 2005; Garraway and Mendell, 2007). Our results could suggest a mechanism for those physiological observations. In addition to TrkB expression in populations of second order nociceptive neurons (Schober et al., 1999), it is expressed robustly throughout the interneuronal circuitry, and also co-expressed along with NT3 in motoneurons (Buck et al., 2000), a finding corroborated in humans (Josephson et al., 2001). Notably, BDNF administration to the injured SC can improve locomotor outcomes, however because of its influence on nociceptive circuitry its therapeutic utility may be limited (Boyce et al., 2012).

In DRG, TrkC is present on medium to large diameter muscle spindle afferents that make monosynaptic connections with motoneurons and cutaneous low threshold mechanoreceptors (Klein et al., 1994; Oakley et al., 1997; Josephson et al., 2001) in the intermediate and ventral horns of the SC. NT3, likely acting via TrkC, exerts a modulatory effect on sensorimotor circuits in both intact (Petruska et al., 2010) and injured preparations (Mendell et al., 2001; Arvanian et al., 2003; Arvanian et al., 2006a,b; García-Alías et al., 2011; Schnell et al., 2011). Locomotor training after SCI is associated with increased expression levels of TrkB and TrkC agonists in rats (Hutchinson et al., 2004; Côté et al., 2011). In addition, co-administration of both BDNF and NT3 to the injury site has been shown to improve hindlimb locomotion after transection in both rats (Blits et al., 2003) and cats (Boyce et al., 2007). Taken together, these findings suggest a potential role for Trk activation in modulation of lumbar sensorimotor circuitry in both intact and injured animals.

The apparent age-related regulation of NGF, BDNF, and NT3 in non-injured SC was unexpected and we made significant efforts to identify possible technical and sampling issues. While those factors that often account for variability did not satisfactorily account for the expression patterns we observed, the single factor of age did appear to fully account for the differences. Expression of the neurotrophins has been examined in the context of embryonic and postnatal development and in aging (e.g., Timmusk et al., 1994; Nosrat, 1998; Bergman et al., 2000). However, to the best of our knowledge, there has been no systematic assessment of the regulation of the neurotrophins at such late postnatal times. If it is indeed borne out that neurotrophins are regulated in the SC over a long postnatal time course, this must be taken into account when designing experiments that may be influenced by the natural progression of this expression.

COORDINATED EXPRESSION OF NEUROTROPHINS AND Trks

Twelve-weeks after injury a coordinated expression pattern existed among the levels of all neurotrophins and Trk receptors regardless of injury severity, and also between the neurotrophins and their cognate Trk receptors in the DRG (**Tables 1** and **2**, **Figures 5** and **6**), a relationship that was not evident at 6 weeks post-SCI. Although there are reports of smaller groups of neurotrophins and/or Trks being regulated in a coordinated fashion (e.g., Widenfalk et al., 1999), to our knowledge this degree of coordination has not been reported, and the mechanism is unclear. One obvious possibility is a feedback/feed-forward relationship between some/all of these genes, and these sorts of relationship do exist (e.g., Michael et al., 1997; Wyatt et al., 1999; Gibbons and Bailey, 2005).

Neurotrophin dependent neurotrophin expression has been demonstrated *in vitro* in NIH3T3 and PC12 cells (Canossa et al., 1997; Mallei et al., 2004), hippocampal neurons (Canossa et al., 1997), and cerebellar granule neurons (Leingärtner et al., 1994). *In vivo*, intrathecal administration of NT3 to intact adult animals for 1 week results in reduced expression of TrkA protein in the DRG, but has no effect on levels of TrkC (Gratto and Verge, 2003). After unilateral axotomy, sub-cutaneous administration of exogenous NT3 similarly causes a decrease in TrkA on the side contralateral to the injury. This contrasts to the increase in TrkA expression seen on the side ipsilateral to the injury; the effect of NT3 on expression levels of TrkB and TrkC however is

not affected by injury in this paradigm, as levels of these transcripts show increased expression up to 4 weeks post axotomy in both ipsi- and contra-lateral DRG (Kuo et al., 2007). Such coordinated expression patterns could potentially result from changes at the epigenetic level or from interactions between the different transcription factors associated with expression of specific transcripts. During development, Runx1 and Runx3 transcription factors play essential roles in cell fate determination of nociceptive (Chen et al., 2006) and proprioceptive (Inoue et al., 2002) neurons, respectively. Much attention regarding transcriptional regulation of neurotrophin expression in the mature nervous system has been given to BDNF, due to its role in activity-dependent mechanisms during long-term potentiation (LTP). Such investigations have revealed several important transcriptional regulators including, CREB, calcium-responsive transcription factor (CaRF), and methyl CpG-binding protein 2 (MeCP2; Tao et al., 1998, 2002; Chen et al., 2003; Reichardt, 2006). Such findings may facilitate future efforts to determine the mechanisms regulating the expression of the neurotrophins and Trk receptors in the injured adult SC and sensory ganglia.

