CELLULAR AND MOLECULAR MECHANISMS OF NEUROTROPHIN FUNCTION IN THE NERVOUS SYSTEM

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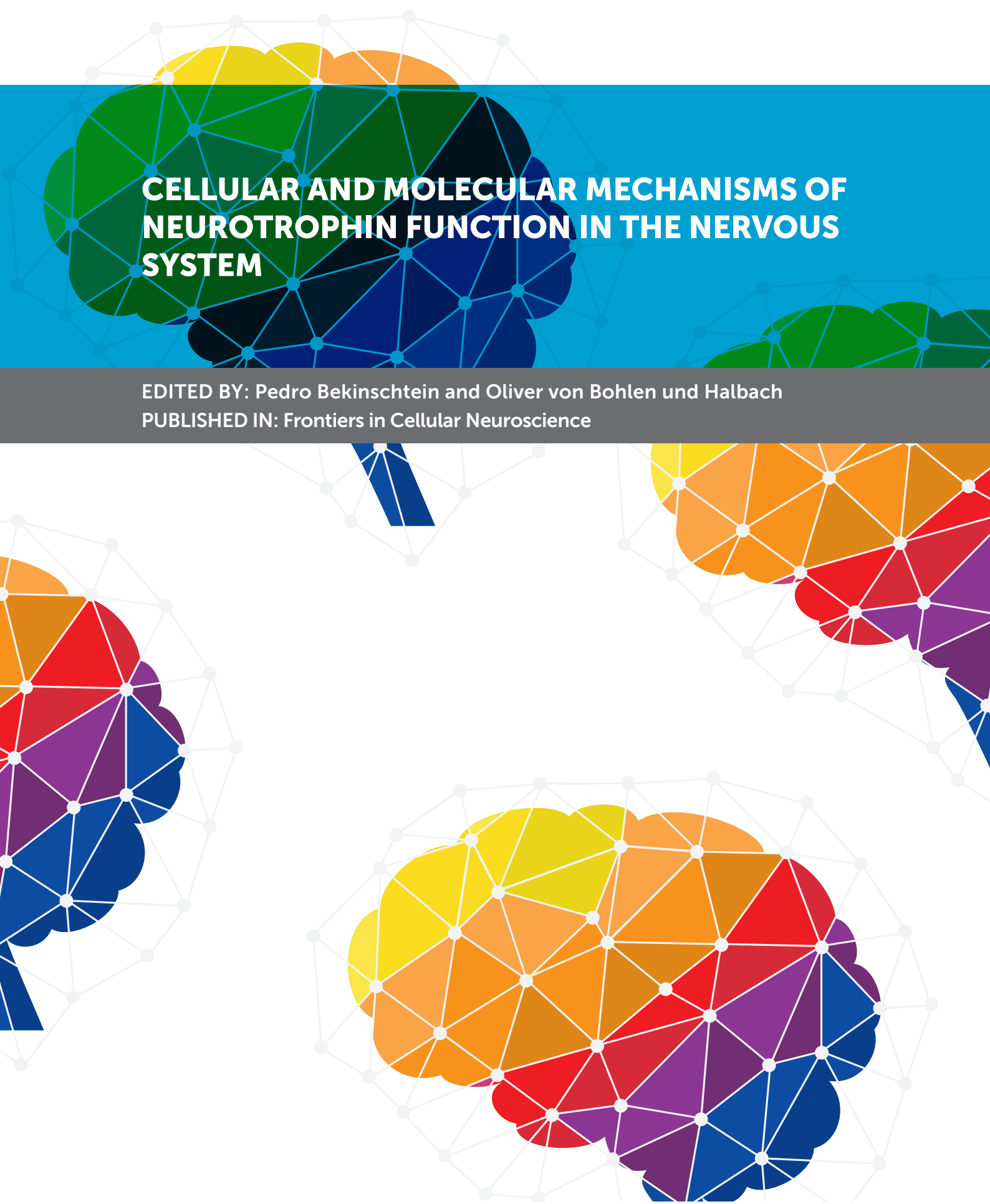

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CELLULAR AND MOLECULAR MECHANISMS OF NEUROTROPHIN FUNCTION IN THE NERVOUS SYSTEM

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Table of Contents

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EDITORIAL
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Editorial: Cellular and Molecular Mechanisms of Neurotrophin Function in the Nervous System

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Keywords: neurotrophic factors, BDNF (brain-derived neurotrophic factor), NGF (nerve growth factor), CNS—central nervous system, neuronal plasticity

Editorial on the Research Topic

Cellular and Molecular Mechanisms of Neurotrophin Function in the Nervous System

The discovery of nerve growth factor (NGF) by Rita Levi-Montalcini in the 1950s represents an important milestone in the processes that led to modern cell biology (Aloe, 2004). NGF is a member of the neurotrophin family. Neurotrophins are a family of proteins that regulate development, maintenance, and function of vertebrate nervous systems. They serve as survival factors to ensure a match between the number of surviving neurons and the requirement for appropriate target innervation and also regulate cell fate decisions, axon growth, dendrite pruning, the patterning of innervation and the expression of proteins crucial for normal neuronal function, such as neurotransmitters and ion channels. They signal through specific tyrosine kinase receptor (trkA, trkB, trkC) and the low affinity receptor p75NTR. Moreover, the precursors of the neurotrophins ("pro-neurotrophins") are discussed to be biologically active by signaling through specific receptors. Brain-derived neurotrophic factor (BDNF), NGF and the neurotrophins 3 and 4 (NT3, NT4) as well as their precursors (pro-neurotrophins) are not only expressed during development, but also in the postnatal brain.

Neurotrophins also have important functions in the mature nervous system. In particular, BDNF is involved in learning and memory and in neuronal plasticity. Changes in BDNF expression especially in the hippocampal formation are associated, among others, with psychiatric disease. BDNF levels are reduced in postmortem brain samples and in the blood of depressed patients, and these reductions are reversible by successful antidepressant treatment (Castren and Rantamaki, 2008). Moreover, in humans, the BDNF val66met polymorphism might represent a biological signature for the neuroanatomical and cognitive abnormalities commonly observed in patients suffering from bipolar disorders (Cao et al., 2016). Moreover, recent data indicate that BDNF concentrations were significantly lower in patients with attempted suicide/ideation and that therefore BDNF concentrations could serve as a response marker for antidepressant treatment in major depressive disorder (Ai et al., 2019).

Interventions like exercise or antidepressant treatment can enhance the expression of BDNF in the brain. In the review article by Miranda et al. the role of BDNF as a key molecule for memory processes in the healthy and the pathological brain is reviewed. While the role of BDNF in synaptic plasticity and in memory formation is well-known, its participation in memory reconsolidation has been less studied. In the Mini-review by Gonzalez et al. the involvement of BDNF signaling in memory reconsolidation is discussed.

The hippocampus is a key structure for spatial and declarative memory formation. Most of our knowledge on synaptic plasticity in mammals comes from hippocampal circuits, including the

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famous paradigm of long-term potentiation (von Bohlen und Halbach et al., 2018). Moreover, adult hippocampal neurogenesis is thought to be related to learning and memory functions. Interestingly, neurotrophins are involved in hippocampal long-term potentiation as well as in adult neurogenesis implicating an important role in neuronal plasticity (von Bohlen und Halbach and von Bohlen und Halbach, 2018). The Mini-review by De Vincenti et al. focuses on mechanisms that modulate and diversify BDNF functions and its implications for hippocampal synaptic plasticity, thereby shedding light to novel mechanisms that are discussed for the rapid, localized, and dynamic control of BDNF release and function. In addition to that Mini-review, the original article by Foltran et al. analyzes the differential hippocampal expression of BDNF isoforms and their receptors under diverse configurations of the serotonergic system in a mice model of increased neuronal survival. This work sheds light on the role of the different BDNF isoforms in the regulation of neurogenic process taking place in the murine hippocampal formation.

Activation of the low affinity receptor p75NTR is known to trigger synapse loss and neuronal death. These pathological features are also caused by the human immunodeficiency virus-1 (HIV) envelope protein gp120, which increases the levels of proBDNF. The research article by Speidell et al. portrays the interaction of p75NTR with the human immunodeficiency virus-1 (HIV) envelope protein gp120 in the brain and suggests that activation of p75NTR is one of the mechanisms crucial for the neurotoxic effect of gp120. Furthermore, the research article by Zhang et al. focuses on the involvement BDNF and NGF in peripheral nerve injury and indicates that increased levels of BDNF and NGF are associated with promoted sciatic nerve regeneration and improved nerve function.

Aside from the neurotrophins further substances have neurotrophic properties. Among them is the well-known family of fibroblast growth factors as well as the insulin like growth factors. The article by Chen W. et al. highlights the capacity of insulin-like growth factor-1, derived from astrocytes, to protect neurons against excitotoxicity. Recently, it has been described that interleukin 1 also plays a role in the nervous system and the article by Chen G. et al. entitled "Interleukin-1β promotes Schwann cells de-differentiation in Wallerian degeneration via the c-JUN/AP-1 pathway" indicates that interleukin 1ß plays an important role in Wallerian degeneration that is associated with alterations in p75NTR expression.

Together, the series of articles, should give a brief overview on the roles and function of neurotrophic substances in the nervous system. Since one of the best-analyzed systems in this context are represented by the neurotrophins and especially by BDNF. Thus, the mini reviews mainly focus on the actions of BDNF. However, since other factors also have been found to have neurotrophic properties or interact with the neurotrophin system, specific original articles were also included in the "Special Issue." We hope that this selection of articles will be helpful to get insight in the fascinating world of the neurotrophins and their actions in the central nervous system.

AUTHOR CONTRIBUTIONS

PB and OB contributed equally to the special issue and the editorial.

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MINI REVIEW
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Mechanisms That Modulate and Diversify BDNF Functions: Implications for Hippocampal Synaptic Plasticity

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Brain-derived neurotrophic factor (BDNF) is a neurotrophin that has pleiotropic effects on neuronal morphology and synaptic plasticity that underlie hippocampal circuit development and cognition. Recent advances established that BDNF function is controlled and diversified by molecular and cellular mechanisms including trafficking and subcellular compartmentalization of different *Bdnf* mRNA species, pre- vs. postsynaptic release of BDNF, control of BDNF signaling by tropomyosin receptor kinase B (TrkB) receptor interactors and conversion of pro-BDNF to mature BDNF and BDNF-propeptide. Defects in these regulatory mechanisms affect dendritic spine formation and morphology of pyramidal neurons as well as synaptic integration of newborn granule cells (GCs) into preexisting circuits of mature hippocampus, compromising the cognitive function. Here, we review recent findings describing novel dynamic mechanisms that diversify and locally control the function of BDNF in hippocampal neurons.

Keywords: BDNF, Pro-BDNF, TrkB, hippocampus, synaptic plasticity

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INTRODUCTION

The correct function of the nervous system depends on the proper establishment of the synaptic contacts achieved by neurons. For this, developing neurons must acquire a correct morphology that allows the formation of neuronal circuits. Alterations in neuronal morphology have been associated with different neuropathological conditions, characterized by cognitive defects (Forrest et al., 2018). Neuronal architecture is regulated by intrinsic and extrinsic factors, among which are neurotrophins (Park and Poo, 2013). Within this family, brain-derived neurotrophic factor (BDNF) is highly expressed in the hippocampus where it supports a variety of functions including regulation of neuronal morphology and synaptic plasticity by binding to the high-affinity receptor tyrosine kinase, tropomyosin receptor kinase B (TrkB; Leal et al., 2015).

In the hippocampus, the most prominent type of cells that determine the tri-synaptic circuitry are the pyramidal neurons that form the pyramidal layer of CA1 and CA3 regions, and the granule cells (GCs) of the dentate gyrus. Hippocampal pyramidal and dentate GCs express both TrkB and BDNF (Drake et al., 1999), and there is a large body of evidence indicating that BDNF is a relevant modulator of structural and functional synaptic plasticity in these type of excitatory neurons (Gonzalez et al., 2016; von Bohlen Und Halbach and von Bohlen Und Halbach, 2018). Although TrkB is also expressed in hippocampal

GABAergic interneurons and important effects of BDNF on these inhibitory neurons have been reported (Porcher et al., 2018), this topic will not be discussed in the present revision.

A morphological correlate of synaptic plasticity is represented by the complexity of the dendritic arbors as well as by the density, shape and size of dendritic spines. The role of BDNF as a modulator of the dendritic structure of hippocampal pyramidal neurons *in vivo* is still unclear. However, a clear role for BDNF in hippocampal dendrite development has been observed in cultured pyramidal neurons (Cheung and Ip, 2007; Ji et al., 2010; Kwon et al., 2011; Lazo et al., 2013). Numerous studies have reported that BDNF increases dendritic spine density and induces long-term potentiation (LTP) in hippocampal pyramidal neurons. Consistently with this, TrkB-deficient mice have significantly fewer dendritic spines and excitatory synapses on CA1 neurons (Luikart et al., 2005; von Bohlen und Halbach et al., 2008; **Figure 1**).

In the adult DG, multiple studies reported a clear contribution of BDNF and TrkB in dendrite morphogenesis of newborn hippocampal neurons. The contribution of BDNF/TrkB signaling in the integration of newborn neurons was studied in conditional mice in which TrkB was deleted specifically in adult progenitors. In this study, the authors demonstrated that dendritic and spine growth of adult-born GCs is reduced in these animals (**Figure 1**). In line with this, a significant reduction in dendritic development, synaptic formation and maturation has been observed in postnatal-born granule neurons in different BDNF-mutant mice (Chan et al., 2008; Gao and Chen, 2009) and BDNF secreted by newborn GCs has been shown to function as an autocrine factor involved in dendrite development and synaptic maturation (Wang et al., 2015).

Since BDNF plays a critical role in the maintenance and refinement of neuronal circuits involved in learning and memory, diverse mechanisms are used to regulate its activity. In this review, we provide new insights into the mechanisms that regulate and diversify BDNF biology in hippocampal neurons, such as trafficking and subcellular compartmentalization of different *Bdnf* mRNA species, conversion of pro-BDNF to mature BDNF and BDNF-propeptide, modulation of BDNF signaling by novel TrkB receptor interactors and pre- vs. postsynaptic release of BDNF. Interestingly, these regulatory mechanisms allow BDNF to exert a rapid and dynamic refinement of the hippocampal connections in response to experience-dependent neuronal activity.

SUBCELLULAR COMPARTMENTALIZATION OF *Bdnf* mRNA CONTRIBUTES TO SYNAPTIC PLASTICITY

The subcellular compartmentalization of *Bdnf* mRNAs and its local secretion are necessary for proper development and plasticity. The synthesis of multiple transcripts is a mechanism that tightly controls BDNF expression. The rat *Bdnf* gene comprises nine exons but the coding sequence (CDS) resides in exon nine (**Figure 2A**). Thus, the eight

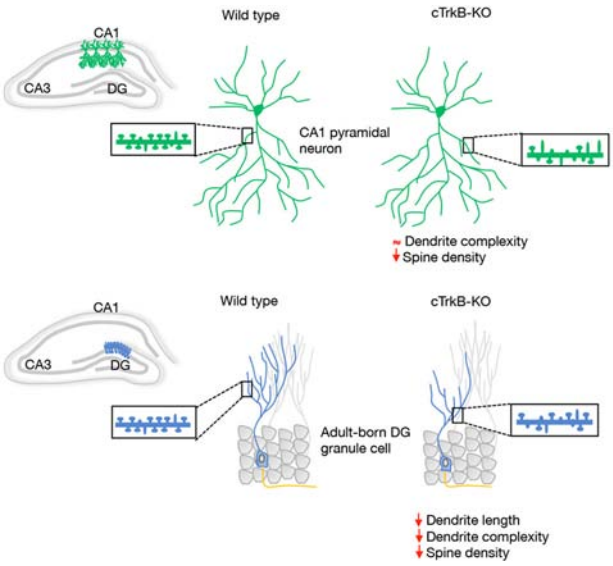

FIGURE 1 | Schematic representation of the dendrite morphology and dendritic spine density reported *in vivo* in hippocampal CA1 pyramidal neurons and adult-born DG granule cells (GCs) from wild type and conditional tropomyosin receptor kinase B knockout (cTrkB-KO) mice. The insert displays examples of dendritic shafts showing an array of mushroom (mature) and thin (immature) dendritic spines.

upstream exons drive transcription of multiple *Bdnf* splice variants that encode an identical BDNF protein in a regional and cell-type specific manner (Liu et al., 2006; Aid et al., 2007). The presence of different *Bdnf* transcripts has led to propose the spatial code hypothesis, which suggests that differential expression of 5′ untranslated region (UTR) molecules allow spatial, temporal and stimulus-specific BDNF expression (Tongiorgi, 2008; Maynard et al., 2017). It is widely accepted that most *Bdnf* transcripts such as those containing exon 1 (Ex1) and Ex4, are found mainly within the cell body and proximal dendrites, whereas selected variants such as Ex2 and Ex6 are located in distal dendrites (**Figure 2A**). Thus, downregulation of *Bdnf* Ex1 and Ex4 transcripts in cultured hippocampal neurons reduces proximal dendrite number, while decreasing Ex2 and Ex6 transcripts alters distal dendrites (Baj et al., 2011). Moreover, mice with a selective disruption in individual *Bdnf* 5′UTR splice variants lead to local deficits in CA1 and CA3 dendrite and spine morphology (Maynard et al., 2017). Interestingly, the dysregulation of *Bdnf* transcripts containing specific 5′UTR exons has been associated with deficits in fear memory (Hill et al., 2016). The *Bdnf* gene also encodes two different 3′UTR, which add a new level of complexity to BDNF biology. The protein can be translated from different mRNA species containing either a short or a long 3′UTR (Timmusk et al., 1993; **Figure 2A**). While the short 3′UTR *Bdnf* mRNA is restricted to cell bodies in hippocampal neurons, the long 3′UTR *Bdnf* mRNA is transported to dendrites for local translation (An et al., 2008). Although BDNF abundance in dendrites is generally low, its dendritic localization is enhanced in response to depolarization (Tongiorgi et al., 1997), or to

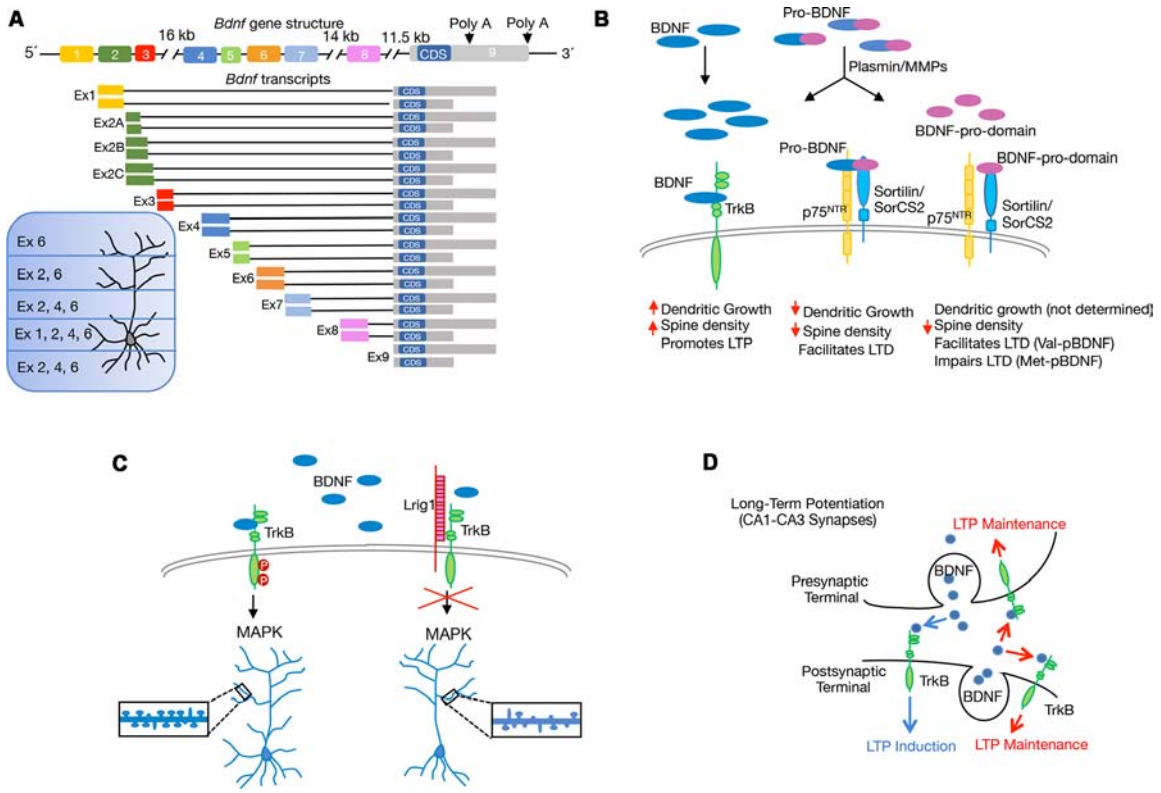

FIGURE 2 | Scheme showing recent molecular and cellular mechanisms through which brain-derived neurotrophic factor (BDNF) regulates structural and functional synaptic plasticity in the hippocampus. **(A)** Schematic representation of rat *Bdnf* gene and splice variants derived from it, with different 5′untranslated region (UTR) and 3′UTR. The coding sequence (CDS) is indicated. Expression of BDNF generated by the different transcripts containing exons (Ex) is indicated according to Baj et al. (2011). **(B)** Illustration shows the extracellular proteolytic cleavage of pro-BDNF to give rise mature BDNF and the BDNF-prodomain, as well as the interaction of the different BDNF isoforms with specific receptors. The differences in the regulation of long-term depression (LTD) between the BDNF-prodomain polymorphisms: Val-pro-BDNF (Val-pBDNF) and Met-pro-BDNF (Met-pBDNF) are stated. **(C)** Model describing the endogenous inhibition of BDNF/TrkB signaling by the transmembrane protein Lrig1 and its implication for proximal dendrite development and spine formation in CA1-CA3 pyramidal neurons. **(D)** Model proposed for pre- vs. postsynaptic BDNF release and their contribution to long-term potentiation (LTP). Postsynaptic BDNF, as well as both presynaptic and postsynaptic TrkB, contribute to LTP maintenance (red arrows). Involvement of presynaptic BDNF and postsynaptic TrkB in LTP induction is also shown (blue arrows).

neurotrophin treatment (Righi et al., 2000; Vicario et al., 2015). Interestingly, *Bdnf*-mutant mice, carrying a truncated long 3′UTR, show impaired differentiation and maturation of adult-born hippocampal neurons (Waterhouse et al., 2012). By using long-term cultures of rat hippocampal neurons Orefice et al. (2013) suggested that somatically synthesized BDNF promotes spine formation, whereas dendritically synthesized BDNF is a key regulator of dendritic spine maturation. These findings indicate that the same protein synthesized in different neuronal compartments controls different aspects of the same biological process.

PRO-BDNF AND BDNF-PROPEPTIDES AS NOVEL REGULATORS OF HIPPOCAMPAL CONNECTIVITY

Neurotrophins, and particularly BDNF, are initially synthesized as precursors, named proneurotrophins, in the endoplasmic reticulum, and can be converted into mature neurotrophins intracellularly by the action of furin or other proconvertases in the trans-golgi network or in secretory granules. Alternatively, these molecules can be secreted in their immature form, cleaved in the extracellular medium and converted into the mature form by plasmin and matrix metalloproteases (Lee et al., 2001). The efficiency of cleavage and the ratio of mature to proneurotrophins varies along different developmental stages (Yang et al., 2014; Hempstead, 2015). Proneurotrophins bind a complex composed by $p75^{NTR}$ and either sortilin or SorCS2 (two members of the Vps10p-domain family). The $p75^{NTR}$ receptor binds to the mature domain region or pro-BDNF whereas the prodomain binds to sortilin or SorCS2 (Teng et al., 2005; Anastasia et al., 2013). Increasing evidence indicates that mature and pro-BDNF exert opposing effects in the central nervous system (CNS). While mature BDNF promotes neuronal survival, differentiation, synaptic plasticity and LTP, the pro-BDNF induces apoptosis, growth cone retraction, reduces dendritic spine density and facilitates long-term depression (LTD) in hippocampal slices (Lu et al., 2005; **Figure 2B**).

Different studies have described secretion of both pro- and mature BDNF in response to depolarization

(Nagappan et al., 2009; Yang et al., 2009; Je et al., 2012). In order to understand the physiological role of the pro-BDNF, Yang et al. (2014) generated a knock-in mouse in which the proconvertase/furin cleavage site of BDNF was mutated and expressed under the control of endogenous BDNF promoter. This work revealed that pro-BDNF secreted endogenously reduces dendrite arborization and spine density of hippocampal GCs *in vivo*. Deficits in $p75^{NTR}$ rescue these dendritic defects, showing the requirement of $p75^{NTR}$ as a mediator of pro-BDNF effects. Hippocampal slices from these pro-BDNF-expressing mice exhibit depressed synaptic activity and LTD. Interestingly, abnormalities in the ratio of pro-BDNF/mature BDNF has been described in the brain of individuals with autism, suggesting that the balance between these isoforms could be associated with the disease (Garcia et al., 2012).

The analysis of the human *Bdnf* gene revealed a single nucleotide polymorphism in the BDNF-prodomain which comprises a valine (Val) to methionine (Met) substitution at position 66 (Val66Met). This mutation disrupts the intracellular trafficking and activity-dependent release of BDNF (Chen et al., 2006) and has been associated to neuropsychiatric disorders (Egan et al., 2003; Soliman et al., 2010; Verhagen et al., 2010; Dincheva et al., 2012; Notaras et al., 2016).

Although for a long time, it was believed that the prodomain resultant from the cleavage of proneurotrophins was degraded after processing, later studies revealed that propeptides are present in brain tissue. Similarly to mature and pro-BDNF, BDNF-prodomain (pBDNF) is also secreted in an activity-dependent manner from hippocampal cultures, suggesting that it may act as an independent ligand (Goodman et al., 1996; Nagappan et al., 2009; Yang et al., 2009; Anastasia et al., 2013). The BDNF-prodomain has been shown to exert different neuronal activities depending on the presence of the 66Met substitution. Anastasia et al. (2013) reported that the Met amino acid induces structural changes within the BDNF-prodomain which allows Met-BDNF-prodomain (Met-pBDNF) to interact with SorCS2 and to trigger growth cone retraction in hippocampal neurons. Although the expression of $p75^{NTR}$ is necessary for Met-pBDNF signaling, this ligand only interacts with SorCS2 (**Figure 2C**). Different groups analyzed the effect of BDNF-propeptide on dendritic spine development. Guo and collaborators tested the effects of the Val-pBDNF in cultured mature hippocampal neurons and observed that this ligand induces a reduction in dendritic spines density (Guo et al., 2016). In another study, it was shown that Met-pBDNF but not Val-pBDNF, can trigger disassembly of mature mushroom spines and synaptic contacts on cultured hippocampal pyramidal neurons exposed to these ligands during shorter periods of time (Giza et al., 2018). The discrepancy between the effects observed with Val-pBDNF could depend on the differences in the exposure time to the ligands used in the two studies. In agreement with the role of Met-pBDNF as a modulator of dendritic development, an *in vivo* assay shows that when the Met-pBNDF was delivered into the ventral CA1 region of the hippocampus, it resulted in a decrease in dendritic spine number and spine head size (Giza et al., 2018). In the same study, the authors showed that injection of the Met-pBDNF, but not Val-pBDNF display acute effects on circuitry and fear extinction behavior. Interestingly, the effects of Met-pBDNF were observed in a developmental period corresponding to the periadolescence, when the prodomain and its receptor are highly expressed in mice (Anastasia et al., 2013). Intriguingly, Mizui and collaborators demonstrated that the addition of Val-pBDNF facilitates LTD induction in hippocampal slices, while Met-pBDNF failed to induce LTD facilitation (Mizui et al., 2015), opening the question of how the different versions of the prodomain exert differential effects (**Figure 2B**). A possible answer is that each peptide triggers different signaling pathways.

CONTROL OF BDNF SIGNALING BY NOVEL CELL-INTRINSIC TrkB RECEPTOR INTERACTORS

Genetically modified mouse models have established that TrkB receptors need to be modulated by different proteins to achieve cell-type-specific responses to its ligand during circuit development. Thus, members of the Vps10p-domain and leucine-rich repeat (LRR)-domain containing proteins have been described to be involved in the regulation of BDNF/TrkB signaling.

In addition, to act as a $p75^{NTR}$ co-receptor required for pro-BDNF binding, SorCS2 was also identified as a physiological TrkB receptor interactor in hippocampal pyramidal neurons. SorCS2 is a type I transmembrane receptor that belongs to a Vps10p-domain family of sorting and signaling receptors which also includes Sortilin, SorLA and SorCS1 and 3 (Glerup et al., 2014). In contrast to the SorCS2/$p75^{NTR}$ interaction, which is not influenced by electrical activity, high-frequency stimulation (HFS) promotes the binding of SorCS2 to TrkB. Notably, the interaction between TrkB and SorCS2 directs TrkB receptors to postsynaptic densities for synaptic tagging and contributes to LTP maintenance in a synapse-specific manner. Furthermore, hippocampal neurons lacking SorCS2 failed to induce TrkB phosphorylation and dendritic spine formation in response to BDNF. SorCS2-deficient mice display deficits in long-term memory and a higher tendency to take risk. Based on this evidence, it has been suggested that SorCS2 could be a possible link between proBDNF/BDNF signaling, synaptic plasticity (LTD/LTP) and mental disorders (Glerup et al., 2016). Interestingly, other members of the Vps10p-domain-containing protein, Sortilin, has been described as an interactor of ARHGAP33 involved in TrkB trafficking which is essential for synapse development (Nakazawa et al., 2016).

Previous evidence also demonstrated that engagement of TrkB with different LRR-domain-containing proteins (i.e., Slitrk5 and Lrig1) is a general mechanism that expands the repertoire of BDNF signaling outputs in specific population of neurons during nervous system development (Song et al., 2015; Alsina et al., 2016; Ledda and Paratcha, 2016). For instance, the LRR transmembrane protein Lrig1 was identified as a physiological regulator of proximal dendritic growth and

BDNF function in CA1-CA3 pyramidal neurons. Deletion of Lrig1 mainly increases proximal complexity of apical dendritic arbors, revealing a novel molecular mechanism involved in the determination of basal vs. apical dendrite development. In line with this, overexpression of Lrig1 blocked dendritic spine formation induced by BDNF (Alsina et al., 2016; **Figure 2C**). The role of Lrig1 in the modulation of TrkB has also been analyzed in newborn neurons of the aged hippocampus. In the aging DG, newborn neurons remain immature for a long period of time, but voluntary exercise triggers their rapid growth and functional synaptic integration (Fan et al., 2017). Interestingly, Trinchero et al. (2017) demonstrated that plasticity of aged granule neurons is mediated in a cell-intrinsic manner by neurotrophin signaling. In this system, the knockdown of Lrig1, which promotes TrkB activation, accelerates neuronal development and potentiates the effects of running in newborn-GCs of aged mice. On the other hand, overexpression of Lrig1 in newly generated granule neurons of middle-aged mice abolished dendritic growth induced by running activity (Trinchero et al., 2017). Together, these findings reveal that physical exercise and neurotrophin signaling maximize plasticity of newborn GCs in the aged hippocampus. These data present physiological relevance since understanding the dynamics of experience-dependent connectivity remodeling throughout life is critical to prevent cognitive decline during aging and neurodegeneration (Mattson, 2012). Further research has extended this concept and showed that adult hippocampal neurogenesis combined with increased BDNF levels mimic the beneficial cognitive effects promoted by exercise in a mouse model of Alzheimer's disease and suggest that enhancing neurogenesis and BDNF levels at early stages of the disease may protect against subsequent neuronal cell death (Choi et al., 2018).

PRE vs. POSTSYNAPTIC RELEASE OF BDNF AND ITS IMPACT ON LTP

Although it is clearly demonstrated that BDNF signaling regulates LTP in the hippocampus (Alder et al., 2005; Waterhouse and Xu, 2009; Panja and Bramham, 2014), the exact contribution of pre vs. postsynaptic BDNF secretion and TrkB signaling for synaptic plasticity remained controversial for several years. To address this, different approaches using genetic deletion of BDNF in specific hippocampal regions have been used (Zakharenko et al., 2003; An et al., 2008). In the last year, Lin et al. (2018) used a viral-mediated approach to delete BDNF or TrkB selectively in either the CA3 or CA1 region of the Schaffer collateral pathway to examine its impact for synaptic plasticity. These experiments revealed that presynaptic BDNF regulates the strength of LTP, while postsynaptic BDNF contributes to LTP maintenance. In addition, these experiments showed that LTP induction is mediated by anterograde BDNF/TrkB signaling, whereas both anterograde and retrograde BDNF/TrkB signaling are essential for LTP maintenance (Lin et al., 2018; **Figure 2D**). In a very elegant study using fluorescence resonance energy transfer (FRET)-based sensor for TrkB and two-photon fluorescent lifetime microscopy, Harward et al. (2016) identified an autocrine BDNF/TrkB signaling within a single stimulated dendritic spine of CA1 pyramidal neurons. In this study, the authors described a spine-autonomous autocrine signaling mechanism that evokes BDNF release from the same stimulated spine. The release of BDNF requires the activation of the NMDAR and CaMKII-dependent pathway. Subsequently, BDNF activates TrkB on the same stimulated spine to promote functional and structural plasticity. Therefore, these findings demonstrate that on one side, increased expression and secretion of BDNF is the result of stimulus-evoked neuronal activity and that on the other side, activity-evoked BDNF secretion strengthens synaptic potentiation and modulates axo-dendritic morphology in a local and synapse-specific manner. This work is in line with a previous study showing that synaptic stimulation of a single spine induces a gradual enlargement of spine heads that is dependent on protein synthesis and mediated by BDNF secretion (Tanaka et al., 2008). These studies provide new insight into the role of axonal and dendritic BDNF and TrkB receptor signaling in hippocampal LTP. Further behavioral experiments will be required to precisely understand the role of pre vs. postsynaptic BDNF/TrkB signaling in different learning and memory paradigms.

PERSPECTIVES

The studies summarized here, describe multiple mechanisms that enable a rapid, localized and dynamic control of BDNF functions in hippocampal neurons. Impairment in these mechanisms leads to defects in synaptic plasticity and memory processes, which highlights their biological relevance.

Despite the recent progress in understanding the mechanisms underlying BDNF biology, many questions still remain to be answered. For instance, the role of pro-BDNF in the control of adult hippocampal neurogenesis has been barely addressed. Future studies undoubtedly are necessary to dissect the physiological contribution of the different BDNF splice variants as well as mature and immature forms of BDNF in specific synapses of the adult hippocampal circuit. For this, it will be important to determine the exact cellular source and the mechanism of biosynthesis and release of BDNF, pro-BDNF and BDNF-prodomain at specific synapses. Indeed, it will be also important to characterize the array of BDNF receptors, co-receptors and endogenous TrkB regulators present *in vivo* at specific synaptic connections of the hippocampus.

Dysfunction of the BDNF/TrkB system is involved in the onset of brain disorders, such as Alzheimer, autism and depression (Sungur et al., 2017; Numakawa et al., 2018). Therefore, understanding the basic biology of the different BDNF isoforms, receptors and interactors at the synapse will provide useful insights for the design of therapeutic tools for different neuropsychiatric diseases.

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Co-transplantation of Epidermal Neural Crest Stem Cells and Olfactory Ensheathing Cells Repairs Sciatic Nerve Defects in Rats

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Cell-based therapy is an alternative strategy to improve outcomes of peripheral nerve injury (PNI). Epidermal neural crest stem cell (EPI-NCSC) is obtained from autologous tissue without immunological rejection, which could expand quickly *in vitro* and is suitable candidate for cell-based therapy. Olfactory ensheathing cell (OEC) could secrete multiple neurotrophic factors (NTFs), which is often used to repair PNI individually. However, whether the combination of EPI-NCSC and OEC have better effects on PNI repair remains unclear. Here we use EPI-NCSC and OEC co-transplantation in a rat sciatic nerve defect model to ascertain the effects and potential mechanisms of cells co-transplantation on PNI. The effect of EPI-NCSC and OEC co-transplantation on PNI is assessed by using a combination of immunohistochemistry (IHC), electrophysiological recording and neural function test. Co-transplantation of EPI-NCSC and OEC exerts a beneficial effect upon PNI such as better organized structure, nerve function recovery, and lower motoneuron apoptosis. IHC and enzyme-linked immuno sorbent assay (ELISA) further demonstrate that cells co-transplantation may improve PNI via the expression of brain derived growth factor (BDNF) and nerve growth factor (NGF) up-regulated by EPI-NCSC and OEC synergistically. Eventually, the results from this study reveal that EPI-NCSC and OEC co-transplantation effectively repairs PNI through enhancing the level of BDNF and NGF, indicating that cells co-transplantation may serve as a fruitful avenue for PNI in clinic treatment.

Keywords: peripheral nerve injury, epidermal neural crest stem cell, olfactory ensheathing cell, co-transplantation, brain derived growth factor, nerve growth factor

Abbreviations: BDNF, brain derived growth factor; cMAPs, compound muscle action potentials; DMEM/F_{12}, dulbecco's modified eagle medium: nutrient mixture F-12; ECM, extracellular matrix; ELISA, enzyme-linked immuno sorbent assay; EPI-NCSCs, epidermal neural crest stem cells; GDNF, glial cell line-derived neurotrophic factor; H&E, hematoxylin and eosin; IHC, immunohistochemistry; NCV, nerve conductive velocity; NGF, nerve growth factor; NTE, nerve tissue engineering; NTFs, neurotrophic factors; OECs, olfactory ensheathing cells; PNI, peripheral nerve injury; PNS, peripheral nerve system; SC or SCL_{4-6}, lumbar spinal cord at L4–L6; SCI, spinal cord injury; SFI, sciatic function index; SN, sciatic nerve; TEM, transmission electron microscopy.

INTRODUCTION

Peripheral nerve injury continues to be a major challenge in reconstructive neurosurgery. Owing to huge clinical demand, peripheral nerve regeneration, particularly larger gap injuries, has become a prime focus of basic and clinical research. Accelerating axonal regeneration to promote reinnervation and improve functional recovery after PNI is a clinical necessity and an experimental challenge (Goel et al., 2009; Cotter et al., 2010; Tang et al., 2013).

For surgical repair of PNI with substantial neural defects, the current gold standard is to bridge the defect with an autologous nerve graft that is obtained from another part of the body. In terms of inevitable drawbacks associated with autologous nerve graft, the development of artificial substitutes for autologous nerve grafts is an urgent need in the field of regenerative medicine. NTE has been shown to satisfy this need. It is typically comprised of a neural scaffold with incorporated biochemical cues. The scaffold is prepared with a variety of synthetic or natural biomaterials through well-defined fabrication techniques. Among a large body of scaffold biomaterials, Poly(lactic-co-glycolic acid) (PLGA) (Bini et al., 2004), comprised of Polylactide (PLA) and Polyglycolide (PGA), shows excellent neural affinity and biocompatibility with cells. PLGA not only can be used to control the release of inosine, NGF and insulin-like growth factor (IGF), but also combine to seed cells such as Schwann cells, bone marrow stromal cells (BMSCs) or neural stem cells (NSCs), or can be processed into a graft for guiding peripheral nerve regeneration.

Seed cells, as an important component of NTE, provide an optional strategy for NTE with an enhanced ability to repair PNI. Attractively, EPI-NCSCs, combined with the advantage of adult stem cell and embryonic stem cell, represent a unusual type of multipotent adult stem cell and is a suitable candidate for cell-based therapy (Hu et al., 2006; Sieber-Blum et al., 2006). Accumulating evidences (Amoh et al., 2005) suggest that EPI-NCSCs can be induced to differentiate into Schwann cells and might secrete growth factors to modulate the behavior of Schwann cells. Our previous reports have showed that EPI-NCSCs could reduce inflammation (Li et al., 2017) and promote the segmental recovery of PNI (Zhang et al., 2014). Moreover, OECs are specialized glial cells somewhat similar to Schwann cells and astrocytes, which secrete NGF and BDNF, suggesting that NTFs produced by OECs might enhance the survival of damaged axons (Marshall et al., 2006). Indeed, abundant studies have reported that OECs are able to promote axonal regeneration and remyelination after SCI (Gomes et al., 2018; Gómez et al., 2018; Wright et al., 2018) and PNI (Radtke et al., 2009; Guérout et al., 2011). Importantly, the synergic effects of EPI-NCSCs and OECs improved locomotor function of contused spinal cord of rats and enhanced the expression of NTFs (Zhang et al., 2015). Based on these, the combination of EPI-NCSCs and OECs may synergistically repair PNI. Therefore, we will probe the mechanism that co-transplantation of EPI-NCSCs and OECs have reparative effects on PNI.

In the present study, we engineered nerve grafts by incorporating EPI-NCSCs and OECs as seed cells into PLGA to bridge a 10 mm long sciatic nerve defect in rats, and investigated therapeutic effects of cells co-transplantation by using IHC, electrophysiological recording *in vivo* and neural function test.

MATERIALS AND METHODS

The Acquisition and Identification of EPI-NCSCs and OECs

Isolation and Culture of EPI-NCSCs and OECs

EPI-NCSCs

Epidermal neural crest stem cells were isolated and cultured following procedures described by Sieber-Blum et al. (2006) and Clewes et al. (2011). Sprague Dawley rats were anaesthetized with 3% pentobarbital sodium (Sigma, United States) (70 mg/kg), cut whisker pads, stripped hair follicle, adhered to collagen coated dish, added culture medium, and emigrated from expants on the third day. The protocol is described in the **Supporting Information** (**Supplementary Material**).

OECs

Olfactory ensheathing cells were isolated from adult green fluorescent protein (GFP)-Sprague Dawley rats by modification of the method described by Sasaki et al. (2004). Sprague Dawley rats were anaesthetized with 3% pentobarbital sodium (Sigma, United States) (70 mg/kg), separated olfactory bulbs, reserved outer nerve layer, minced tissue, digested with 0.25% trypsin for 30 min at 37°C, transferred to culture dish, and grew up on the 7th day. The protocol is described in the **Supporting Information** (**Supplementary Material**).

Identification of EPI-NCSCs and OECs

Washed cells with 0.01 M phosphate buffer (PBS) for three times, fixed with 4% Polyoxymethylene (PFA) for 30 min, permeated with 0.3% Triton X-100 for 30 min, blocked with 1% BSA for 30 min, incubated with primary antibody overnight at 4°C, washed with PBS for three times (5 min each time), then incubated with second antibody for 1 h at RT, washed with PBS for three times (10 min each time), dried at RT, covered with Fluoromount anti-fade reagent (Sigma, United States), and observed with fluorescence microscope. The protocol is described in the **Supporting Information** (**Supplementary Material**).

Fabrication and degradation of nerve conduits

Nerve conduits were fabricated and degraded following procedures described by Moore et al. (2006) and Li et al. (2010). The nerve conduits were fabricated from 10% PLGA dissolved in $CHCl_3$ (Sigma, United States), cut into 15 mm length, and sterilized by gamma irradiation for 30 min. The nerve conduits were put in heat-sealed pouch, vacuum-dried for 24 h to obtain tare weights. Then, samples were put in 0.01 M PBS, and incubated at 37°C in 5% CO_2 incubator. At regular intervals, monitored pH and weighed. Details are provided in the **Supporting Information** (**Supplementary Material**).

Animal model and transplantation

Fifty Sprague-Dawley rats (Laboratory Animal Center, Third Military Medical University, Chongqing, China) weighing

220–250 g were used in all groups. Experiment is divided into five groups: (1) DMEM/F_{12} (n = 10); (2) EPI-NCSC (n = 10); (3) OEC (n = 10); (4) EPI-NCSC+OEC (n = 10); (5) Control (n = 10). The right sciatic nerve as experimental side, the left sciatic nerve or normal animals as control. All experimental procedures with animals were approved by the local institution review board and were carried out according to the guidelines of the Third Military Medical University (Chongqing, China) for the care and use of laboratory animals. Details are provided in the **Supporting Information** (**Supplementary Material**).

Histological observation and survival of transplanted cells in vivo

The H&E staining protocol was used to assess organization of structures. The graft was harvested and fixed with 4% PFA 8 weeks after transplantation. Samples derived from the middle portion of the graft were longitudinally sectioned into 20 μm thickness section for H&E staining. Details are provided in the **Supporting Information** (**Supplementary Material**).

Electrophysiology and Retrograde Tracing

Electrophysiology

To evaluate nerve regeneration, *in vivo* electrophysiology was performed. Stimuli electrodes (strength: 3 mA; interval: 0.25 ms) were placed under the sciatic nerve trunk 5 mm proximal to the suturing point and recording electrodes were inserted in the gastrocnemius. cMAPs was gathered by data acquisition software (Power lab, Australia). NCV was calculated by recording latency time of proximal and distal end. Normal nerve as control. Eight weeks after transplantation, five rats in each group were randomly chosen for electrophysiology.

Retrograde Tracing

Retrograde tracing was used to assess nerve regeneration. Eight weeks after transplantation, the sciatic nerve was exposed under anesthetization, and 2% DiI solution (15 μL) was injected into the nerve trunk 10 mm proximal to the suturing point with a microinjector. After the injection, the needle was kept *in situ* for 5 min. After 24 h, gathered samples, sectioned transversely into 15 μm sections, and observed by fluorescence microscope (BX 51WI, Olympus). Details are provided in the **Supporting Information** (**Supplementary Material**).

Analysis of the Expression of BDNF and NGF

Brain derived growth factor and NGF play a vital role in repairing PNI, therefore we test the expression of BDNF and NGF by IHC and ELISA eight after transplantation. The rats were anesthetized with 3% pentobarbital sodium (70 mg/kg). Samples were harvested and sectioned into 15 μm sections. Incubated with primary antibody overnight at 4°C, then incubated with second antibody for 1 h at 37°C, and washed three times with PBST. Samples were observed by fluorescence microscope (BX 51WI, Olympus).

Eight weeks after transplantation, the expression of BDNF and NGF was quantified by ELISA. Samples were harvested. The concentration of BDNF and NGF was measured by ELISA kit (Abcam, United States) according to the manufacturer's instructions. Details are provided in the **Supporting Information** (**Supplementary Material**).

Evaluation of Myelination and Cell Apoptosis

Transmission electron microscopy and toluidine blue staining were used to evaluated the myelination and cell apoptosis was assessed by TUNEL staining. Eight weeks after transplantation, the graft was harvested, cut into ultrathin sections to stain with lead citrate and uranyl acetate, and followed by observation under transmission electron microscope. TUNEL staining was carried out by *in situ* Cell Death Detection Kit (Roche, Germany) according to the manufacturer's instructions. Details are provided in the **Supporting Information** (**Supplementary Material**).

Behavior Test and Gastrocnemius Assessment

Sciatic Function Index

The evaluation of locomotor function was performed by the SFI according to methods described by Bain et al. (1989). Rats were acclimatized experiments before surgery. Foot prints from the normal (N, right side) and experimental (E, left side) were recorded 2, 4, and 8 weeks after transplantation. SFI ranged from −100 to 0. The value 0 describes normal function and the value −100 shows complete transaction of sciatic nerve. Details are provided in the **Supporting Information** (**Supplementary Material**).