The lack of any TFs for trkC after the filtering process is more a reflection of the relative amount of data available than reality. The filtering step in the bioinformatic analysis involved the use of annotations, which, valuable though they are, have inherent limitations. Certainly there are published data regarding factors involved in regulating the expression of trkC, particularly Runx3 (Levanon et al., 2002), Brn3a/Pou4f1 and Runx1 (Zou et al., 2012), and REST/NRSF (Nakatani et al., 2005).

In spite of the lack of any result related to TrkC, four TFs did emerge as possibly interacting with all of the remaining genes. The majority of published information related to these genes and their involvement in regulation of neurotrophins and Trks is in the context of development or cancer. This does not imply that they function exclusively in those contexts, but only that those contexts are the most studied. We could not identify any studies examining Pax3, NeuroD, or MafB in SC or DRG in the context of SCI. Maf has been studied in relation to neurodegeneration (Kobayashi et al., 2011) and in stress (Machiya et al., 2007). Pax3 was studied in relation to nerve injury, where it was found to not be regulated (though this does not imply it not being active; Vogelaar et al., 2004).

There are studies examining CREB in SC (Crown et al., 2005, 2006; Yu and Yezierski, 2005; Yune et al., 2008) or DRG (Qiao and Vizzard, 2005) in the context of SCI, with the latter study examining TrkA, TrkB, and CREB, though not in direct relation to each other. Interestingly, the expression of activated CREB in the DRG changed over the course of the first 6 weeks after SCI, with the levels at 6 weeks being significantly greater than controls, though not in the DRG we examined here. Other studies demonstrate induction of CREB in injured/stressed neurons and also in neurons post-synaptic to stressed sensory neurons (e.g., Ji and Rupp, 1997; Bedogni et al., 2003; Choi et al., 2003; He et al., 2003; Zhu et al., 2004a), while others demonstrate CREB regulating multiple NTs (Bender et al., 2001), in at least one case by interacting with cytokines (Otten et al., 2000).

Unlike the results of the analysis of the gene promoter regions using the TransFac database, the set of miRNAs that emerged from the TargetScan analysis of gene 3′-UTRs were not shared across multiple genes. It should be noted that these analyses necessarily have certain differences that certainly impacted the results. Most notable is that TargetScan returns only those miRNA targets that have experimental confirmation. The data in **Table 7** could therefore be considered a snapshot of the current experimental data regarding which miRNA species interact with those genes (Saba et al., 2008; Guidi et al., 2010; Natera-Naranjo et al., 2010; Rau et al., 2010; Smith et al., 2010; Benoit and Tenner, 2011; Farrell et al., 2011; Kawahara et al., 2011, 2012; Yu et al., 2011, 2012; Brandenburger et al., 2012; Hamada et al., 2012; Ryan et al., 2012; Wang et al., 2012). Thus, although our analysis revealed no common miRNA species that interacted with multiple genes (representing a possible common regulatory mechanism), this may yet be the case.

Because our data are derived from homogenized tissue, we cannot make any conclusions about the cellular basis of this apparent coordinated expression. That is, we cannot determine which aspect, if any, of this coordination is occurring within single cells, or if it is simply occurring within the same tissue but arises through expression of different combinations of genes across different cells. Considerations of this issue here are at best speculative as there are virtually no studies that can provide information directly relevant to the question. Relevant information would include (1) an indication of which types of cells were expressing the genes, or at least if they were neural, non-neural, or both, (2) an indication of whether or not any combination of the genes were expressed in any single cells, and both of these would (3) have to be sampled from DRG or SC 12 weeks after SCI. We are not aware of any studies fitting these criteria (**Table 1**). Although the relationship is not direct, we can nonetheless draw from a number of sources to make inferences about what may be happening.