Withdrawal Latency

The assessment of sensorial function was carried out by hot bath (Hargreaves et al., 1988; Alleva et al., 1997; Inoue et al., 2004). The hind paw was immersed in 50 ± 1°C hot water bath to measure the withdrawal latency. Rats were acclimatized experiments before surgery. In test session, each rat was tested in five trials with an interval of 5 min. The hind paw withdrawal latencies were calculated as the mean of five trials. The maximum of withdrawal latency was set at 5 s to prevent tissue damage.

Gastrocnemius Assessment

Samples were harvested and weighed 2, 4, and 8 weeks after transplantation. The weight rate of the gastrocnemius (right/left) was used to assess nerve regeneration.

Statistical Analysis

All data were expressed as mean ± SD. All statistical analyses were carried out in SPSS17.0 software. Differences among groups were assessed by one-way ANOVA test. Two-way ANOVA test was used to analyze SFI and withdrawal latency in different groups and 2, 4, and 8 weeks after transplantation. One or two-way ANOVA tests were followed by the Bonferroni *post hoc* test. A p-value of <0.05 was set as the criteria for statistical significance.

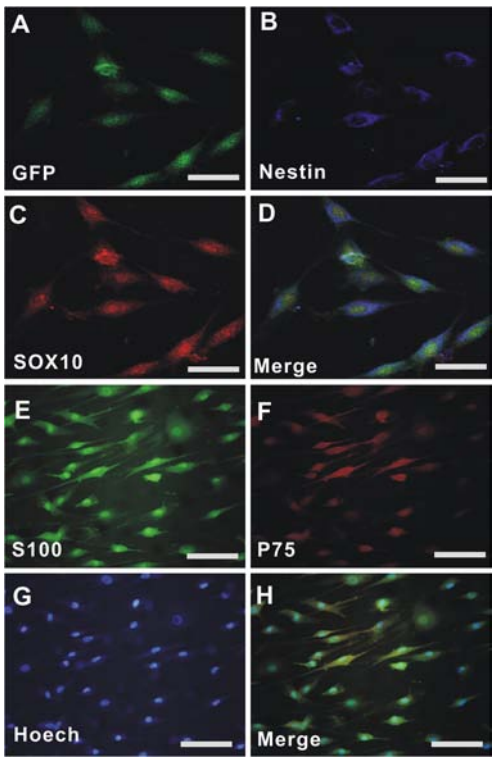

FIGURE 1 | The culture and identification of EPI-NCSCs and OECs. **(A)** GFP-EPI-NCSC. **(B)** Nestin. **(C)** SOX10. **(D)** Merged GFP/Nestin/SOX10. **(E)** S-100. **(F)** p75. **(G)** Hoechst 33342. **(H)** Merged S-100/p75/Hoechst 33342. Nuclei were stained by Hoechst 33342 (blue). Scale for **A–D**, 15 μm; Scale for **E–H**, 25 μm.

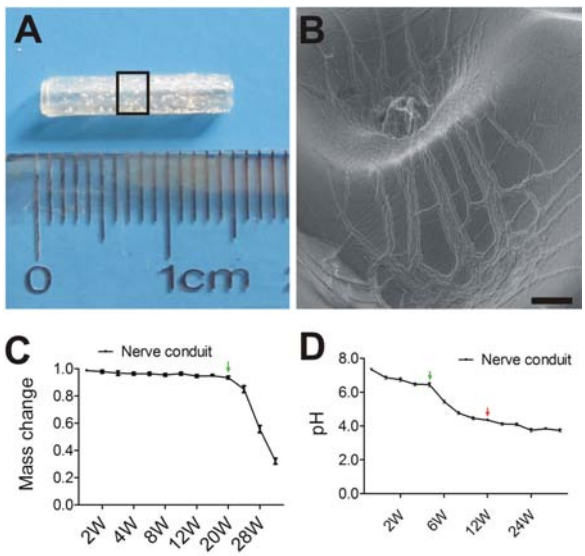

FIGURE 2 | Tissue engineered nerve conduits for peripheral nerve regeneration. **(A)** The nerve conduit. **(B)** SEM of longitudinal sections of nerve conduit in enlarged rectangle box. **(C)** The profile of Mass change. **(D)** The profile of pH change. **B**, Scale = 100 μm; Values are shown as mean ± SD (n = 3).

RESULTS

Characterization of EPI-NCSCs and OECs

Epidermal neural crest stem cells migrated from the bulge of hair follicles of GFP-rats and subcultured for 3 passages (P_3), and the cells displayed a spindle-like shape (Green, **Figure 1A**) with green fluorescent protein (GFP). Double immunofluorescent staining demonstrated that the cells were positive for Nestin (Blue, **Figure 1B**), SOX10 (Red, **Figure 1C**), and Nestin/SOX10/GFP (Merged, **Figure 1D**). OECs were isolated from olfactory bulb of rats and cultured for P3. Immunofluorescent staining demonstrated that the cells were positive for S-100 (Green, **Figure 1E**), p75 (Red, **Figure 1F**), Hoechst 33342 (Blue, **Figure 1G**), and S-100/p75/ Hoechst 33342 (Merged, **Figure 1H**). The purity of EPI-NCSCs and OECs is above 95%.

The Feature of the Nerve Conduits

To explore the potential therapeutic effects of EPI-NCSC and OEC co-transplantation on PNI, we constructed a 15-mm nerve conduit to repair the rat sciatic nerve defect (**Figure 2A**). The internal surface of nerve conduit was a little rough (**Figure 2B**) and had a few micropores for cell adhesion and migration.

The degradation of the nerve conduits under simulated physiologic conditions was estimated as expected for PLGA with the given copolymer ratio. As shown by the pH change curve in **Figure 2D**, pH decreased from 7.4 to 3.8 up to 28 weeks. Markedly, pH change from 4 weeks (green arrows) to 12 weeks (red arrows). In contrast, little or no mass loss was observed at 20 weeks (**Figure 2C**), followed by a period of more precipitous mass loss from 20 weeks (green arrows) to 34 weeks. The curve of mass change is typical for PLGA degradation.

Histological Observation

Eight weeks after transplantation, the graft was exposed (**Figure 3A**). It was not degraded with intact shape and it distributed some blood vessels on the surface of the graft. H&E staining showed a sparse organized structure with few cells in vehicle group ($DMEM/F_{12}$, **Figure 3B**), whereas the organized structure was compact and with greater cell numbers in cell transplantation groups (**Figures 3C–E**). The organized structure in control group (**Figure 3F**) was more regular than cell transplantation groups and $DMEM/F_{12}$ group. Although EPI-NCSCs (**Figure 3C**) or OECs (**Figure 3D**) transplantation alone can integrate into the donor, co-transplantation of EPI-NCSCs and OECs (EPI-NCSC+OEC, **Figure 3E**) displayed better effects, as reflected by much more surviving cells in EPI-NCSC+OEC group compared to EPI-NCSCs or OECs transplantation alone ($^{***}P < 0.001$; **Figure 3H**). However, it showed that there is no significant difference of survival number between single transplantation of OEC and co-transplantation of EPI-NCSC and OEC, which indicated that transplantation microenvironment may be more suitable for OECs growth rather than EPI-NCSCs.

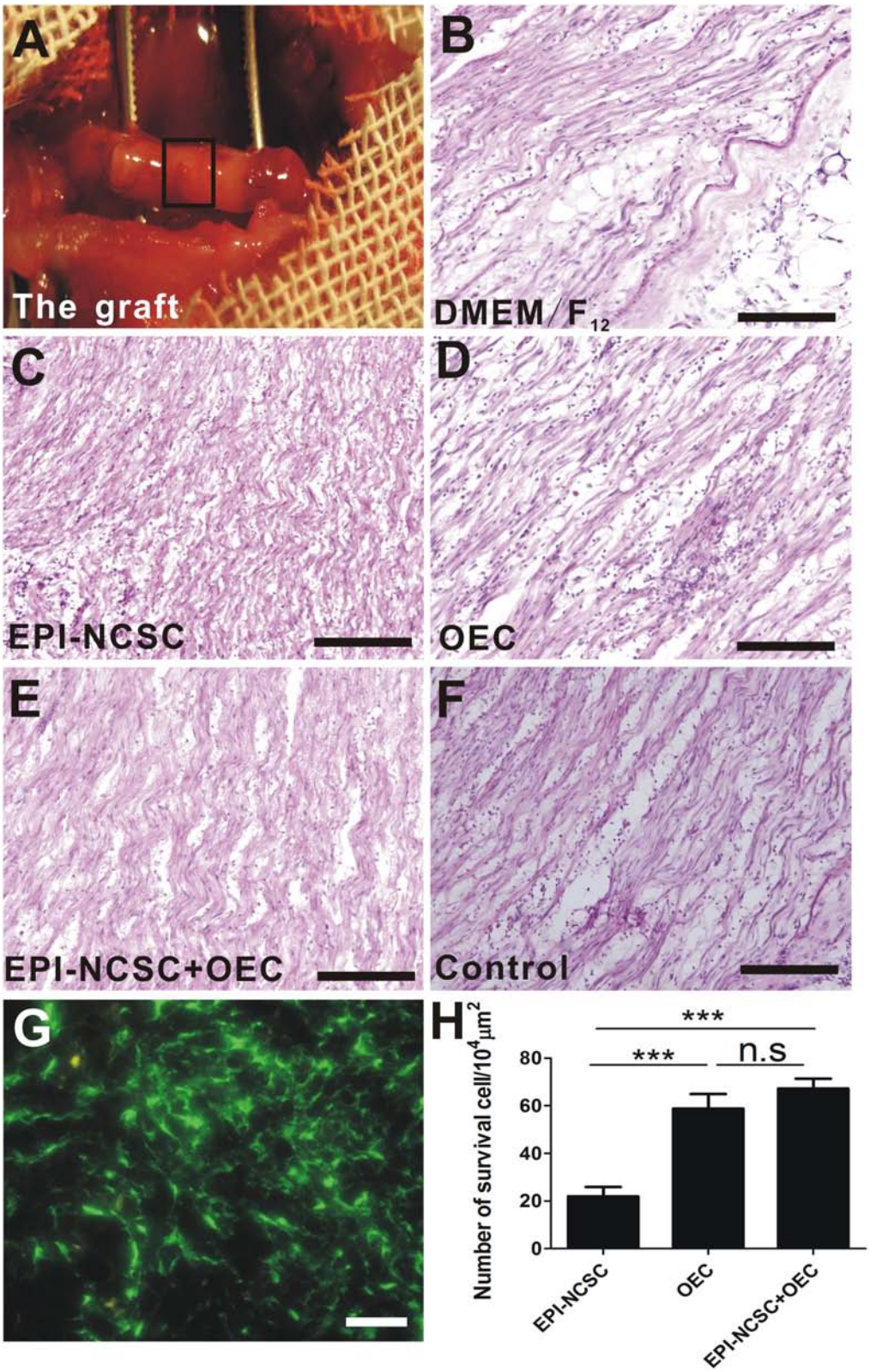

FIGURE 3 | The histological observation of the graft and cell survival *in vivo* 8 weeks after transplantation. **(A)** The graft. HE staining of longitudinal sections from **(B)** the DMEM/F_{12} group, **(C)** the EPI-NCSC group, **(D)** the OEC group, **(E)** the EPI-NCSC+OEC group, **(F)** the Control group. **(G)** Transplanted GFP-cells, **(H)** the number of surviving cells. **(B–F)** Scale, 100 μm; **(G)** Scale, 15 μm; Values are shown as mean ± SD (n = 6); ***P < 0.001; n.s., not significant.

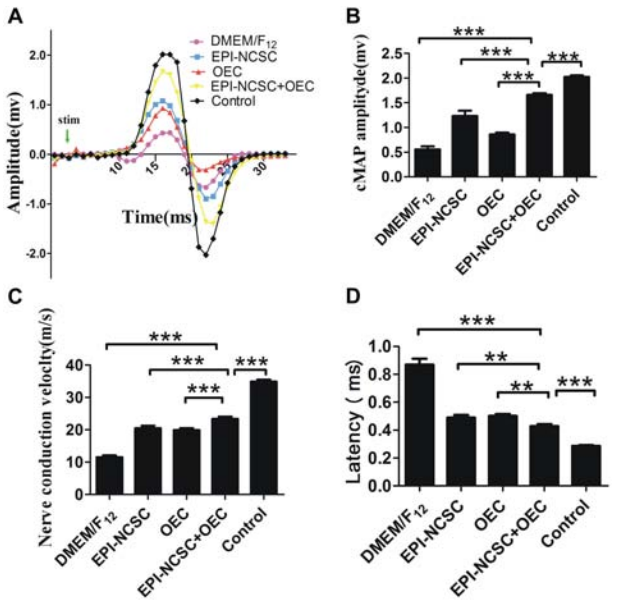

FIGURE 4 | Examination of cMAPs and NCV 8 weeks after transplantation. **(A)** cMAPs traces of the DMEM/F_{12}, EPI-NCSC, OEC, EPI-NCSC+OEC, and control groups. **(B)** Histogram shows latency in the different groups. **(C)** Histogram shows NCVs in the different groups. **(D)** Histogram shows cMAPs in the different groups. Values are shown as mean ± SD ($n = 5$); $^{***}P < 0.001$, $^{**}P < 0.01$.

These results indicate that transplanted cells are able to survive, migrate at the graft, and partly make up for nerve defects.

EPI-NCSC and OEC Co-transplantation Promotes Nerve Regeneration

Electrophysiology

Eight weeks after transplantation, cMAPs was recorded in the different groups (**Figure 4A**). The results showed that the amplitude of cMAPs was much bigger in the EPI-NCSC, OEC, and EPI-NCSC+OEC groups than that recorded in the DMEM/F_{12} group ($^{***}P < 0.001$; **Figure 4B**). Notably, although the amplitude of cMAPs in EPI-NCSC+OEC group was much bigger than that recorded in the EPI-NCSC or OEC group ($^{***}P < 0.001$, **Figure 4B**), it was still smaller than that recorded in the control group ($^{***}P < 0.001$, **Figure 4B**). The latency in the EPI-NCSC+OEC group was less than in the EPI-NCSC ($^{**}P < 0.01$, **Figure 4D**), OEC ($^{**}P < 0.01$, **Figure 4D**), and DMEM/F_{12} groups ($^{***}P < 0.001$, **Figure 4D**). Similarly, the NCVs in EPI-NCSC+OEC group was faster than that in the EPI-NCSC or OEC group ($^{***}P < 0.001$, **Figure 4C**). Taken together, these results indicate that co-transplantation of EPI-NCSCs and OECs improves nerve regeneration better than single EPI-NCSCs or single OECs.

Retrograde Tracing and Behavior Assessment

DiI-labeled SCL_{4-6} motoneurons were examined 8 weeks after transplantation in DMEM/F_{12} (**Figure 5A**), EPI-NCSC (**Figure 5B**), OEC (**Figure 5C**), EPI-NCSC+OEC (**Figure 5D**) and control groups (**Figure 5E**). The ratio of DiI-labeled SCL_{4-6} motoneurons in the EPI-NCSC+OEC group was higher than that in the DMEM/F_{12} ($^{***}P < 0.001$), but lower than the Control group ($^{***}P < 0.001$, **Figure 5F**). The ratio of DiI-labeled SCL_{4-6} motoneurons in the EPI-NCSC+OEC group had no significant difference compared with the EPI-NCSC or OEC group (**Figure 5F**). Locomotor and sensorial functions were assessed by the SFI and limb withdrawal latency from a hot water bath at 2, 4, and 8 weeks after transplantation (**Figures 5G,H**). Although the SFI displayed no difference among these cell transplantation groups at 2 and 4 weeks, the SFI in the EPI-NCSC+OEC group was much bigger than that in the DMEM/F_{12} ($^{***}P < 0.001$, **Figure 5G**), OEC ($^{***}P < 0.001$, **Figure 5G**) and EPI-NCSC groups ($^{*}P < 0.05$, **Figure 5G**) at 8 weeks after transplantation. Notably, the SFI in the EPI-NCSC+OEC group was still lower than that in the control group ($^{***}P < 0.001$, **Figure 5G**). Similarly, the withdrawal latency in the EPI-NCSC+OEC group was shorter than that in the DMEM/F_{12} ($^{***}P < 0.001$, **Figure 5H**), EPI-NCSC ($P < 0.001$, **Figure 5H**) and OEC groups ($^{***}P < 0.001$, **Figure 5H**), but still shorter than that in the control group ($^{***}P < 0.001$, **Figure 5H**). Altogether, these behavioral results indicate that co-transplantation of EPI-NCSCs and OECs has better effect on improving motor and sensory functions in rats subjected to PNI.

Toluidine Blue Staining and TEM

Eight weeks after transplantation, toluidine blue staining was performed to assess the remyelination of injured nerves. The results showed that the density of myelinated axons in the EPI-NCSC+OEC group was greater than that in the EPI-NCSC ($^{*}P < 0.05$, **Figure 6F**), and DMEM/F_{12} groups ($^{***}P < 0.001$, **Figure 6F**), but still less than that in the control group ($^{***}P < 0.001$, **Figure 6F**). In addition, the density of myelinated axons in the EPI-NCSC+OEC group has no significant difference vs. the OEC group. Further TEM analysis showed that the mean thickness of myelinated nerve fibers in the EPI-NCSC+OEC group was much bigger than that in the EPI-NCSC ($^{***}P < 0.001$, **Figure 6L**), OEC ($^{***}P < 0.001$, **Figure 6L**) and DMEM/F_{12} groups ($^{***}P < 0.001$, **Figure 6L**), but still less than that in the control group ($^{***}P < 0.001$, **Figure 6L**). In general, toluidine blue staining and TEM demonstrate that co-transplantation EPI-NCSC and OEC effectively facilitate the myelinated axons regeneration compared to single transplantation of EPI-NCSC or OEC.

TUNEL Staining and Gastrocnemius Recovery

TUNEL staining displays that the percentage of motoneuron apoptosis in the EPI-NCSC+OEC group is lower than that in the DMEM/F_{12} ($^{***}P < 0.001$, **Figure 7F**), EPI-NCSC ($^{***}P < 0.001$, **Figure 7F**) and OEC groups ($^{*}P < 0.05$, **Figure 7F**), but has no significant difference compared to the control group (**Figure 7F**). Neurons with green fluorescence are apoptotic (**Figures 7A–E**), and puce cells are also apoptotic neurons (**Figures 7G–K**). The resuming ratio of gastrocnemius in the EPI-NCSC+OEC group is bigger than that in the DMEM/F_{12} group ($^{***}P < 0.001$, **Figure 7L**), EPI-NCSC ($^{***}P < 0.001$, **Figure 7L**), and OEC groups ($^{***}P < 0.001$, **Figure 7L**), but lower than in the control

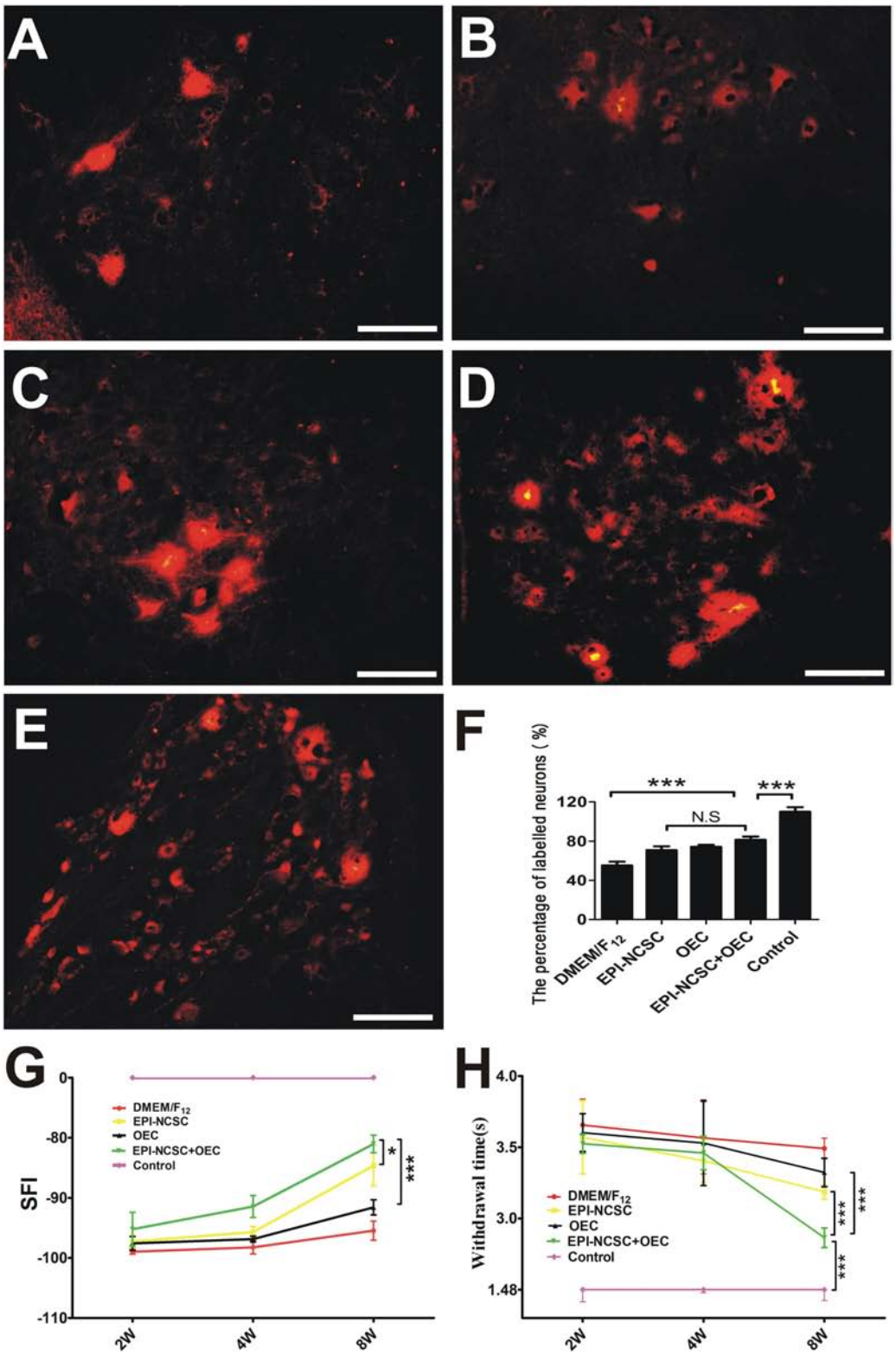

FIGURE 5 | The assessment of nerve function 8 weeks after transplantation. Dil-labeled motoneurons of SCL_{4-6} in **(A)** the DMEM/F_{12} group, **(B)** the EPI-NCSC group, **(C)** the OEC group, **(D)** the EPI-NCSC+OEC group, **(E)** the Control group, **(F)** histogram showing the ratio of Dil-labeled motoneurons in all groups, **(G)** motor function assessment by SFI 2, 4, and 8 weeks after transplantation, **(H)** sensory function assessment by withdrawal time from a hot water bath 2, 4, and 8 weeks after transplantation. Scale, 15 μm, Values are shown as mean ± SD ($n = 5$); N.S: no significant difference, $^{*}P < 0.05$, $^{***}P < 0.001$.