(1) There is some evidence that at 6 weeks after SCI Trk receptors are expressed almost exclusively in DRG neurons, much as before the SCI (Qiao and Vizzard, 2002, 2005). However, it must be noted that there is a plethora of evidence of expression of NTs and Trks in non-neuronal cells (e.g., Funakoshi et al., 1993; Elkabes et al., 1998; Nemoto et al., 1998; Noga et al., 2002; Hess et al., 2007), although much of this is in the context of cancer (e.g., Tacconelli et al., 2005; Howe et al., 2011; Jin et al., 2011). Studies which identify the cell types expressing the NTs or Trks are necessary as it is possible that at least a portion of the tissue-level regulation could be due to invading cells. Certainly the complement of immune cells in the SC is affected by injury, even in segments spatially remote from the injury (e.g., Popovich et al., 1997). Immune cells invade the DRG after nerve injury (e.g., Nguyen et al., 2007; Vega-Avelaira et al., 2009; Kim and Moalem-Taylor, 2011), but there is no indication that this possibility has been examined in DRG at any time after SCI. However, evidence suggests that the immune cells and their functions throughout the body may be affected by SCI (e.g., Popovich et al., 2001), and some express Trk receptors and/or neurotrophins (e.g., Noga et al., 2002; Nassenstein et al., 2004; Tabakman et al., 2004).

(2) There is evidence that single neurons can express certain limited combinations of the genes examined here, though to our knowledge there has been no examination of all together that could distinguish each of the Trk receptors and neurotrophins (e.g., McMahon et al., 1994; Obata et al., 2004).
(3) There are certainly studies which examine the chronic post-SCI condition, but we could not identify any that could provide data relevant to these specific considerations (i.e., they examined other readouts).

Almost irrespective of the outcome of the above considerations, there is still another consideration that can be brought to bear. Although there are a number of papers describing co-expression of some of these genes in single cells where common genetic/molecular regulation could possibly be exerted, it is highly unlikely that all the coordinated expression is accounted for by single cells. Even in the feasible condition where expression is limited to neurons, and perhaps even to the same population of neurons that expressed these genes in the intact system (i.e., differences in expression would be based on volume regulation in any given cell and not on recruitment/de-recruitment of cell populations), what is the likelihood that this degree and scope of coordinated expression could occur across different cell types independently? It seems highly unlikely that each of the genes considered here would change in a single cell type independent of its regulation in any other cell type, and still give rise to this result. However, because there is little-to-no cellular expression data here or in the literature from which to extrapolate the identity of the cells expressing these genes (i.e., immunocytochemical or *in situ* hybridization assessment of SC or DRG 12 weeks post-SCI), we must acknowledge that this is indeed possible in principle. There is, however, virtually no reason to expect that individual cells would express all of the "coordinated" genes and thus have the mechanism of coordinated regulation exist fully inside of those given single cells. Therefore, at least some of the coordination must arise *across* cells which express one or more of the "coordinated" genes.

It is possible that coordinated regulation/expression may arise due to shared direct molecular mechanisms, but the literature and our bioinformatic analyses provide little evidence for a simple mechanism of this sort. There may yet be coordinated transcriptional regulation that is indeed shared across cell types, but may reside at a level above our analyses (i.e., shared factors may be directing the actions of separate factors that then individually act on the different genes). Alternatively, there may be a shared biological process(es) or response(es) that is being executed in the various different cells – a process that has similar outcomes in terms of gene regulation but arrives there through the actions of different specific molecular entities. For "simplicity," let us consider that only the neurons of the DRG are involved. Even this cell population is not homogeneous in function, form, or sensitivity. Each of the Trk receptors is largely separately expressed. Given the dissimilarities of their regulatory sequences, they may each be directly regulated by distinct factors. However, conditions may arise that induce the non-homogeneous neuronal types, regardless of the specific Trk they express (and thus which specific factors will act on the DNA and/or mRNA), to coordinately regulate the expression of their Trk receptor. It is possible that the regulation of those specific factors may be under a control mechanism that is itself shared across the different neuron types. Our analysis would not detect this. As an example, consider cellular stress or injury. Numerous authors have reported on the regulation of Trks and neurotrophins in response to nerve injury, and the change in expression over time (e.g., Ernfors et al., 1993; Sebert and Shooter, 1993; Krekoski et al., 1996; Yamamoto et al., 1996; Bergman et al., 1999; Lee et al., 2001; Kuo et al., 2007), and many aspects of our data coincide with the reported regulation after nerve injury or neuronal stress. Intriguingly, there was another report of "coordinated regulation" associated with DRG neurons and glia in conditions of injury and/or stress (Cameron et al., 2003).