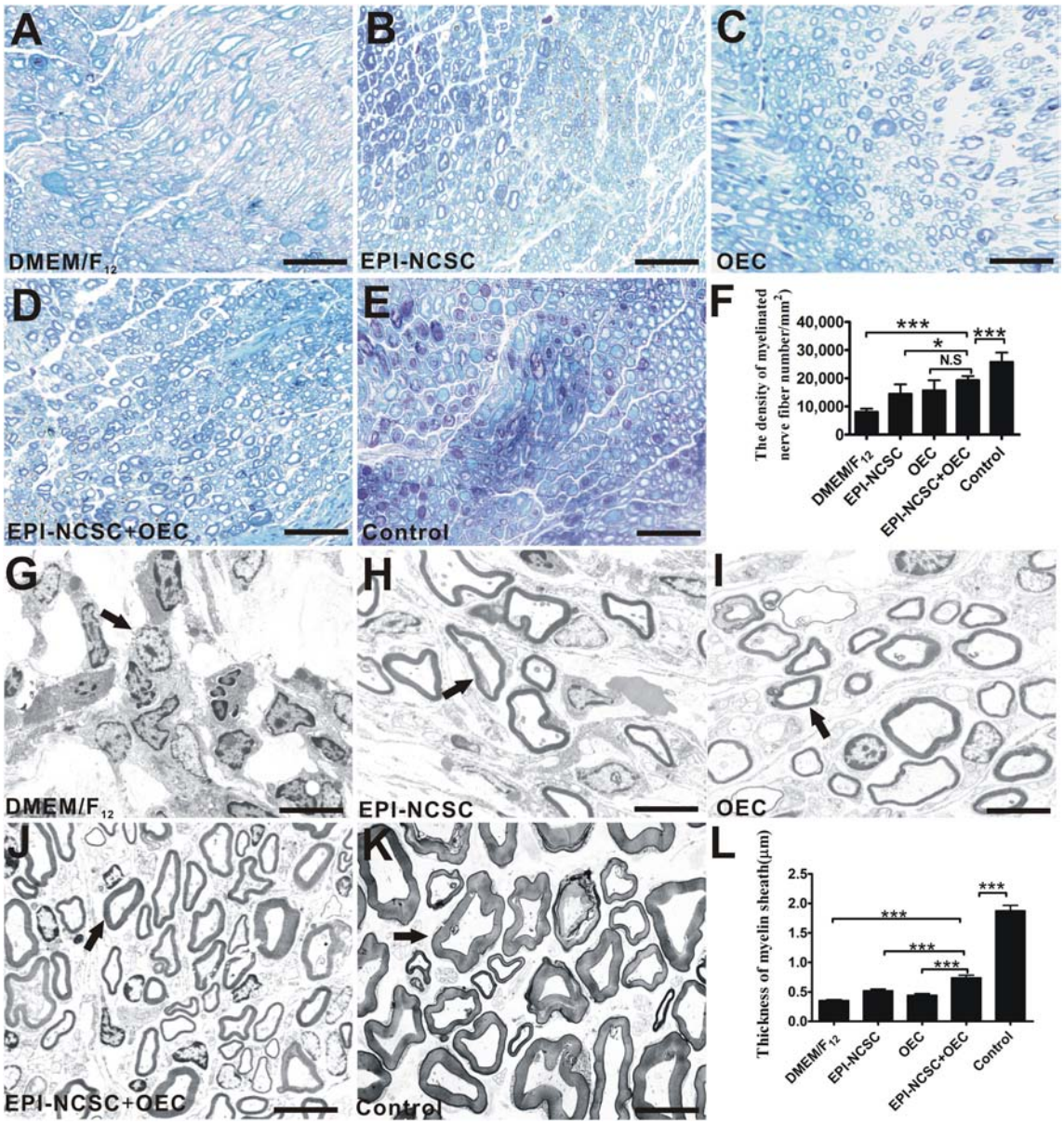

FIGURE 6 | Toluidine blue staining and TEM of regenerated nerves 8 weeks after transplantation. Light microscopy images of toluidine blue staining of **(A)** the DMEM/F_{12} group. **(B)** The EPI-NCSC group. **(C)** The OEC group, **(D)** the EPI-NCSC+OEC group, and **(E)** the Control group. **(F)** Analysis of the density of myelinated nerve fibers. TEM images of ultrathin sections showing myelinated nerve fibers in **(G)** the DMEM/F_{12} group, **(H)** the EPI-NCSC group, **(I)** the OEC group, **(J)** the EPI-NCSC+OEC group, and **(K)** the Control group. **(L)** Analysis of the thickness of myelinated nerve fibers. **A–D**: Scale, 50 μm; **E–H**: Scale, 10 μm; Values are shown as mean ± SD ($n = 5$); N.S: no significant difference, $^{*}P < 0.05$; $^{***}P < 0.001$.

group ($^{***}P < 0.001$, **Figure 7L**) 8 weeks after transplantation. In conclusion, these results indicate that the co-transplantation of EPI-NCSCs and OECs decreases motoneuron apoptosis and improves gastrocnemius recovery after PNI.

Analysis of BDNF and NGF

To elucidate the potential mechanisms by which co-transplantation of EPI-NCSCs and OECs promoted the regeneration of peripheral nerves, we next measured the levels of BDNF and NGF. IHC for BDNF showed that the number of BDNF positive cells in the EPI-NCSC+OEC group was greater than that in the EPI-NCSC ($^{**}P < 0.01$, **Figure 8F**), OEC ($^{*}P < 0.05$, **Figure 8F**), and DMEM/F_{12} groups ($^{***}P < 0.001$, **Figure 8F**), but has no significant difference compared to the control group (**Figure 8F**). The number of NGF positive cells in the EPI-NCSC+OEC group is greater than that in all other groups including the DMEM/F_{12} ($^{***}P < 0.001$, **Figure 8L**), OEC ($^{***}P < 0.001$, **Figure 8L**), and control groups ($^{**}P < 0.01$, **Figure 8L**), but has no significant difference compared to the EPI-NCSC group.

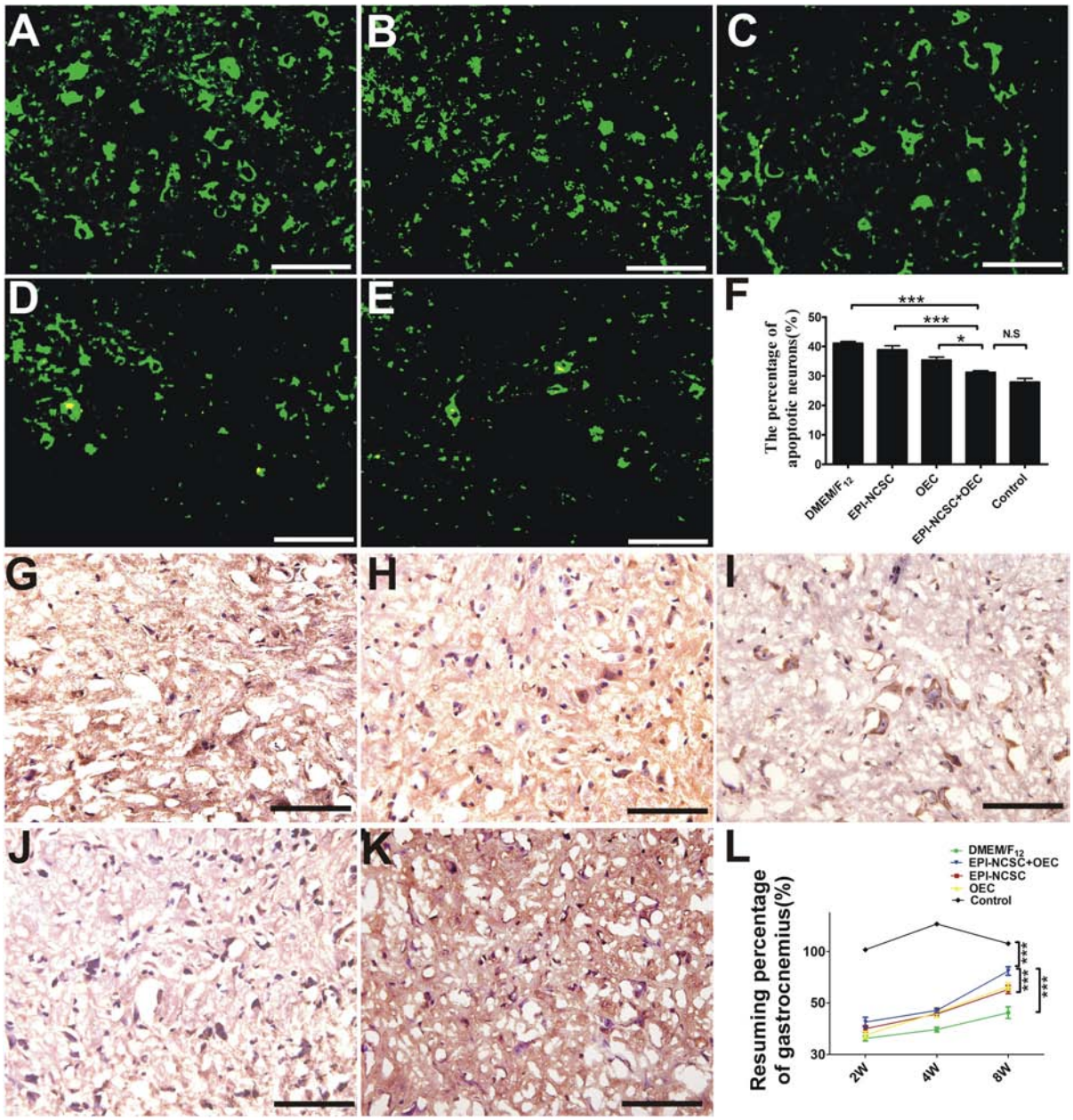

FIGURE 7 | TUNEL staining and gastrocnemius recovery after transplantation. **(A)** Fluorescent image of motoneurons apoptosis in the DMEM/F_{12} group. **(B)** The EPI-NCSC group. **(C)** The OEC group. **(D)** The EPI-NCSC+OEC group. **(E)** The Control group. TNUEL staining of motoneurons apoptosis in **(F)** histogram of the percentage of apoptosis motoneuron. **(G)** The DMEM/F_{12} group. **(H)** The EPI-NCSC group. **(I)** The OEC group. **(J)** The EPI-NCSC+OEC group. **(K)** The Control group **(L)** the assessment of gastrocnemius 2, 4, and 8 weeks after transplantation. Scale = 25 μm; Values are shown as mean ± SD (n = 5); N.S: no significant difference, $^{*}P < 0.05$, $^{***}P < 0.001$.

We next directly detected the expression level of BDNF and NGF both in the SN and in the SC 8 weeks after transplantation by ELISA. The results showed that the amount of BDNF in the SN and SC was much higher in the EPI-NCSC+OEC group, compared with the EPI-NCSC ($^{***}P < 0.001$, **Figure 8M**), OEC ($^{***}P < 0.001$, **Figure 8M**), and DMEM/F_{12} groups ($^{***}P < 0.001$, **Figure 8M**). Similarly, the amount of NGF in the SN and SC was also much higher in the EPI-NCSC+OEC group, compared with the EPI-NCSC ($^{***}P < 0.001$, **Figure 8N**), OEC ($^{***}P < 0.001$, **Figure 8N**) and DMEM/F_{12} groups ($^{***}P < 0.001$, **Figure 8N**).

In short, these results indicate that co-transplantation of EPI-NCSCs and OECs significantly increases the expression of BDNF and NGF, and then may consequently promote nerve regeneration.

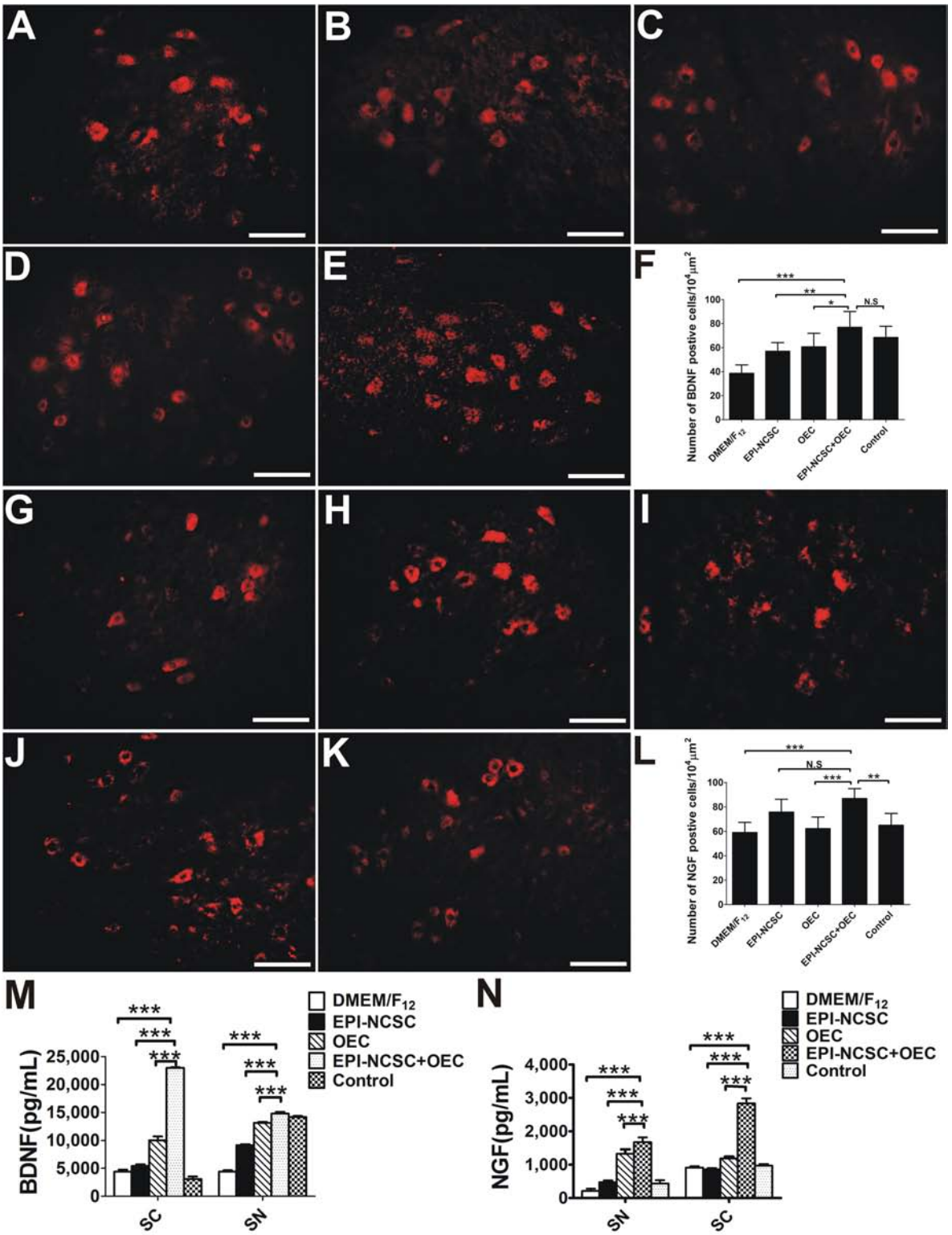

FIGURE 8 | The expression analysis of BDNF and NGF 8 weeks after transplantation. Immunofluorescent staining of BDNF in **(A)** the DMEM/F_{12} group, **(B)** the EPI-NCSC group, **(C)** the OEC group, **(D)** the EPI-NCSC+OEC group, and **(E)** the Control group. **(F)** Histogram of the number of BDNF positive cells in each group. Immunofluorescent staining of NGF in **(G)** the DMEM/F_{12} group, **(H)** the EPI-NCSC group, **(I)** the OEC group, **(J)** the EPI-NCSC+OEC group, and **(K)** the Control group. **(L)** Histogram of the number of NGF positive cells in each group, **(M)** expression levels of BDNF in SC and SN, **(N)** expression levels of NGF in SC and SN. Scale, 20 μm; values are shown as mean ± SD ($n = 5$); N.S: no significant difference, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

DISCUSSION

The poor outcome after PNI has provoked researchers to improve methodologies for peripheral nerve regeneration (Xu et al., 2012; Hu et al., 2013). In this study, we used a rat sciatic nerve defect model to demonstrate that EPI-NCSC and OEC co-transplantation may ameliorate PNI. We further employed histology (TEM, IHC), electrophysiology *in vivo*, behavioral tests and retrograde tracing methods to identify the facilitation of peripheral nerve regeneration and nerve function recovery after co-transplantation.

Peripheral nerve system has a great regeneration potential, particularly when there is an appropriate microenvironment, such nerve conduits applied to a nerve defect that provide a guide and a biological environment for nerve regeneration. During the past few years, studies have been focused on various conduit materials, particularly biodegradable polymers such as poly(glycolic acid) (PGA) (Waitayawinyu et al., 2007), poly(L-lactic acid) (PLLA) (Hood et al., 2009), polycaprolactone (PCL) (Mligiliche et al., 2003) and Poly(lactic-co-glycolic acid) (PLGA) (Bini et al., 2004). These studies have indicated that the conduit itself does not have a pronounced effect on nerve repair. Thus, approaches to nerve repair are now focused on the optimization of the combination of nerve conduits such as NTFs (Tang et al., 2013), ECM (Li et al., 2010, 2017), and seed cells (Gu et al., 2014). We have reported that PLGA is strong (**Figures 2C**, **3A**), and has neural affinity and biocompatibility with cells (Zhang et al., 2014), which are ideal graft properties for nerve regeneration (Li et al., 2010; Zhang et al., 2014). Interestingly, ECM provides well microenvironment, which regulate cell adhesion, spreading, and proliferation, and promote cell survival and myelination (Chernousov et al., 2008). Therefore, in this study transplanted cells combined with ECM may facilitate transplanted cell adhesion, spreading, proliferation, and cell survival. These are in line with the results in our study (**Figures 3G,H**).

Cell transplantation provides great potential for enhancement of nerve regeneration in view of NTFs, anti-inflammatory effect, as well as axon regeneration. EPI-NCSCs is a preferred candidate for cell transplantation, due to multipotent potential, autologous tissue without immune issue, and easy obtainment. What's more, it combines the virtue of adult stem cells and embryonic stem cells. EPI-NCSCs, originated from the embryonic neural crest, can be easily obtained from the bulge of hair follicles without harmlessness for the donor (**Figures 1A–D**), which expand quickly *in vitro*. Recent studies have demonstrated the utilization of stem cells for peripheral nerve regeneration (Amoh et al., 2005). OECs secrete many NTFs including BDNF and NGF, which is often used to repair PNI (Guérout et al., 2011; Zhu et al., 2014; Ruiz-Mendoza et al., 2016; Wright et al., 2018). Based on the above, we co-implanted EPI-NCSCs and OECs into nerve conduits that were applied to a rat sciatic nerve defect model, demonstrating that EPI-NCSC and OEC co-transplantation increased the number of myelin sheathes (**Figures 6D,I,K**) and facilitated functional nerve recovery (**Figures 5G,H**).

The recovery of locomotor function is assessed by the SFI (de Medinaceli et al., 1982). In the present study, the SFI of the EPI-NCSC+OEC group was higher than that the DMEM/F_{12} group 8 weeks after transplantation (**Figure 5G**). This is consistent with report that transplanted OECs in a transected sciatic nerve model improved the motor function 3 weeks after surgery (Radtke et al., 2009). In the sensory function test, withdrawal time from hot water was employed to assess the recovery of sensory function (Röyttä et al., 1999). A previous study demonstrated that transplanted EPI-NCSCs promoted sensory recovery in response to warm water withdrawal (Li et al., 2017); we showed a similar but modest effect, whereas we observed a quicker withdrawal response in the EPI-NCSC+OEC group when compared with the other groups, except that the withdrawal response never came to reaching the control group (**Figure 5H**). This indicated that EPI-NCSC+OEC co-transplantation promoted the partial recovery of motor and sensory function. We further assessed the function of the regenerating sciatic nerves by electrophysiology *in vivo*. The results indicated that NCV, cMAPs and latency in the EPI-NCSC+OEC group were improved compared with the individual cell group (**Figure 4**). Previous studies have reported that transplanted neural stem cells improve NCV in a rat sciatic nerve transection model (Xu et al., 2012), that EPI-NCSCs promote the recovery of sciatic nerve cMAPs (Li et al., 2017), and that transplanted OECs improved sciatic nerve NCV and cMAPs in a rat sciatic nerve defect model (Li et al., 2010). We further performed gross observations and histological analyses by HE and TEM, which showed that the number, and thickness of myelin sheathes in the EPI-NCSC+OEC group were greater than in the individual cell group (**Figure 6**), similar to a previous study showing that transplanted OECs promoted the recovery of myelin sheathes in a rat sciatic nerve defect model (Franklin et al., 1996; Li et al., 2010). However, it reported that OECs was capable of remyelinating demyelinated CNS axons following transplantation into rat spinal cord injuries (Barnett et al., 2000; Kato et al., 2000) and promoted axon sprouting in the lesioned spinal cord (Richter et al., 2005), which indicated that OECs might play a crucial role in the regeneration of myelin sheathes and axon. To assess the accuracy of axon regeneration by EPI-NCSC+OEC co-transplantation, we used DiI retrograde tracing methods. The results indicated that the number of DiI-labeled motoneurons in SCL_{4-6} in the EPI-NCSC+OEC group was greater than in the DMEM/F_{12} group (**Figure 5**). Further, EPI-NCSC+OEC co-transplantation reduced motoneuron apoptosis in SCL_{4-6} 8 weeks after implantation (**Figure 7**), similar to a previous study using horseradish peroxidase retrograde tracing that demonstrated greater neuron survival following NCSCs transplantation than in the control group 52 weeks after transplantation (Lin et al., 2009). Strikingly, triple fluorescent retrograde tracing determined that OECs promoted the recovery of facial motor nerves by stimulating axonal sprouting (Guntinas-Lichius et al., 2001). Additionally, the resuming ratio of gastrocnemius in the EPI-NCSC+OEC group was higher than the EPI-NCSC, OEC, and DMEM/F_{12} groups (**Figure 7L**) which further indicated that EPI-NCSC+OEC co-transplantation promoted neurotrophic support to the recovery of gastrocnemius. Overall, our results indicate that EPI-NCSC+OEC co-transplantation may promote axonal regeneration and the recovery of nerve function.

However, how can functional nerve and axon regeneration be improved? Research to date indicates the importance of NTFs, particularly BDNF and NGF, during recovery after PNI (Sendtner et al., 1992; Zhang et al., 2000; Shakhbazau et al., 2012a; Tang et al., 2013). A growing amount of evidence indicates that, in addition to providing structural support for growing axons by the expression of NTFs (Stoll and Müller, 1999), OECs release many NTFs, including NGF, BDNF and GDNF (Bunge et al., 1989; Brown et al., 1991). NGF is known to guide axons (Yu et al., 2010), promote axonal sprouting (Tuszynski et al., 1996), and cell migration (Cao et al., 2007; **Figures 3G**, **8N**), stimulate myelination (Chan et al., 2004), improve the regeneration of sensory neurons and reduce denervated muscle atrophy (Crowley et al., 1994; Shakhbazau et al., 2012a; **Figures 7L**, **8N**), and eventually to improve functional recovery after injury (Röyttä et al., 1999; Kemp et al., 2011). In our study, the expression of NGF in the EPI-NCSC+OEC group was higher than in the other group (**Figures 8G–L,N**), indicating that NGF might participate in nerve repair. Up-regulation of NGF might also play a vital role in nerve repair by increasing the number of myelin sheathes, which was greater in the EPI-NCSC+OEC group than in the DMEM/F_{12}, EPI-NCSC or OEC groups (**Figure 6F**). Besides, BDNF is important for stimulating axonal elongation (Acheson et al., 1991; Zhang et al., 2000; English et al., 2005; Wilhelm et al., 2012) and for survival of motoneurons (Sendtner et al., 1992; Yan et al., 1992; Koliatsos et al., 1993). We also found that the expression of BDNF in the EPI-NCSC+OEC group was greater than in the other group (**Figures 8A–F,M**). Likewise, it reported that OEC-M treatment after contusive SCI increased BDNF levels and then improved the function recovery and promoted the axonal regeneration (Pastrana et al., 2007; Gu et al., 2017). Herein, high expression level of BDNF and NGF in the EPI-NCSC+OEC group might improve nerve function and promote the axonal regeneration. Therefore, in this study we speculated that the mechanism that EPI-NCSC+OEC co-transplantation repaired PNI might be up-regulation of BDNF and NGF, but BDNF and NGF might derive from: (1) OEC might secrete, which have been reported to express BDNF and NGF and facilitate axonal regeneration after SCI (Lipson et al., 2003); (2) Schwann cells might secrete BDNF and NGF after PNI *in vivo* (Shakhbazau et al., 2012b); (3) EPI-NCSC might secrete little BDNF and NGF (Sieber-Blum et al., 2006), but the combination of EPI-NCSC and OEC might heighten the expression level of BDNF and NGF, which have been reported that co-culture of Schwann cells and adult stem cells led to synergistic neurotrophic effects (BDNG and NGF) in PNI (Dai et al., 2013) and synergic effects of EPI-NCSCs and OECs increased the expression of BDNF and GDNF in SCI (Zhang et al., 2015). One maybe that the combination of cells promote the secretion of BDNF and NGF from Schwann cells of donor; another maybe that EPI-NCSC prompts the expression of BDNF and NGF from OECs. However, specific origin of BDNF and NGF remain to further investigate.

Overall, our results unveiled a beneficial effect of co-transplantation of EPI-NCSCs and OECs after PNI, whereas many issues remained to be determined such as the differentiation of stem cells *in vivo*. EPI-NCSC and OEC co-transplantation was a promising and easily transformable approach that could lead to significant amelioration of patients suffering from PNI.

CONCLUSION

Our findings indicated that EPI-NCSC and OEC co-transplantation promoted sciatic nerve regeneration and improves nerve function. Moreover, the mechanism of PNI improved by EPI-NCSC and OEC co-transplantation was likely to up-regulate the expression of BDNF and NGF. The application of EPI-NCSC and OEC co-transplantation in clinical trials might improve clinical outcomes and provided a new methods for PNI.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All experimental procedures with animals were approved by the local institution review board and were carried out according to the guidelines of the Third Military Medical University (Chongqing, China) for the care and use of laboratory animals.

AUTHOR CONTRIBUTIONS

LZ performed the experiment, conceived the study design, analyzed the data, and drafted the manuscript. BLi and BLiu participated in conception and design of the experiments. ZD modified the manuscript.

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SUPPLEMENTARY MATERIAL

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Interleukin-1β Promotes Schwann Cells De-Differentiation in Wallerian Degeneration via the c-JUN/AP-1 Pathway

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Schwann cells (SCs) de-differentiate in Wallerian degeneration (WD) following nerve injury and, by doing so, can actively promote nerve repair and functional recovery. An innate-immune response is an important component of the complex of events referred to as WD. Damaged peripheral nervous system SCs produce IL-1β and other inflammatory cytokines. We hypothesized that, in addition to a role in immune responses, IL-1β participates in de-differentiation and proliferation of SCs. qPCR and ELISA demonstrated that expression of IL-1β mRNAs and protein increased after nerve injury. Immunofluorescent staining and western blotting demonstrated that expression of the p75 neurotrophin receptor (p75NTR) was significantly increased and levels of myelin protein zero (MPZ) were significantly decreased after IL-1β exposure compared with control groups *in vitro* WD. Additionally, qPCR demonstrated that IL-1β elevated expression of the de-differentiation gene p75NTR and decreased expression of myelination locus MPZ and promoted SCs de-differentiation. Furthermore, immunofluorescent staining, western blotting, qPCR and ELISA revealed that IL-1β promoted c-JUN expression and activation of AP-1 activity of SCs in an *in vitro* WD model. Finally, Immunofluorescent staining illustrated that IL-1β elevated expression of Ki67 in SCs nuclei, the apoptosis of SCs were detected by TUNEL. SCs of WD produce IL-1β which promotes SCs de-differentiation and proliferation.

Keywords: Wallerian degeneration, Schwann cells, IL-1β, MPZ, p75NTR, c-JUN/AP-1, de-differentiation

INTRODUCTION

The ultimate goal of regenerative medicine research is to enable replacement of lost or damaged tissues or organs. Regeneration can potentially be accomplished using the processes of de-differentiation, trans-differentiation or reprogramming. Humans have a limited capacity to regenerate tissues or organs, including liver and the peripheral nervous system (PNS). These tissues respond to injury through cellular reprogramming, producing cells that specifically promote repair and regeneration (Jessen and Arthur-Farraj, 2019). In some cases, the process of regeneration

Abbreviations: DAPI, 4′6-diamino-2-phenyl indole; PNS, peripheral nervous system; RT, reverse transcription; SCs, Schwann cells; SNs, sciatic nerves; WD, Wallerian degeneration.

involves de-differentiation of mature cells. De-differentiation is a mechanism in which terminally differentiated cells revert to a less-differentiated stage within the same lineage and allows cells to proliferate before re-differentiating, leading to the replacement of lost cells (Jopling et al., 2011).

Schwann cells (SCs), myelinated glial cells of the PNS, de-differentiate and convert to denervated SCs in Wallerian degeneration (WD) following nerve injury and, by doing so, can actively promote nerve repair and functional recovery (Jessen and Mirsky, 2008; Tricaud and Park, 2017) So, it is also described as repair SCs. Meanwhile, they activate a series of repair-related phenotypes (Jessen and Mirsky, 2016; Jessen and Arthur-Farraj, 2019). Following de-differentiation, SCs clear myelin debris by autophagy, in addition, SCs contribute to macrophage-mediated myelin removal and re-enter the cell cycle, proliferate, and then form bands of Büngner, which support and direct outgrowing axons to sites of innervation (Martini et al., 2008; Rotshenker, 2011; Brosius et al., 2017). Moreover, these cells express and secrete a large number of axonal growth promoting factors, then re-differentiate and myelinate regenerated axons, which eventually leads to substantial functional recovery (Woszczycka-Korczynska et al., 2013; Jessen and Mirsky, 2016). Damaged SCs may induce cell apoptosis and limit functional recovery of peripheral nerves (Zhao et al., 2017). This sequence emphasizes the central function of SC de-differentiation in PNS regeneration.

An innate-immune response is an important component of the complex of events referred to as WD. PNS injury induces immune and non-immune cells to produce cytokines and develop an efficient cytokine network during WD (Rotshenker et al., 1992; Be′Eri et al., 1998; Shamash et al., 2002). Before macrophage recruitment, injured PNS produce IL-1β and other inflammatory cytokines (Rotshenker, 2011). These inflammatory cytokines have an irreplaceable effect on the initiation and regulation of inflammation during injury (Bastien and Lacroix, 2014). Certain inflammatory cytokines can influence de-differentiation in some types of terminally differentiated cells. For example, IL-1β induces chondrocyte de-differentiation (Montaseri et al., 2011; Hong et al., 2014), and some scholars demonstrate that IL-1β also increases vascular smooth muscle cells de-differentiation and proliferation (Sasu and Beasley, 2000; Clement et al., 2006; Zhu et al., 2007). We hypothesized that, in addition to a role in immune responses, IL-1β participates in SC de-differentiation and proliferation. We used a rodent *in vitro* WD model to investigate effects of IL-1β on SC de-differentiation, excluding effects of immune cells and other inflammatory cytokines.

MATERIALS AND METHODS

In vitro WD Model

The Ethics Committee for Animal Research at the Ninth People's Hospital affiliated to Shanghai Jiao Tong University approved all experimental protocols involving the use of rats. Sciatic nerve (SN) explant cultures were performed as previously reported by Thomson et al. (1993) with minor modifications (Thomson et al., 1993; Lee et al., 2009). Eight-week old male Sprague–Dawley rats, obtained from the Ninth People's Hospital Animal Center, Shanghai, China, were euthanized with 10% chloral hydrate. SNs were exposed and carefully cut, and WD induced by nerve injury. Connective tissues surrounding the SNs and epineurium were carefully detached in DMEM under a stereomicroscope (Carl Zeiss). SNs were dissected into explants 1 cm in length. SN explants were established by loosely separating small bundles of fibers from the isolated nerve. The bundles of nerve were maintained in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B at 37°C with 5% CO_2. Cell culture reagents were obtained from Invitrogen. Nerve bundles were treated with or without various concentrations of recombinant rat IL-1β (401-ML-025, R&D Systems).

Schwann cells and fibroblasts compose most of the non-neuronal cell population in intact PNS, whereas macrophages, which are scarce in intact PNS, are recruited in large numbers from the third day after injury (Perry et al., 1987; Reichert et al., 1994). In our modified *in vitro* WD model, in which epineurium were carefully detached, SCs comprise the majority of the cell population in this model and are the primary object of this study.

RNA Extraction, RT-qPCR, and qPCR Analysis

Total RNA was isolated from SNs in the *in vitro* WD model. Briefly, SNs were washed with PBS and lysed with TRIzol reagent (Invitrogen), according to the manufacturer's protocol. 2 mg of total RNA was used for reverse transcription (RT), and the products used in qPCR. Target genes were quantified with an ABI 7500 Real-Time PCR System (Life Technologies). PCR primer pairs were designed based on sequences of different exons of the corresponding genes (**Table 1**). All PCR amplifications were performed with an initial denaturation at 95°C for 10 s, followed by 40 cycles at 95°C, 30 s, 60°C, 30 s, followed by melting curve analysis at 95°C, 60 s and 60°C, 30 s.

Quantification of IL-1β by ELISA

Fifteen 30 mm SN segments without epineurium were harvested from rats. Three SN segments were frozen immediately in liquid nitrogen. The remainder were cultured as per the *in vitro* WD model. These nerve segments were harvested after 12, 24, 36, and 48 h. Nerve segments were extracted in 1 ml PBS containing a mixture of protease inhibitors (Roche Molecular Biochemicals).

TABLE 1 | List of oligonucleotides used for quantitative real time PCR.

Target gene	Sequence	References
Rat IL-1β	5′ AGT GTG TGA TGT TCC CAT TAG 3′ 5′ GCT TAT GTT CTG TCC ATT GAG 3′	NM_031512.2
Rat p75NTR	5′ GAG GAT TAC GGA CCT ATC TGA 3′ 5′ TGC CTT TCT CTG GGT TTT AC 3′	NM_012610.2
Rat MPZ	5′ CAT TGT GGT TTA CAC GGA CAG 3′ 5′ CTT GGC ATA GTG GAA GAT TGA 3′	NM_017027.1
Rat c-JUN	5′ TGA AGT GAC CGA CTG TTC TAT 3′ 5′ CTT AGG GTT ACT GTA GCC GTA G 3′	NM_021835.3

We used two-site sandwich ELISA to identify and quantify IL-1β in SNs, according to the manufacturer's instructions (Duo-Set; R&D Systems). SNs were rinsed in ice-cold PBS to remove excess blood. Tissues were minced and homogenized in 1 ml PBS with a glass tissue grinder on ice. The resulting suspension was subjected to ultrasonication to further disrupt cell membranes. Homogenates were centrifuged for 15 min at 1500 × *g* and supernatants used for ELISA.

Immunofluorescent Staining

Teased nerve fibers mounted on slides were treated with PBS containing 4% Paraformaldehyde for 30 min and blocked with PBS containing 0.2% Triton X-100 and 2% BSA for 60 min. Nerve fibers were incubated with primary antibody (anti-p75NTR antibody 1:1000, ab52987, Abcam; anti-myelin protein zero (MPZ) antibody, 1:1000, ab31851, Abcam; anti-c-JUN antibody, 1:1000, #9165, Cell Signaling; anti-Ki67 antibody,1:100, ab16667, Abcam) for 16 h at 4℃ and washed three times with PBS. Next, slides were incubated with Alexa 549- or 488-conjugated secondary antibody (1:800, Alexa Fluor) for 2 h at room temperature and washed three times with PBS. Finally, slides were incubated with PBS counterstained with 4′6-diamino-2-phenyl indole (DAPI; Vectashield, Vector Laboratories) to visualize nuclei. DAPI staining was used for enumeration and identification of nuclei. The slides were visualized using a 20×/0.50 Plan-Neofluar lens (Carl Zeiss). c-JUN-positive endonuclear cells were counted to analyze percent of c-JUN (+)in three independent experiments. Ki67-positive endonuclear cells were counted to analyze percent of Ki67 (+)in three independent experiments.

Western Blotting

Proteins were extracted with RIPA lysis buffer containing 1 mM PMSF (Beyotime) and 40 mM protease inhibitor (Roche Molecular Biochemicals). Lysates were cleared by centrifugation at 14,000 rpm for 5 min at 4°C. Protein concentrations were measured using a BCA protein assay kit (Pierce Chemicals) according to the manufacturer's protocol. Reducing buffer was added to each protein extract and samples heated to 100°C for 5 min. Reduced samples containing equal amounts of protein were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in 5% BSA and probed with anti-p75NTR, anti-MPZ, and anti-c-JUN antibodies. Blots were washed, incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), and developed with ECL Plus (Amersham Biosciences). Filters were stripped and probed with a goat polyclonal antibody against β-actin (Santa Cruz Biotechnology) to assess equivalent protein loading and to normalize protein levels. Protein bands were analyzed using a chemiluminescence kit (Santa Cruz Biotechnology) and visualized using BandScan 5.0 software western immunoblotting detection system.

AP-1 Activity Assay

The AP-1 activity assay was used to examine AP-1 activity of SCs in the *in vitro* WD model with or without 5 ng/ml at 6, 12, and 24 h. The DNA binding activity of AP-1 was determined using an AP-1 enzyme-linked immunosorbent assay kit essentially as instructed by the manufacture (Active Motif North America). Briefly, samples in each group were lysed in 10 mM HEPES buffer, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and inhibitors of proteases as described above. After the addition of 0.6% (v/v) Nonidet P-40, the samples were incubated for 15 s on ice and then centrifuged at 13,000 × *g* for 30 s at 4℃. The pellet was suspended in the supplied nuclear lysis buffer and centrifuged at 13,000 × *g* for 10 min at 4℃. Nuclear protein (10 μg) was loaded into the 96-wells of an enzyme-linked immunosorbent assay plate pre-coated with an oligonucleotide containing the sequence 5′-TGAGTCAG-3′ and incubated for 60 min at room temperature. Mutated c-JUN oligonucleotides supplied in the kit were used as specificity controls. AP-1 binding to the nucleotide was detected with an anti-phospho-c-JUN antibody and horseradish peroxidase-conjugated secondary antibody followed by colorimetric analysis.

TUNEL Staining of Apoptosis

Teased nerve fibers mounted on slides were treated with PBS containing 4% Paraformaldehyde for 15 min, discarded with fixative solution and washed with PBS for 3 times. Next, slides were incubated with 0.1% sodium citrate buffer solution and 0.1% Trion × 100 for 2 min on ice. Rinse slides three times with PBS and add TUNEL reaction mixture (Roche Molecular Biochemicals) on nerve fibers for 60 min in the dark. Rinse slides three times with PBS. Finally, slides were incubated with PBS counterstained with 4′6-diamino-2-phenyl indole (DAPI; Vectashield, Vector Laboratories) to visualize nuclei. DAPI staining was used for enumeration and identification of nuclei. The slides were visualized using a 20 × /0.50 Plan-Neofluar lens (Carl Zeiss). The TUNEL labeled red and DAPI labeled blue were positive at the same time as apoptosis.

Statistical Analysis

Data are expressed as the means ± SE. Each independent experiment was repeated three times. The significance of differences between two independent samples was statistically assessed using Student's *t*-test. The statistical significance of differences between groups was determined by one-way ANOVA followed by the Least Significant Difference (LSD) test. p-values < 0.05 were considered significant.

RESULTS

IL-1β mRNA Expression and IL-1β Protein Production by SCs in *in vitro* WD Model

Sciatic nerves without epineurium were frozen immediately after removal from rats, as a control group. SNs were cultured as per *in vitro* WD model and harvested at different times. These SN tissues were used thereafter as sources for the detection of IL-1β mRNA expression and IL-1β protein production by SCs. Relative quantification of IL-1β mRNA was performed by qPCR, and results were analyzed by one-way ANOVA followed by LSD test (**Figure 1A**). The analysis revealed expression of IL-1β mRNA by SCs increased after SN injury, peaking at 12 h.

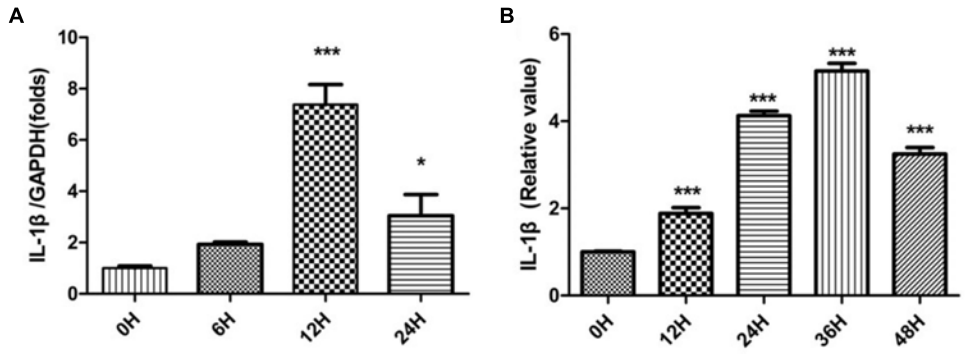

FIGURE 1 | IL-1β mRNA expression and IL-1β protein production. The relative quantification of IL-1β mRNA was performed by qPCR **(A)** and IL-1β protein levels were determined by ELISA **(B)** in SCs. The control group is labeled as 0 h. SCs harvested from SNs at different times were compared to the control group. $^{*}p < 0.05$, $^{***}p < 0.001$.

IL-1β exerts its biological activity as a soluble molecule only. We further tested for the presence of soluble IL-1β using ELISA. Expression levels of soluble IL-1β by SCs were compared with quantities in SNs. Relative values of IL-1β were analyzed by one-way ANOVA followed by LSD test (**Figure 1B**). Production of soluble IL-1β protein by SCs increased after nerve damage, peaking at 36 h.

Effect of IL-1β on De-Differentiation of SCs

Sciatic nerve SCs were treated with various concentrations of recombinant rat IL-1β (0, 5, and 50 ng/ml) in this *in vitro* WD model and harvested after 48 h. We detected expression of p75NTR, a marker of SC de-differentiation (Jessen and Mirsky, 2008; Shin et al., 2013), and MPZ, an essential factor in myelination in these SNs to assess SCs de-differentiation (Warner et al., 1998). Immunofluorescent staining demonstrated that expression of p75NTR increased and levels of MPZ decreased in the 5 ng/ml IL-1β group compared with 0 and 50 ng/ml groups (**Figures 2A,B**) at 48 h. Additionally, the difference of p75NTR and MPZ expression between the 50 and 0 ng/ml IL-1β groups is not obvious. These were quantitatively verified by western blotting, with results analyzed by one-way ANOVA followed by LSD test (**Figures 2C–E**). 5 ng/ml IL-1β promoted SCs de-differentiation. Nevertheless, a high concentration (50 ng/ml) IL-1β did have the similar effect.

Effect of IL-1β on Expression of p75NTR and MPZ mRNAs by SCs in *in vitro* WD Model

As 5 ng/ml IL-1β promoted SC de-differentiation, we further quantified expression of p75NTR and MPZ mRNAs by SCs in the present *in vitro* WD model. We harvested SCs from SNs with or without 5 ng/ml IL-1β at 6, 12, and 24 h. qPCR demonstrated that expression of p75NTR mRNA increased in a time-dependent manner in each concentration group, and was elevated more significantly in the 5 ng/ml group compared with the 0 ng/ml group (**Figure 3A**). Additionally, expression of MPZ mRNA decreased in a temporal manner in both concentration groups, and further decreased in the 5 ng/ml group compared with the 0 ng/ml group (**Figure 3B**). This suggested that 5 ng/ml IL-1β increased expression of the de-differentiation gene p75ntr and decreased expression of myelination locus MPZ, promoting SCs de-differentiation.

Effect of IL-1β on c-JUN and AP-1 Activity in SCs

The transcription factor c-JUN is a global regulator of WD and SCs de-differentiation (Arthur-Farraj et al., 2012; Jessen and Mirsky, 2016) and plays a role in demyelination after PNS injury (Parkinson et al., 2008; Lee et al., 2014). Additionally, these functions of c-JUN were localized in SC nuclei. Therefore, we firstly used immunofluorescent staining to detect the expression of c-JUN in SC nuclei. SCs exposed or not to 5 ng/ml IL-1β were harvested after 24 h. Immunofluorescent staining demonstrated that levels of c-JUN in SC nuclei increased in the 5 ng/ml group compared with the 0 ng/ml group. This was quantitatively verified by percent analysis of endonuclear c-JUN(+) cells (**Figures 4A,B**). In addition, western blot analysis also revealed that expression of c-JUN in SCs was significantly increased in the 5 ng/ml group in comparison to the 0 ng/ml group (**Figures 4C,D**). To further verify effects of IL-1β on c-JUN expression, we used qPCR to detect expression of c-JUN mRNA in SCs in our *in vitro* WD model with or without 5 ng/ml IL-1β at 6, 12, and 24 h. qPCR demonstrated that expression of c-JUN mRNA increased in a time-dependent manner in both concentration groups, and increased more significantly in the 5 ng/ml group compared with the 0 ng/ml group at 24 h (**Figure 4E**). These results suggested that 5 ng/ml IL-1β increased expression of c-JUN and c-JUN transcription factor, and elevated the proportion of endonuclear c-JUN(+) cells in SCs.

The transcription factor c-JUN is a component of the heterodimeric AP-1 transcription factor complex and c-JUN/AP-1 are highly expressed in response to neuronal

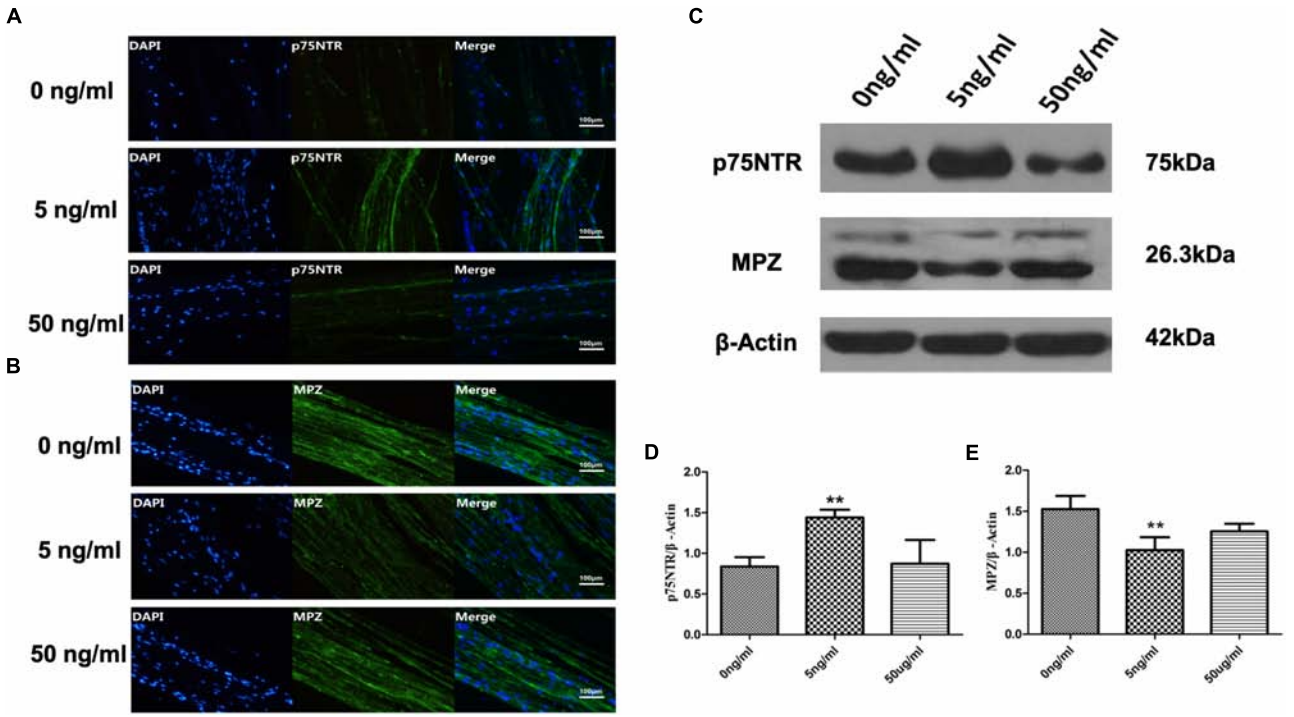

FIGURE 2 | Expression of p75NTR and MPZ in the *in vitro* WD model at 48 h. Immunofluorescent staining of p75nNTR **(A)** and MPZ **(B)**, and western blotting to quantitatively analyze expression levels **(C–E)** in SCs with various concentrations of IL-1β at 48 h. Results are means ± SE (n = 3 per group) from independent experiments. $^{**}p < 0.01$.