It is not clear if SCI induces any long-term injury or stress on DRG neurons. Certainly the central axons of some DRG neurons are damaged in the SCI, particularly those terminating in the affected cord, or with long axons ascending through the dorsal columns (Huang et al., 2006). However, the effect of injury to central axons differs from that of injury to peripheral axons (e.g., Stam et al., 2007), and the long-term effects on expression of neurotrophins and Trk receptors has not been examined. Injury to central axons is not the only possible source of stress to the sensory neurons, however. The inflammatory condition of the SC and continued spread of damage may induce injury or stress in the sensory neurons at times remote from the acute SCI, and at locations remote from the lesion (e.g., Popovich et al., 1997; Popovich, 2000; Bao et al., 2004, 2011; Fleming et al., 2006; Gris et al., 2008; Kwon et al., 2010; Lubieniecka et al., 2011; Ng et al., 2011; Stammers et al., 2012). There is a systemic inflammatory condition (Fleming et al., 2006; Gris et al., 2008; Bao et al., 2012) that has unknown effects on these neurons. Additionally, one must consider the effects of SCI on the peripheral tissues innervated by the sensory and motor neurons. The inflammation and altered activity/mobility/use state can impact these tissues (e.g., Edwards-Beckett and King, 1996; Lynch et al., 2000; Gris et al., 2008) with uncertain consequences for the innervating neurons. The increased expression of galanin, a neuropeptide induced in DRG neurons by stress/injury (Suarez et al., 2006), in the DRG innervating bladder and bowel (but not other DRG) after SCI (Zvarova et al., 2004) suggests that the histopathology secondary to SCI may stress the sensory neurons innervating those tissues. Tissue damage has been shown to induce stress/injury responses in sensory neurons (e.g., Ivanavicius et al., 2007; Hill et al., 2010; Thakur et al., 2012), and has been shown to affect regulation of multiple neurotrophins in the injured tissue (Vizzard, 2000).

REGULATION OF NEUROTROPHINS AND Trks AFTER SCI: ENOUGH ASSESSMENT OR NOT?

Although there are many reports examining the expression of neurotrophins and/or Trk receptors after SCI, there is relatively little overlap of the data (**Table 1**), and general principles have yet to be identified. That is not to say that the data disagree, but more that the studies have largely produced different data. Indeed, given the number of factors that influence gene regulation after SCI, much work is yet to be done. A matrix of variables demonstrated by our study and others to significantly impact the regulation of these genes suggests that over 1000 assessments would be

Table 8 | Matrix of factors influencing outcomes in SCI research.

Injury type/severity	Injury location	Region investigated	Post-SCI time
Hemisection – lateral	Cervical	Cervical	1–3 days
Hemisection – D/V	Brachial plexus	Brachial plexus	3–7 days
Transection	Thoracic	Thoracic	1–3 weeks
Contusion – mild	Lumbar	Lumbar	3–6 weeks
Contusion – moderate	Lumbar plexus	Lumbar plexus	6–12 weeks
Contusion – severe	Sacral	Sacral	12–24 weeks

required to provide relatively thorough coverage (**Table 8**). This matrix relates only to natural progression and does not include variables for the two sexes, different species and strains, and outcome measures (e.g., protein, mRNA, behavior, etc.). It would thus only be expanded when considering treatments, sex/species/strain-differences, and multiple outcome measures (some of which are mutually exclusive), each of which has been shown to influence the data (e.g., Popovich et al., 1997; Sroga et al., 2003; Kigerl et al., 2006; Beck et al., 2010).

This study examined only mRNA expression levels, which could change due to a limited set of non-mutually exclusive scenarios. Cells already expressing the specific transcripts could up- or down-regulate expression, or a different population of cells – resident or infiltrating – could begin expressing these transcripts *de novo*. Our data cannot speak to the relative contribution of these possibilities as they come from homogenized tissue. Although the literature provides some insight for the 6-week post-SCI data, this is not true for the 12-week data as neurotrophins and Trk receptors have not been examined at 12 weeks post-SCI (**Table 1**). Further, we are not aware of any work examining whether cells infiltrate the DRG after SCI.

CONCLUSION

Despite the limitations of examining only mRNA expression, this study has established that different injury severities within the same model can result in different forms of regulation of these important genes in neural tissue. It has also demonstrated that expression of these genes in neural structures providing innervation to the hindlimb changes over a time course important for experiments examining activity-dependent plasticity and also for modeling the human condition. Thus, this study (1) offers insight for interpreting published data and for designing future studies; (2) serves as a reference for mechanistic studies that manipulate the neurotrophin-Trk signaling systems, (3) indicates that injury severity, post injury time, and tissue sampled all influence the assessments of gene regulation, (4) suggests that regulation of these genes continues to occur as late as 12 weeks post-SCI, and (5) suggests that some common factor or process may be influencing expression of these genes at later times after SCI.

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