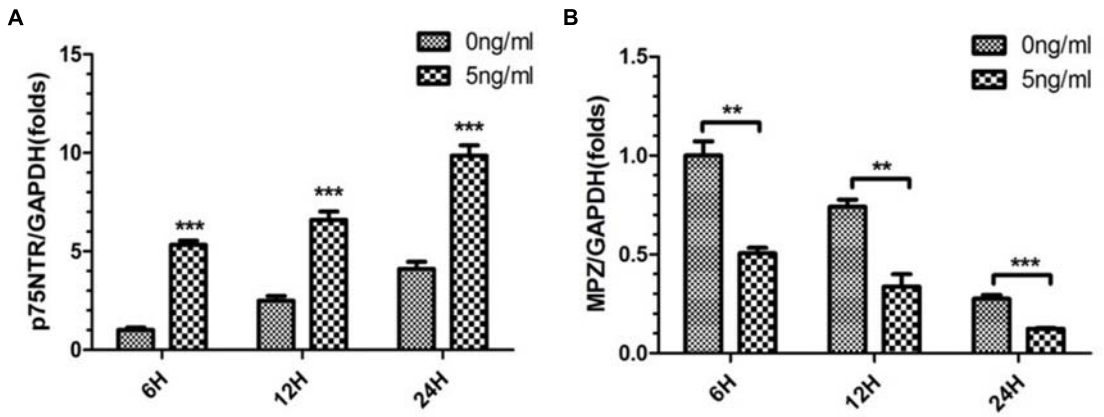

FIGURE 3 | Expression of p75NTR and MPZ mRNA in the *in vitro* WD model. qPCR was used to quantitatively analyze expression of p75NTR **(A)** and MPZ mRNAs **(B)** with or without 5 ng/ml IL-1β at 6, 12, and 24 h. Results are means ± SE (n = 3 per group) from independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

trauma (Raivich et al., 2004). We therefore examined AP-1 activity of SCs in the *in vitro* WD model with or without 5 ng/ml IL-1β at 6, 12, and 24 h using an AP-1 activity assay. This indicated that AP-1 activity increased in a time-dependent manner in both concentration groups, and was more significantly increased at 12 and 24 h in the 5 ng/ml group compared with the non-exposed group (**Figure 4F**). AP-1 activity assay results, combined with analyses of c-JUN, suggested that 5 ng/ml IL-1β promoted endonuclear c-JUN expression and stimulation of AP-1 activity in our *in vitro* WD model.

Effect of IL-1β on Proliferation and Apoptosis of SCs

We harvested SCs from SNs with or without 5 ng/ml IL-1β after 24 h. Then, we firstly used immunofluorescent staining to detect the expression of Ki67 in SC nuclei. This demonstrated

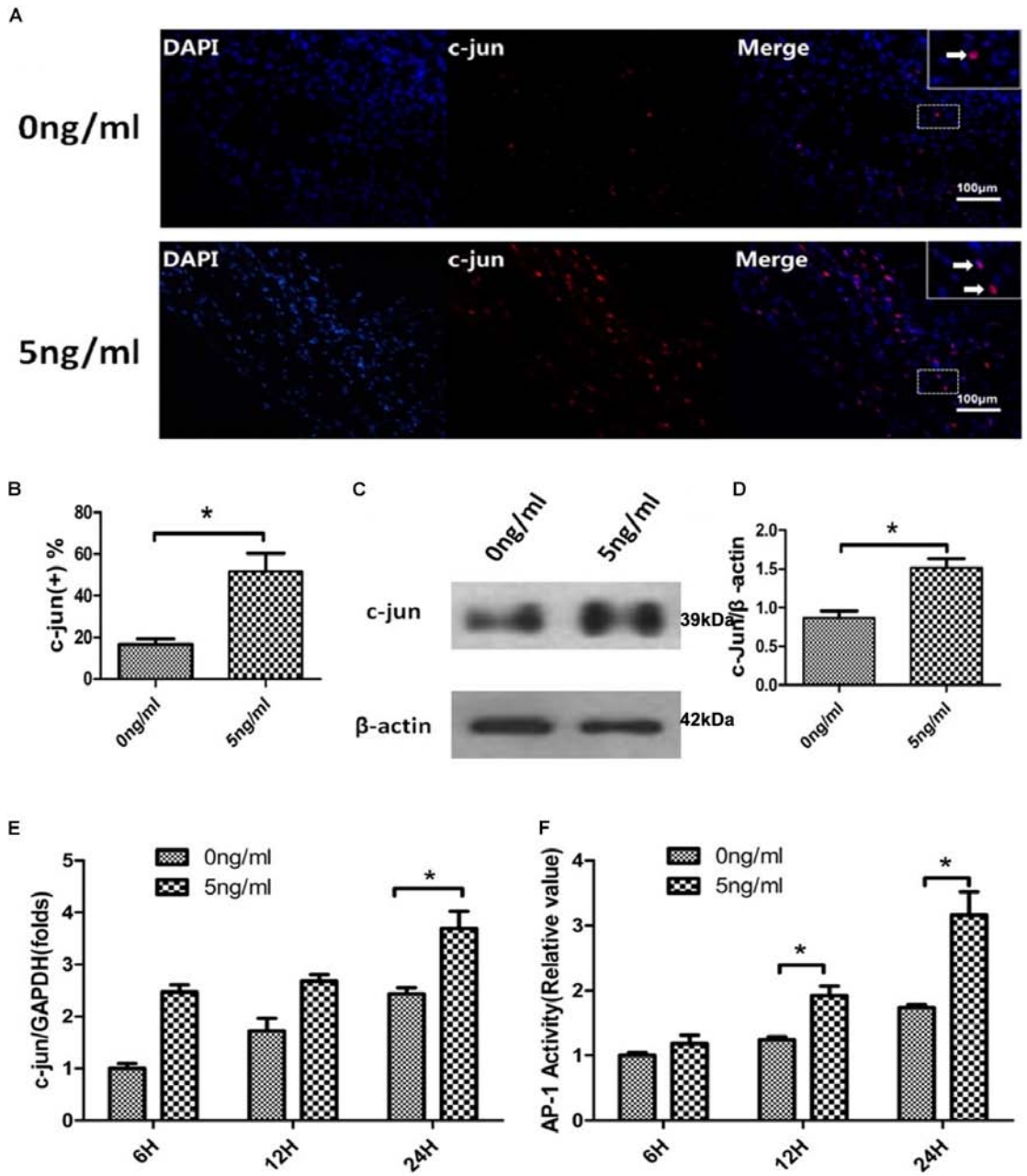

FIGURE 4 | c-JUN and AP-1 activity assay in the *in vitro* WD model. Immunofluorescent staining of c-JUN **(A,** arrows indicate c-JUN(+) cells**)**, analysis of percentage of endonuclear c-JUN(+) cells **(B),** and western blotting to quantitatively analyze expression of c-JUN **(C,D)** in SCs with or without 5 ng/ml IL-1β at 24 h. qPCR was used to quantitatively analyze expression of c-JUN mRNA **(E)**, and AP-1 activity assay to quantitatively analyze activity **(F)** of SCs in the *in vitro* WD model with or without 5 ng/ml IL-1β at 6, 12 and 24 h. Results are means ± SE (n = 3 per group) from independent experiments. *$p < 0.05$.

that levels of Ki67 in SCs nuclei increased in the 5 ng/ml group with the 0 ng/ml group. This was quantitatively verified by percent analysis of endonuclear Ki67(+) cells, and the percent was increased in the 5 ng/ml group compared with the 0 ng/ml group at 48 h (**Figures 5A,C**). After that, we used TUNEL to detect the percentage of SCs apoptosis. SCs harvested from *in vitro* WD model treated with or without 5 ng/ml IL-1β after 48 h. TUNEL demonstrated that percentage of SCs apoptosis decreased in the 5 ng/ml group compared with the 0 ng/ml group (**Figures 5B,D**).

DISCUSSION

In a reminiscent process of the injury responses of zebrafish cardiomyocytes or pigment cells of the newt iris, nerve injury

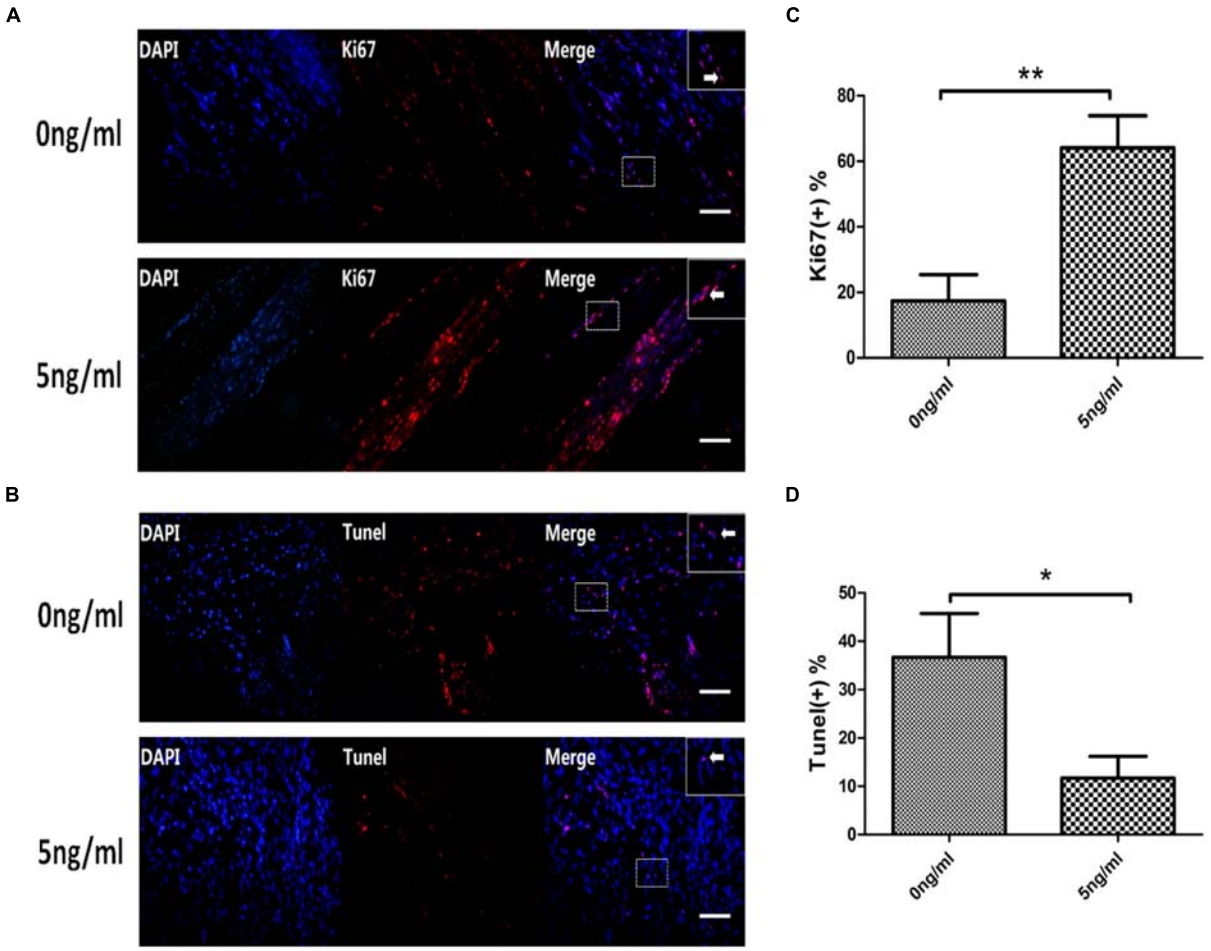

FIGURE 5 | Expression of Ki67 and TUNEL in the *in vitro* WD model. Immunofluorescent staining of Ki67 **(A**, arrows indicate Ki67(+) cells**)**, analysis of percentage of endonuclear Ki67(+) cells **(C)** in SCs with or without 5 ng/ml IL-1β at 48 h. TUNEL staining of apoptosis cells (**B**, arrows indicate apoptosis cells),analysis of percentage of TUNEL(+) cells **(D)** in SCs in the *in vitro* WD model with or without 5 ng/ml IL-1β at 48 h. Results are means ± SE (n = 3 per group) from independent experiments. *$p < 0.05$, **$p < 0.01$.

and loss of axonal contact causes mammalian SCs to lose their differentiated morphology, down-regulate myelin gene expression, up-regulate markers of immature SCs, and re-enter the cell cycle (Arthur-Farraj et al., 2012). During WD, SCs de-differentiate and up-regulate genes implicated in promoting axon growth, neuronal survival, and macrophage invasion, and break down their myelin sheaths by autophagy and phagocytosis, and morphologically transform into cells with long, parallel neural processes (Chen et al., 2007; Vargas and Barres, 2007; Gordon et al., 2009; Brosius et al., 2017). SCs de-differentiation allows them to form uninterrupted regeneration tracks (Bands of Büngner) that guide axons back to their targets, and provides a permissive environment for nerve regeneration (Vargas and Barres, 2007; Allodi et al., 2012). Although WD responses, including those resulting from SC injury, are key to damage repair, the molecular mechanisms that control these processes has remained uncertain.

Here, our research showed that the expression of IL-1β mRNA and protein increased in SCs after nerve injury, with IL-1β mRNA peaking at 12 h and IL-1β protein maximal by 36 h in this *in vitro* WD model. These results of our study are similar to previous reports for *in vivo* WD.

To test the hypothesis that, in addition to a role in immune responses, IL-1β participates in SC de-differentiation, we used the *in vitro* WD model treated with various concentrations of IL-1β to investigate effects on SC de-differentiation. Our study suggested that a specific concentration of IL-1β (5 ng/ml) increased expression of p75NTR and decreased levels of MPZ. Nevertheless, a high concentration (50 ng/ml) IL-1β did have the similar effect. SCs in immature states re-express p75NTR, which is a marker of SC de-differentiation (Jessen and Mirsky, 2008; Shin et al., 2013). During de-differentiation, SCs cease to express myelin genes, including MPZ (Warner et al., 1998; Jang et al., 2017). Up-regulation of p75NTR and down-regulation of MPZ have been implicated in SCs de-differentiation and regeneration. Thus, our study suggested that an appropriate concentration of IL-1β promoted SCs de-differentiation and regeneration in WD. On the contrary, a too high concentration of IL-1β might

deactivate SCs and could not promote SCs de-differentiation and regeneration in WD.

Previous studies indicate that IL-1β causes de-differentiation of primary cultured articular chondrocytes via the c-JUN/AP-1 pathway (Hwang et al., 2005). On the other hand, IL-1β can induce differentiation of precursor cells. For example, IL-1β is an essential factor for maturation of endothelial precursor cells to ECs (Voronov et al., 2014). However, our research is the first to report that IL-1β promotes SCs de-differentiation in WD. At the same time, there are some reports indicating that IL-1β promotes neurite outgrowth by deactivating RhoA via the p38 MAPK pathway (Temporin et al., 2008a) and sensory nerve regeneration after SN injury (Temporin et al., 2008b). Before and after macrophage recruitment, WD can be defined as two phases characterized by cytokine protein production profiles. The first phase is characterized by the synthesis of IL-1β and other inflammatory cytokines (such as TNF-α, IL-1α, GM-CSF and IL-6). The second phase is characterized by the production of Il-10, Il-6, and a GM-CSF inhibitor molecule, and furthermore, by the diminished production of IL-1β. Therefore, the first phase is largely inflammatory and the second is predominantly anti-inflammatory (Rotshenker, 2011). Thus, based on our research and previous reports, we interpret that appropriate concentrations of IL-1β are conductive to de-differentiation and regeneration of SCs during the first phase of WD.

The transcription factor c-JUN is a key regulator of WD, governs major aspects of injury response, determines the expression of trophic factors, adhesion molecules, the formation of regeneration tracks and myelin clearance and controls the distinctive regenerative potential of peripheral nerves. A key function of c-JUN is the activation of a repair program in SCs and the creation of a cell specialized to support regeneration (Arthur-Farraj et al., 2012; Boerboom et al., 2017). In our study, immunofluorescent microscopy revealed that expressed c-JUN was localized mainly in SC nuclei, consistent with its function as a component of the AP-1 transcription factor. In addition, the endonuclear c-JUN(+) cells fraction of IL-1β (5 ng/ml) treated group was significantly higher than ration of control group. Furthermore, expression of c-JUN protein and mRNA each significantly increased in IL-1β treated cells compared to control cells. Therefore, in our *in vitro* WD model, appropriate concentrations of IL-1β mainly increased expression of c-JUN in SCs nucleus, and promoted de-differentiation and regeneration of SCs.

Among potential intracellular activators of c-JUN is the AP-1 transcription complex, of which c-JUN is a key component (Arthur-Farraj et al., 2012). In this study, we analyzed AP-1 activity and found that IL-1β also promoted activation of SC AP-1 in our *in vitro* WD model. Thus, our findings further reinforce that appropriate concentrations of IL-1β promote de-differentiation and regeneration of SCs via the c-JUN/AP-1 pathway during the first phase of WD. The c-JUN/AP-1 complex is a prominent downstream nuclear target of ERK, JNK and PI3/AKT pathways (Eriksson et al., 2007; North et al., 2010; Mruthyunjaya et al., 2011). Further studies are required to verify whether IL-1β regulates c-JUN/AP-1 via the ERK, JNK and PI3/AKT pathways or others.

Ki67 is a nuclear antigen that acts as a specific and sensitive marker of cell proliferation, and it is a reliable indicator for detecting cell proliferation. Ki67 was not expressed in G_0 phase and G_1 early, and began to express in the middle and late G_1. It was located in the peri-nuclear region, and the expression in S phase and G_2 phase increased gradually, and M phase peaked (Miller et al., 2018). Therefore, Ki67 is also widely used for pathological evaluation of the proliferation and differentiation of different tumor cells (Berlin et al., 2017). Through Immunofluorescent staining of Ki67 and analysis of percentage of endonuclear Ki67(+) cells in SCs with or without 5 ng/ml IL-1β at 48 h, we found that the appropriate concentration of IL-1β(5 ng/ml) increased the expression of Ki67 in SCs, and increased the positive rate of Ki67. It can be seen that IL-1β can promote the proliferation of SCs during the process of WD. Furthermore, we used TUNEL to detect and quantify the apoptosis rate in SCs, our study suggested that a specific concentration of IL-1β (5 ng/ml) inhibited apoptosis in SCs during WD.

CONCLUSION

Schwann cells of WD produce IL-1β which promotes SCs de-differentiation and regeneration via the c -JUN/AP-1 signaling pathway. SCs of WD produce IL-1β which promotes SCs proliferation and induces inhibition of SCs. The precise molecular mechanisms of IL-1β regulation of c-JUN/AP-1 activity are not fully understood, and further studies are required.

ETHICS STATEMENT

The study was approved by the Ethics Committee for Animal Research at Shanghai Ninth People's Hospital affiliated to Shanghai JiaoTong University, School of Medicine. In the meantime, in accordance with the approved institutional guidelines and regulations.

AUTHOR CONTRIBUTIONS

GC conducted this project and wrote the manuscript. XL executed the experiments. WnW and YW participated in data analysis. FZ conceived the plan. WiW initiated this project and proposed the fundamental frame of this project. All authors read and approved the final manuscript.

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Astrocytic Insulin-Like Growth Factor-1 Protects Neurons Against Excitotoxicity

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Background: Exogenous insulin like growth factor-1 (IGF-1) is known to be neuroprotective in animal models with brain insults, while it can also cause hyperexcitability in rodents. In this regard, the role of endogenous IGF-1 in brain responses to brain insults like excitotoxicity, a common pathology in brain injuries, remains to be elucidated. Here, we investigated the potential role of cell-specific endogenous IGF-1 in the kainic acid (KA) -induced degeneration of the neurons.

Methods: Kainic acid was given to primary cultured cortical neurons and co-cultured astrocytes were added as a supportive system. We evaluated the cell proliferation rate, IGF-1 level in different groups and applied the PCR-Chip assay to explore the downstream of IGF-1. In addition, we applied the viral transfer of astrocytic IGF-1 to rodents treated with KA and assessed the associated molecular marker and behavioral outcomes in these rodents.

Results: We found KA induced increased cell death and hyperphosphorylated tau in neurons; co-cultured astrocytes could prevent these pathologies, and this rescuing effect was abrogated with blockade of the astrocytic IGF-1 with AG1024 (IGF-1R inhibitor). PCR-Chip assay identified that astrocytic IGF-1 could decrease the p-GSK-3 at Tyr 216 in neurons treated with KA and this effect was abrogated with AG1024 as well. In addition, *in vivo* study showed that gene transfer of astrocytic IGF-1 decreased p-tau and cognitive dysfunction in KA mice.

Conclusion: Our results show astrocytic IGF-1 exhibits neuroprotective properties in neurodegenerative processes in the CNS.

Keywords: insulin-like growth factor-1, kainic acid, excitotoxicity, hyperphosphorylated tau, astrocytes, neurons

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INTRODUCTION

Excitotoxicity is involved in neurodegenerative diseases such as traumatic brain injury, stroke and epilepsy. Following neurotoxic lesions such as ischemia (Aberg et al., 2006), or injury to the cortex (Pansiot et al., 2016) or the spinal cord (Sisti et al., 2019), insulin growth factor-1 (IGF-1), and its receptors are found to increase in associated brain lesions, implying a role of IGF-1 in the

Abbreviations: GSK-3β, glycogen synthase kinase; IGF-1, insulin-like growth factor-1; KA, kainic acid; MLPT, modified limb preference test; MTT, thiazolyl blue tetrazolium bromide.

pathological response to these brain insults. IGF-1 has been shown to exert its neuroprotective effects on different cells after brain injury.

Insulin like growth factor-1 is demonstrated to be neuroprotective after brain trauma or stroke. In a traumatic brain injury (TBI) rat model, IGF-1 is shown to increase in the ipsilateral side of the brain injury (Santi et al., 2018). In addition, exogenous IGF-1 has been found to protect the cerebral parenchyma in stroke models and to result in improved neurological outcomes (Liang et al., 2018). Generally, IGF-1 combines with IGF-1 receptor (IGF-1R) to exert functions. IGF-1R is widely distributed in both neurons and astrocytes in the brain. Neuronal IGF-1R is thought to affect neuronal polarity during cerebral cortical migration (Gontier et al., 2015), while reduced IGF1 signaling in astrocytes impairs their support for neurons under conditions of stress and this is associated with defects in the mitochondrial respiratory chain in astrocytes (Nieto Guil et al., 2017). In translational sessions, researchers found exogenous IGF-1 decreases brain lesions and prevents neurological deficits in a rodent stroke model (Liu et al., 2004).

Moreover, the pharmaceutical role of IGF-1 has been confirmed in other animal models of neurodegeneration, such as multiple sclerosis (Akcali et al., 2017), Alzheimer's (Zheng and Tong, 2017) and Parkinson's disease (Xiao et al., 2017), which have a common neuropathology in excitotoxicity. Most interestingly, IGF-1 has been tested both in preclinical (Yao et al., 2008) and in open-label clinical trials for the treatment of cerebellar ataxia with encouraging results (Sanz-Gallego et al., 2014). By contrast, several controversial studies have reported exogenous IGF-1 can induce cellular hyperexcitability regardless of neuronal protection *(12)*. A potential reason for the obviously contradictory findings can be that different responses of IGF-1 to excitotoxicity are cell-dependent (Quesada et al., 2007). Until now, few studies have been specific in this aspect. In addition, astrocytes are shown to protect neurons in response to excitotoxicity (Chen et al., 2019). In this way, it would be interesting to investigate the effect of cell-specific endogenous IGF-1, and we report here that co-cultured astrocytes protect neurons against excitotoxicity and, very importantly, gene delivery of IGF-1 in astrocytes shows a neuroprotection *in vivo* following excitotoxicity.

MATERIALS AND METHODS

Animals and Reagents

We used postnatal Sprague-Dawley rats for *in vitro* cultures (P3 days for astrocytes and E18 for neurons). 40 C57*B6 mice at 6–8 weeks old were used for *in vivo* study (10 for each group). All experiments were carried out based on medical ethics guidelines (20170223-001). KA and AG1024 (IGF-1R antagonist) were purchased from Sigma (Steinheim, Germany).

Cell Culture

Cortical cultures were obtained from either E18 or P3 rats (P3 days for astrocytes and E18 for neurons). In brief, harvested cerebral cortex was digested in HBSS and re-suspended in Neurobasal medium supplemented with 2% B-27 and 1% Glutamine to inhibit the glial division. Cells were plated onto 6 dishes coated with poly-l-lysine (1 μg/ml) at a final density of 1.5×10^6/well for neurons or 0.45×10^6/well for astrocytes according to previous reports *(14)*. The cultured neurons showed neurite extensions after 5–7 days (**Supplementary Figure S1**). On the day of the experiment, KA at doses of 0.1, 1, and 10 μmol/L was added to the medium. We applied KA as an excitoxic stimulus due to our previous reports that it can hyperphosphorylated tau and result in neurodegeneration *(15)*. Astrocyte were taken from P3 rats. Cells were grown in DMEM-F12 medium. Co-cultures of astrocyte and neurons were performed in a transwell system, as previously described in detailed reports. We treated neurons with KA at doses of 0.1, 1, and 10 μmol/L for 8 h in the down chamber, while cocultured astrocytes stayed in the upper chamber. Three repeated experiments were carried out in duplicate wells.

Thiazolyl Blue Tetrazolium Bromide Assay (MTT) – Cell Viability

We applied the MTT assay to assess the cell viability according to a previous report *(5)*. Viability of vehicle-treated control cells without KA exposure was taken as standard, with optical density value determined on the fluorescence reader.

Brain Excitotoxicity

Four weeks after the virus injection, 6- to 8-week-old male mice (10 per group) were given one ip injection with KA (5 mg/KG) and the same amount of PBS was administered to controls. Following surgery, mice were returned to their cages, kept at room temperature and allowed free access to food and water. After 6 weeks from the injection, we analyzed the p-tau levels in hippocampus and cortex in experimental mice.

Sample Preparation and Western Blot Analysis

Western blotting was performed as described. Cells were washed once with ice-cold PBS and artificial cerebro-spinal fluid (Sigma-Aldrich, CA, United States). To normalize for protein load, membranes were reblotted (Re-Blot, Chemicon, United States) and incubated with an appropriate control antibody (see section "Results"). BSA method was applied to quantify the basic expression of loading proteins (Bio-Rad, United States). Blotting images were carried out using Image J (MIT, Boston, MA, United States). The representative image is taken from three repeated trials. Most western blot studies were in neurons, except IGF-1 analysis in astrocytes.

Immunofluorescence

Animals were perfused transcardially with dPBS (Thermo Fisher Scientific, MA, United States) followed by 4% paraformaldehyde. The brains were removed from the cranial vault and post fixed in 4% paraformaldehyde overnight at 4°C. Brains were transferred to 15% sucrose overnight at 4°C and subsequently embedded in Tissue-teq (OCT, MA, United States) and then sectioned

(20 microns) using a cryostat (Microm HM550, Thermo Fisher Scientific, MA, United States).

Sections were collected on superfrost slides (Thermo Fisher Scientific, MA, United States), air dried, and fixed with 4% paraformaldehyde for 30 min and blocked in blocking buffer (2% normal goat serum or rabbit serum and 0.2% triton X-100 in dPBS) for 1 h at room temperature. Sections were then incubated with primary antibodies for hIGF-1 (Sigma, 1:80 dilution) or GFAP (Sigma, CA, United States, 1:80 dilution) overnight for 16 h, followed by a 1 h incubation with fluorescent-labeled secondary antibodies (Oregon green 488 donkey anti-goat, 1:500 dilution and Oregon green 488 goat anti-rabbit, 1:500 dilution, respectively). Fluorescent labeling was visualized on the Nikon ZR1500 microscopy and captured digitally by Nikon customized software (Waltham, MA, United States).

As for quantitative assessment of neuronal loss (NeuN) and PS-198, images (10 times) were captured from coronal sections at the ipsilateral cortex by a researcher who was blinded to the experimental conditions. With Image J software (NIH, United States), 16-bit pictures were transferred manually to quantify the mean optical density of fluorescence of each sample. For GFAP and IGF-1 fluorescence, we did a colocalization study to check the expression of IGF-1 in astrocytes as we used a GFAP-promoter for *in vivo* virus transduction as previously reported (Okoreeh et al., 2017).

Antibodies

Goat anti-IGF-1 antibody (ab106836), rabbit anti-ps198 antibody (ab79540), chicken anti-NeuN antibody (ab134014), and rabbit anti-GFAP (ab33922) were purchased from Abcam (Cambridge, MA, United States). Rabbit Polyclonal GSK3 Beta Antibody (22104-1-AP) was purchased from Proteintech (Cambridge, CA, United Kingdom). Rabbit Anti-phospho-GSK3 Beta (Tyr216) antibody (bs-4079R) was provided by Bioss (Danvers, MA, United States). Donkey anti goat HRP and goat anti rabbit HRP antibody (PAB0012 and PAB0011) were purchased from Bioswamp (Waltham, MA, United States). For secondary antibodies used in immunofluorescence, we used the Alexa Fluor 488 and 594 from Abcam (Cambridge, MA, United States).

PI3K-Akt PCR Array

An RT2 Profiler PI3K-Akt PCR Array (WCgene, Biotechnology, Shanghai) was utilized to screen a battery of downstream factors of IGF-1 in neurons according to previous reports (Chen et al., 2019).

Gene Transfer and Virus Constructs

Recombinant adeno associated virus serotype 8 (AAV2/8) was packaged (Obio, Shanghai) with the open reading frame (ORF) of human (h) IGF-1 gene downstream of the astrocyte-specific promotor, GFAP (GFAP-AAV8-hIGF-1). This construct contained the EGFP reporter gene as well under the GFAP promoter to visually detect those transfected cells. The control construct is composed of an identical shuttle vector without the hIGF-1 gene (GFAP-AAV8-control).

Animals were anesthetized (medical oxygen 3% and isoflurane 1.5%) and fixed in a stereotaxic device (RWD Instruments, Shanghai). One small hole was drilled into the skull for the Bregma reference of lateral ventricle: 2 mm posterior, 1.5 mm lateral, and a depth of 2.5 mm beneath the dura. In each case, a Hamilton syringe with an injection needle touched the associated region and the virus was gradually injected into the lateral ventricle at the rate of 0.25 μl/min for a total of 2 ml at around 8–10 min. All animals received one injection with a left side of the brain with either the GFAP-AAV8-hIGF-1 or the GFAP-AAV8-control construct. Animals were cared for 4 weeks to allow recovery and permit the viral expression in associated brain areas, followed by the KA or saline injection.

Behavior Tests

Modified limb preference (MLPT) test is applied to test the motor function of rodents with a scale from 0 to 5 in three items. The higher score means more severe motor function, and normal mice show zero. First, the rat is suspended 10 cm above a table, and the stretch of the forelimbs toward the table is observed and evaluated: a normal stretch is scored as 0 points; abnormal flexion is scored as 1 point. Next, the rat is positioned along the edge of the table, with its forelimbs suspended over the edge, and is then allowed to move freely. Each forelimb (forelimb-second task, hindlimb-third task) is gently pulled down, and retrieval and placement are evaluated. Finally, the rat is placed near the table edge, in order to assess the lateral placement of the forelimb. The three tasks are scored in the following manner: normal performance is scored as 0 points; delayed (at least 2 s) and/or incomplete performance is scored as 1 point; no performance is scored as 2 points. Total score 5 points indicate maximal neurological deficit, and a score of 0 points denotes normal performance (Lee et al., 2008).

Y-Maze is used to assess the cognitive function of rodents. As previously reported, Y-maze experiments were performed in a Y-shaped device with three arms of the same size (38 cm in length, 8 cm in width, and 13 cm in height; San Diego Instruments, San Diego, CA, United States). A visual cue with different figures was put above the distal end of each arm. First, mice underwent a training session for 15 min. During this session, a novel arm was initially blocked, and then the mouse was placed in the distal end of one arm and allowed to freely explore the two arms for 15 min. After a 2-h recovery interval, all mice underwent a testing session for 5 min. In the testing session, the novel arm was open, and the mouse was put in the same end of the previous arm and permitted to freely explore all three arms for 5 min. The arm and visual cues were randomized between, but not within mice. An overhead camera recorded each trial, and the time spent into each one of the arms was quantified using Ethovision tracking software (Noldus, Wageningen, Netherlands). Shorter duration in novel arms demonstrates cognitive dysfunction for rodents.

Statistical Analysis

Experimental data were mostly expressed as mean ± SEM, and statistical differences were compared by two-way ANOVA followed or a one-way ANOVA in different

studies with GraphPad Prism software (La Jolla, CA, United States). Differences with a p-value <0.05 were considered statistically different.

RESULTS

KA Induced Neuronal Death in a Concentration-Dependent Manner

Neuronal death assessed with MTT is shown to be gradually increased after KA treatment for 8 h ranging from 0.1 to 10 μM when compared with the controls at concentrations post-treatment using MTT and cell counting (**Figures 1A,B**). The neuronal viability was reduced from the dose of 0.1 μM ($P < 0.05$, $n = 6$), and when the dose was increased to over 10 μM, the viability decreased significantly to 45 ± 5.89% ($P < 0.01$, $n = 6$). KA treatment also decreased the neuronal counts between coculture group and neuron alone group from 19750 to 18750, 19250 to 13000, 15500 to 8500/mm^3, when KA treatment at 0.1, 1, and 10 μmol/L, respectively ($P < 0.05$, **Figure 1C**). In the coculture system with different KA treatment groups, there is no difference in OD value at 24 h ($P > 0.05$); however, KA treatment

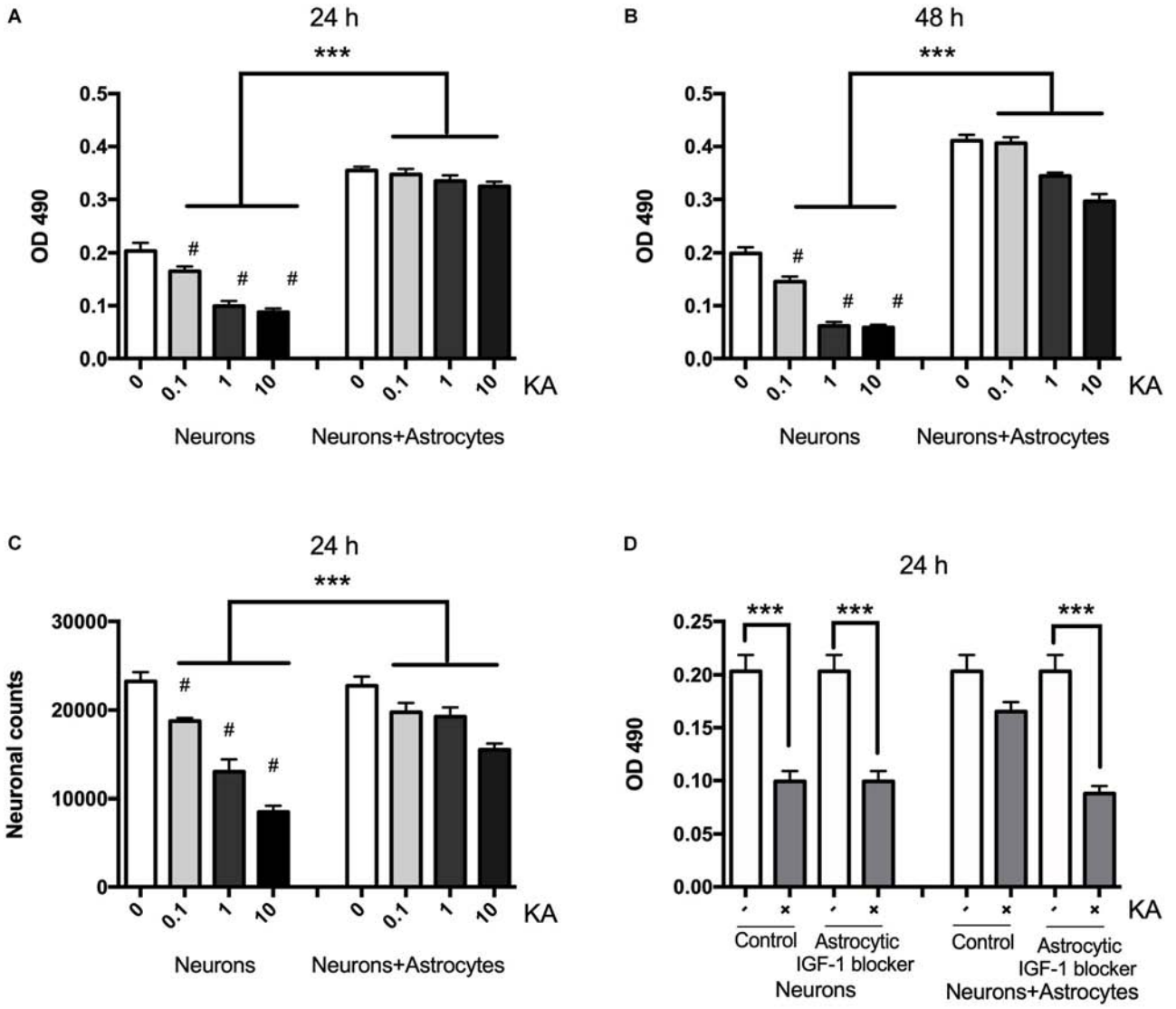

FIGURE 1 | Neurons are protected from excitotoxic injury in the presence of astrocytes whereas when cultured alone they rapidly die with a dose-response effect. Viability of neurons was measured by MTT at OD 490 1 and 2 days after KA treatment in the presence or absence of wild type astrocytes (**A,B**, $F_{2,24} = 78.35$, $^{***}P < 0.0001$ and $F_{2,24} = 294.2$, $^{***}P < 0.0001$, neurons+KA vs. neurons+astrocytes+KA; $^{\#}P < 0.0001$ compared to neurons in KA_0 group, $n = 6$ in each group). Cell counts from KA treated or saline showed that KA increased neuronal loss at 24 h with a dose-dependent manner (**C**, $^{***}P < 0.05$, neurons+KA vs. neurons+astrocytes+KA; $^{\#}P < 0.05$ compared between neurons+KA and cocultured neurons+KA at the same KA concentration, $n = 3$ in each group). IGF-1 blocker does not change KA inducing neuronal loss, but it diminishes the neuroprotective effect of astrocytic IGF-1 in the coculture system (**D**, $F = 8.90$; $^{***}P < 0.05$ compared with non-KA group).

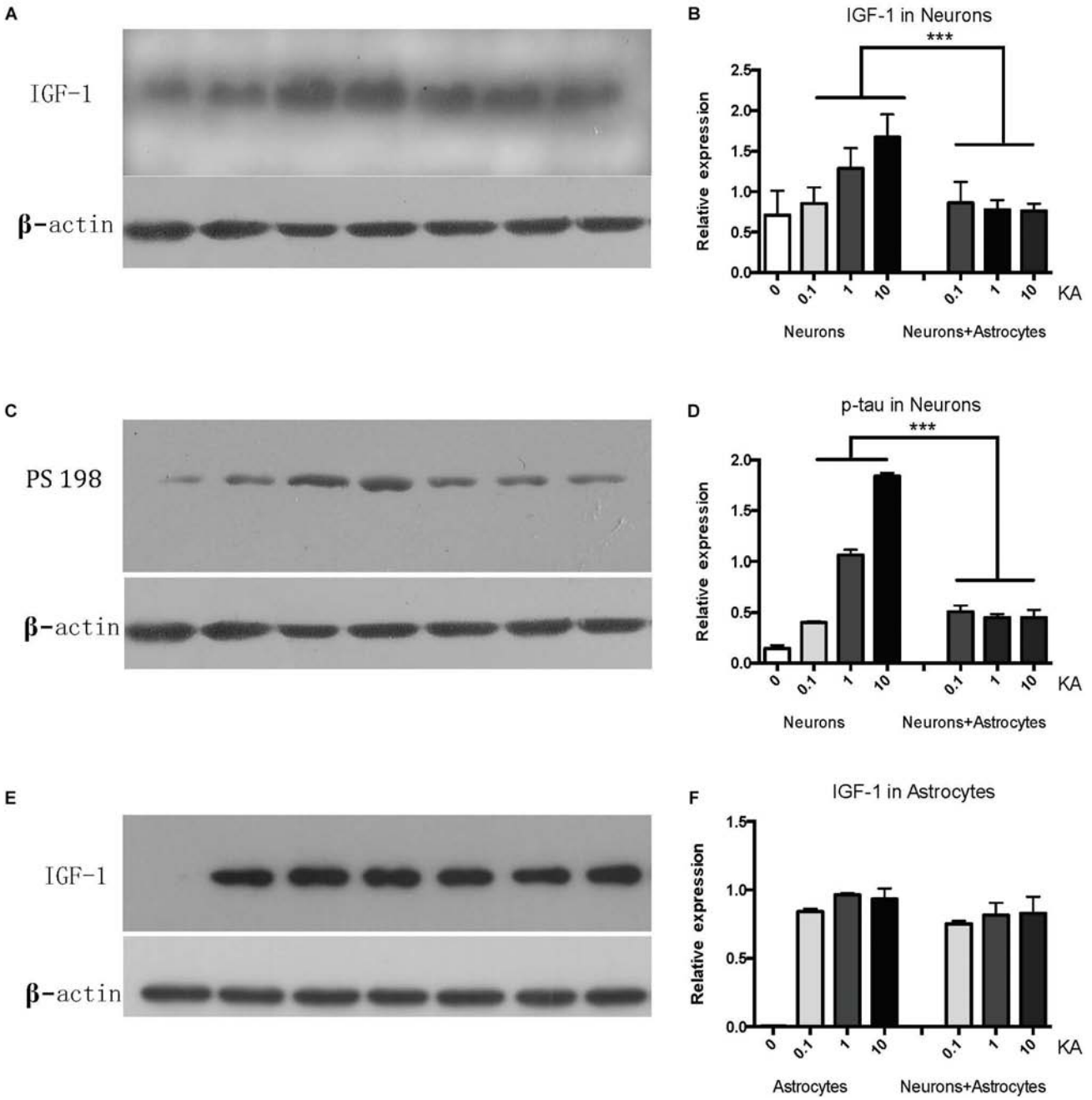

FIGURE 2 | The neuronal IGF-1 and phosphorylated tau (PS198) increase in response to KA with a dose-response effect while it remains lower level in the presence of astrocytes **(A–D)**. The astrocytic IGF-1 was secreted substantial and a dose-independent manner after KA **(E,F)**. ***$P < 0.05$, compared between neurons+KA and neurons +KA+astrocytes, $n = 3$ in each group). We blotted the IGF-1 and PS-198 on the same membrane as both were taken from the neurons, while the astrocytic IGF-1 was blotted to astrocytes medium. The concentration of IGF-1 secreted by neurons and associated expression of p-tau is positively correlated with KA concentration ($R^2 = 0.4008$, $P = 0.0271$; $R^2 = 0.8145$, $P < 0.0001$), by Pearson analysis.

at 1 and 10 μmol/L could reduce the OD value in neurons even in the co-culture system at 48 h after the KA treatment. This might indicate that the supportive function of astrocytes is transient. To confirm the different role of neuronal and astrocytic IGF-1, we specifically blocked the astrocytic IGF-1 with AG1024 (IGF-1R inhibitor differently added in the neuronal or astrocyte medium) in co-culture medium. We found there was no effect of astrocytic IGF-1 blocker in sole cultured neurons with KA (1 μmol/L for 8 h), but it diminished the neuroprotective effect of astrocytic IGF-1 in the co-culture system. Hence, endogenous production of IGF-1 from astrocyte but not neurons is necessary and sufficient to protect neurons (**Figure 1D**).

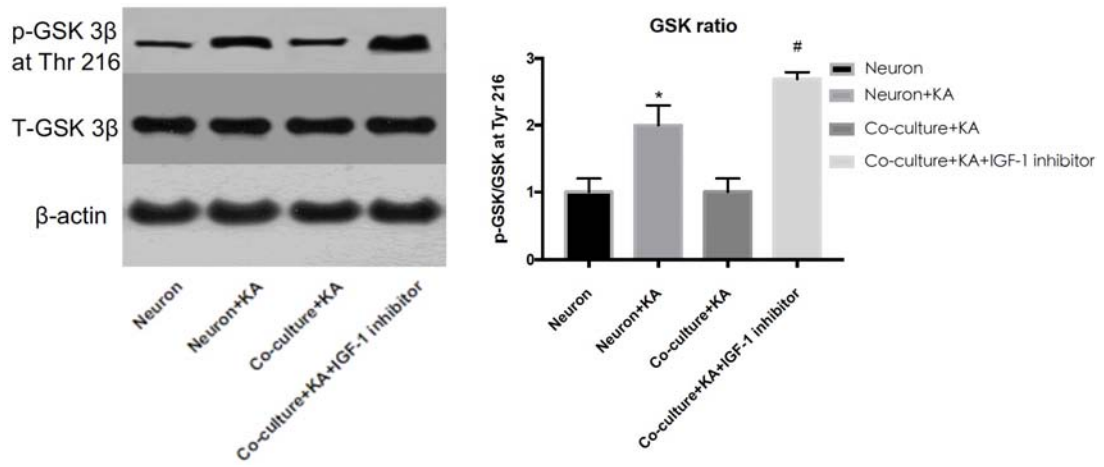

FIGURE 3 | The GSK-3β activity is indicated by the ratio of p-GSK-3β (Tyr 216)/Total-GSK-3β. KA induced the increased GSK-3β activity, while co-cultured astrocyte decreased the GSK-3β activity, and this effect was blocked with astrocytic IGF-1 inhibitor (*$P < 0.05$ compared to the isolated neurons; $^{\#}P < 0.05$, compared to co-culture+KA group).

Astrocytic IGF-1 Signaling Is Essential in Astrocyte Neuroprotection Against Neuronal Excitotoxicity

While cultured neurons without astrocytes are very sensitive to acute excitotoxic insult elicited by KA (**Figure 1A**), when cultured with astrocytes, neurons become very resilient (**Figure 1B**). To confirm whether IGF-1 is involved in this effect, we first found that endogenous IGF-1 increases after KA treatment. As shown in **Figure 2**, only neurons secrete IGF-1 into the culture medium in a dose-dependent way according to the amount of KA ($R^2 = 0.4008$, $P = 0.0271$); while astrocytes secrete substantial and dose-independent amounts of IGF-1 after KA (**Figures 2A,B,E,F**). As neuronal loss by KA has been found to be associated with hyperphosphorylated tau, and here we found that KA increased p-tau expression with a dose-dependent effect as well ($R^2 = 0.8145$, $P < 0.0001$) and the cocultured astrocyte could reduce the p-tau regardless of KA doses (**Figures 2C,D**).

To explore the distinct role of neuronal and astrocytic IGF-1, we applied a PI3K-Akt chipset to KA treated neurons and co-culture system (**Supplementary Figure S2**).

Previous studies showed tau hyperphosphorylation is induced by increased activity of GSK3β (Xue et al., 2017), which is a main kinase in the body to phosphorylate tau proteins (Liu et al., 2016). Based on this, we could consider that co-cultured astrocytes decrease the expression of p-GSK3 at Tyr 216 site to decrease the GSK3β activity and further decrease hyperphosphorylated tau and protects neurons from excitotoxicity, which is confirmed by WB analysis (**Figure 3** and **Table 1**).

Impact of rAAV8-GFAP-hIGF-1 in a Brain Excitotoxicity Model

Considering the role of astrocytic IGF-1 in *in vitro* studies, we accordingly explored whether increasing astrocytic IGF-1 would be essential in a brain excitotoxicity *in vivo* model. As shown in **Figure 4**, GFAP-AAV8 -hIGF-1 transfer reduced the p-tau in both cortex and hippocampus of KA mice at different phosphorylated sites (PS-198, PS-262, and PS396), which is associated with the decreased GSK-3β expression.

Gene transfer of IGF-1 also decreases the neuronal loss and PS-198 in cortex (indicated by fluorescence mean density, **Figure 5**) and most IGF-1 is colocalized with the GFAP expression (**Figure 6**). The increased IGF-1 might come from both viral infection and cell secretion.

TABLE 1 | Gene changes in excitotoxic neurons with cocultured astrocytes.

Upregulate genes	Log value	Downregulated genes	Log value
Mapk14	3.200262919	Fkbp1a	−1.035214322
Mapk8	2.984302712	Csnk2a1	−1.145787057
Prkcz	2.444014809	Grb10	−1.218625033
Rps6ka1	1.925751768	Rheb	−1.285215569
Ilk	1.882264841	Pabpc1	−1.361158017
Btk	1.81595143	Rasa1	−1.367858696
Nfkbia	1.719726027	Wasl	−1.519555394
Myd88	1.445691899	Rac1	−1.554536649
Pik3cg	1.406636297	**GSK-3β**	**−1.567745615**
Tirap	1.151062562	Eif4ebp1	−2.126020794

Selective Overexpression of IGF-1 in Astrocytes Improves Neuronal Dysfunction

To explore the effect of replenishing astrocytic IGF-1 on neurological outcome, motor function was evaluated by modified limb placing test (MLPT) and cognitive function by a Y-Maze at 28 days post-injury. Excitotoxic brain injury lead to significant motor dysfunction as demonstrated by higher MLPT scores after KA treatment in both control and IGF-1 treated mice compared to the sham group with

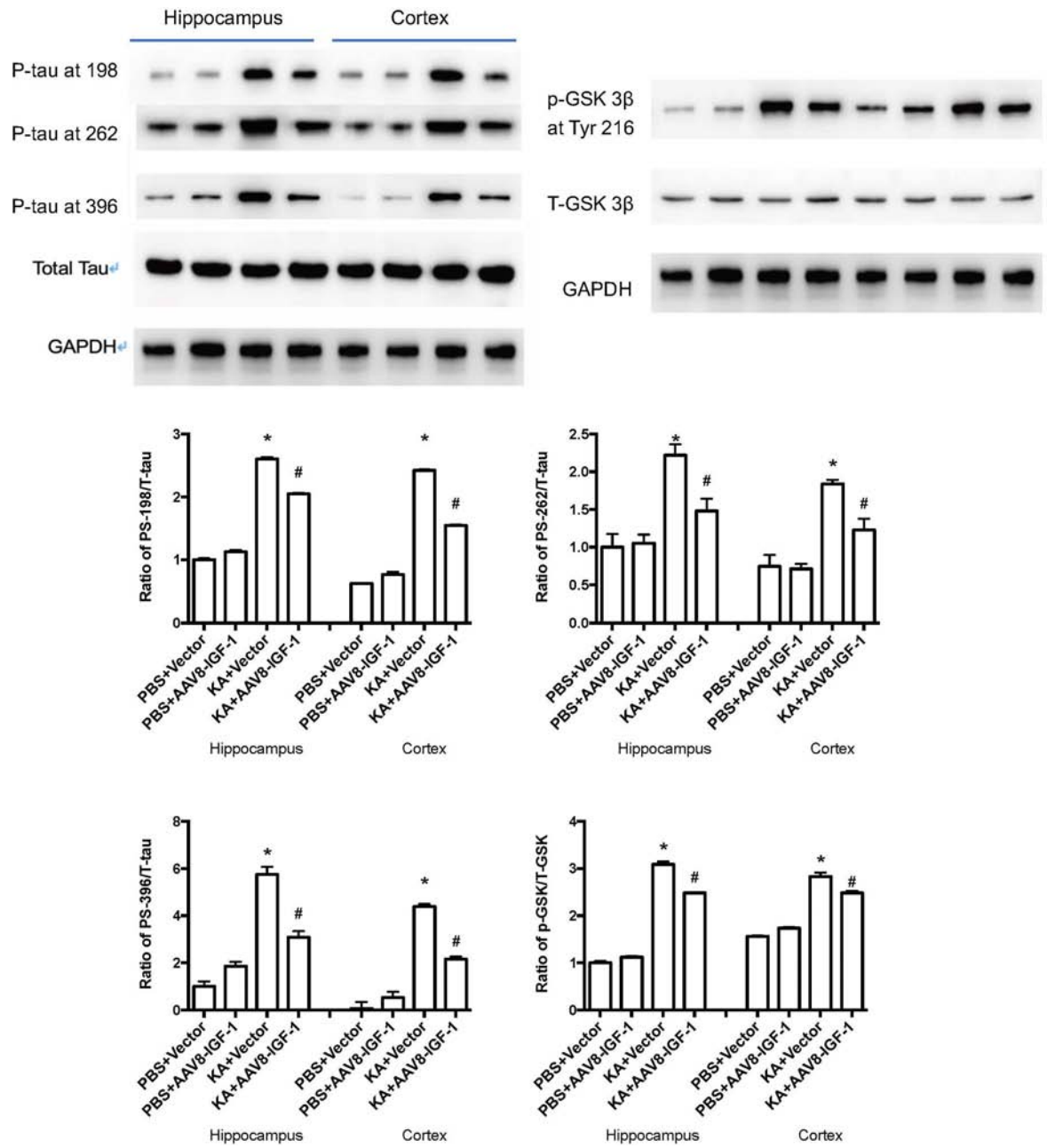

FIGURE 4 | Gene transfer of astrocytic IGF-1 decreases p-tau expression at different phosphorylated sites (PS-198, 262, and 396) and reduces the ratio of GSK-3β at Tyr 216 of total GSK-3β in both cortex and hippocampus via reducing the phosphorylated GSK-3β. We calculated the ratio of both p-tau to total tau and p-GSK to total GSK, therefore, we used the same GAPDH image for the control protein loading comparison. Up panel, representative blot images. Down panel, quantification of WB results. *Comparison between KA+Vector to PBS+vector ($P < 0.05$); #comparison between KA+AAV8-IGF-1 to KA+Vector ($P < 0.05$). Data shown as mean SEM ($n = 3$ in each group).

PBS treatment (**Figure 7**). Nevertheless, IGF-1 treated KA mice showed statistically lower MPLT scores compared to those mice with control vector (**Figure 7A**), indicating that astrocytic IGF-1 overexpression attenuated excitotoxic motor dysfunction.

Excitotoxic injury also caused an obvious cognitive deficit in KA treated mice with a 50% less preference for a novel arm (**Figure 7B**), while excitotoxic IGF-1 mice without a cognitive dysfunction. In addition, excitotoxic IGF-1 mice demonstrated longer duration of the novel arm compared to control mice (**Figure 7B**), indicating astrocytic IGF-1 replenishment prevented spatial learning dysfunction. Motor performance of all mice was equivalent, indicating the motor dysfunction was not a bias during the cognitive test (**Figure 7C**).

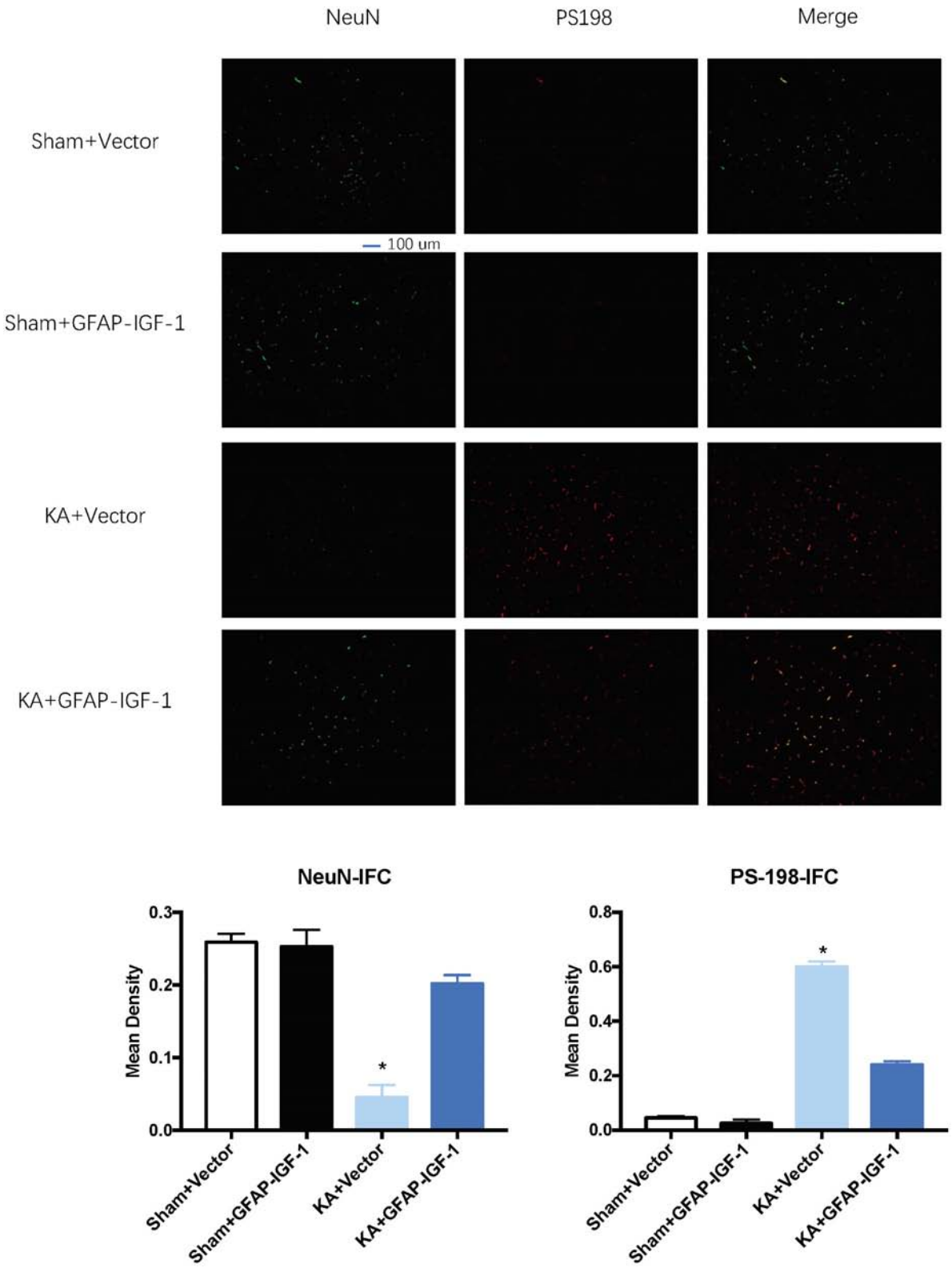

FIGURE 5 | Gene transfer of astrocytic IGF-1 decreases the p-tau expression at the cortex (PS-198 fluorescence) and increases neuronal cell loss (NeuN). KA reduces the mean density of NeuN compared to sham mice, while the astrocytic IGF-1 increases the neuronal mean density. The mean fluorescence density of PS-198 also increases in KA mice, and astrocytic IGF-1 transfer decreases it. $^{*}P < 0.001$, opposed to other groups. $n = 3$ in each group, and data is indicated as mean ± SEM.

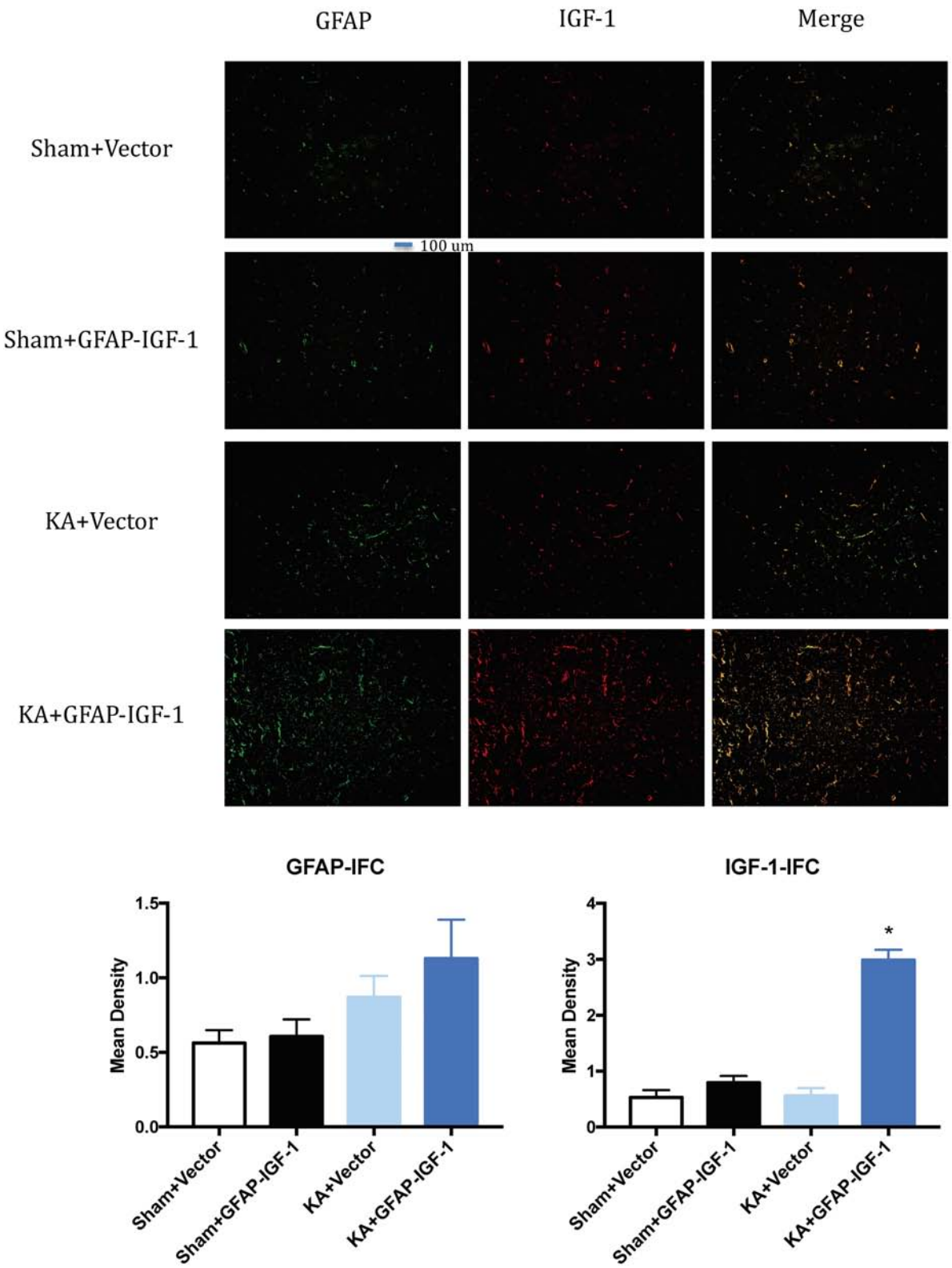

FIGURE 6 | Gene transfer of astrocytic IGF-1 shows most IGF-1 co-localized with the astrocytes. There is no difference in GFAP expression among these groups. The IGF-1 is mostly increased in KA+GFAP-IGF-1 group, showing that increased IGF-1 might be due to both IGF-1 secretion from astrocytes and IGF-1 transfer from virus. $^{*}P < 0.001$, compared to other groups, $n = 3$ in each group, and data is indicated as mean ± SEM.

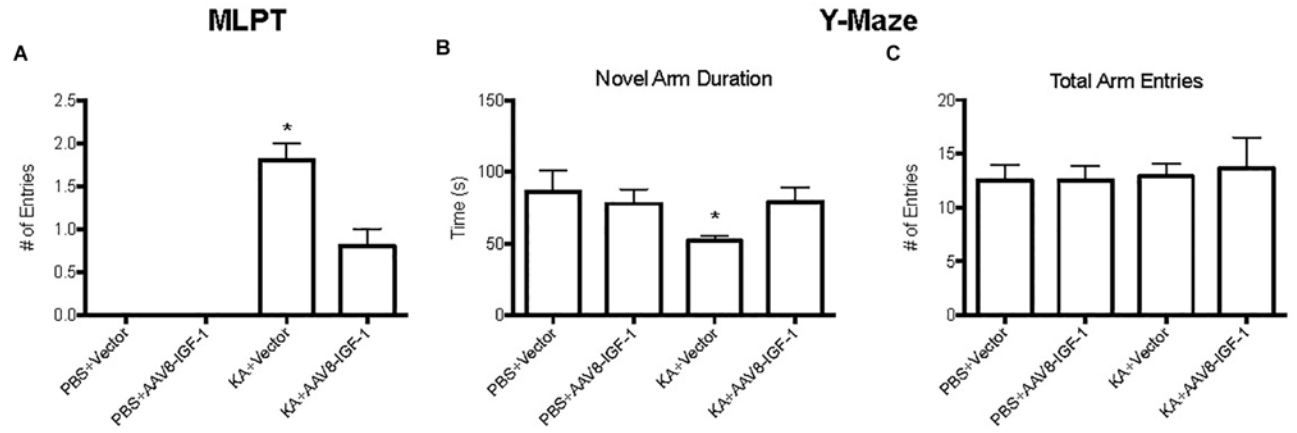

FIGURE 7 | Behavior test of KA mice treated with empty vectors or hIGF-1. KA mice with empty vector showed higher MPLT score and hIGF-1 could reduce it; while KA mice with empty vector showed decreased less novel arm duration, and hIGF-1 could increase it **(A,B)**. No significant differences in total arm entries in all four groups **(C)**. $^*P < 0.05$, statistically different to other three groups, $n = 10$ in each group).

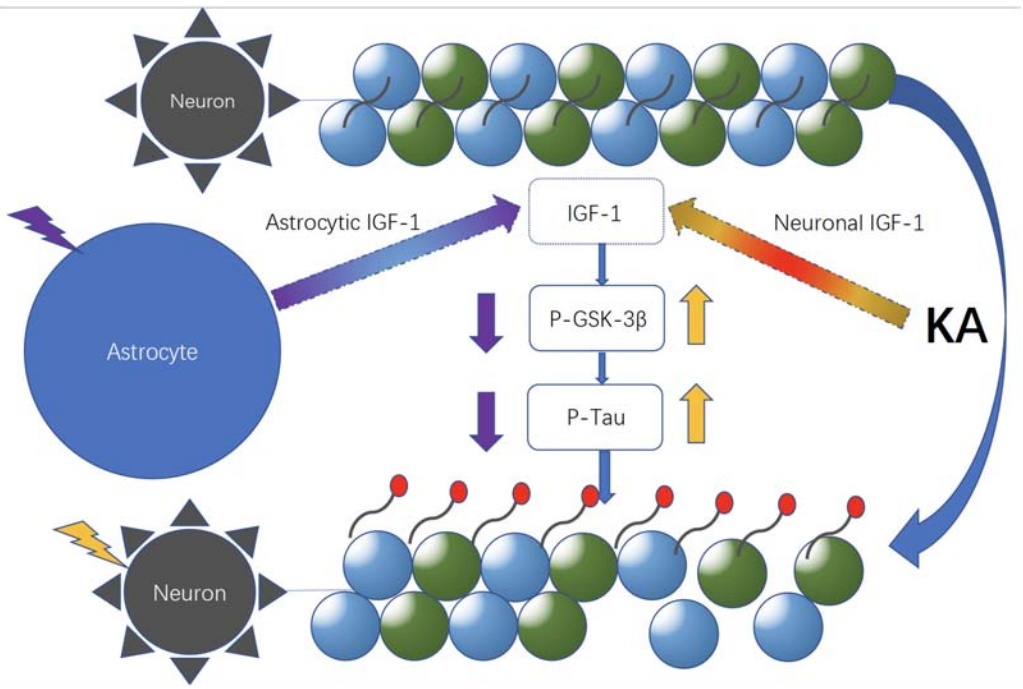

FIGURE 8 | Neuronal and astrocytic IGF-1 might have different roles in excitotoxicity. Neuronal and astrocytic IGF-1 seem to have different effects in excitotoxicity. When neurons alone are treated with KA, they are prone to be hyperphosphorylated with increased GSK-3β activity; in contrast, while neurons are co-cultured with astrocytes, they are resilient to KA and become less phosphorylated due to secretion of astrocytic IGF-1.

DISCUSSION

In the present study, we analyzed the neuroprotective role of endogenous astrocytic IGF-1 after KA - induced neurodegeneration. We found KA resulted in hyperphosphorylation of tau and neuronal death in both *in vivo* and *in vitro* studies. When neurons were co-cultured with astrocytes, there was a lesser degree of neuronal loss and decreased tau phosphorylation and this rescuing effect was blocked with astrocytic IGF-1R antagonist from *in vitro* studies.

Our findings show that neurons in the presence of astrocytes are less sensitive to KA excitotoxicity, as evidenced by decreased MTT in co-cultured neurons. This finding extended the recent finding of cell-specific neuroprotection of IGF-1 signals. These observations highlight the importance of cell-specific IGF-1 against excitotoxic challenge. Therefore,

a better understanding of the cell-specific role of IGF-1 in the brain requires considering its effects on other cells in brains which need further studies to investigate the relationship between astrocytes and microglia and oligodendrocytes.

A lower sensitivity of cocultured neurons with astrocytes to KA provided by IGF-1 allows these cells to survive against excitotoxicity challenge. While in response to excitotoxic injury, the production of IGF-1 by cultured astrocytes and neurons is increased, consistent with the report that after brain ischemia IGF-1 levels are actually higher due to increased synthesis and accumulation in microglia, vessels and astrocytes (Beilharz et al., 1998). Therefore, *in vivo*, astrocytes and neurons will receive IGF-1 input from various local sources, suggesting that the response of increased IGF-1 after brain insults reflects an endogenous neuroprotective mechanism against brain insults (Zheng and Tong, 2017). This conclusion is apparently consistent with previous evidence that genetic ablation of IGF-1 in mice, increases p-tau levels in the brain (Cheng et al., 2005). However, mice with reduced IGF-1 activity (hemizygous for the IGF-1 receptor) have lower levels of Aβ and diminished neuroinflammation in the brain (Kappeler et al., 2008; Nadjar et al., 2009). And these mice exhibited improved spatial memory and reduced anxiety (De Magalhaes Filho et al., 2017). Conceivably, the effects of modulating IGF-1 signaling prior to brain insult (as when using genetic models) may not be the same as after an insult. For example IGF-1 protects nerve cells and/or the brain against diverse types of excitotoxicity-related insults (Puche et al., 2008; Grinberg et al., 2012). However, exogenous IGF-1 applied in TBI could induce hyperactivity in rodents (Song et al., 2016). In this regard, we emphasize the importance not only of cell type but also of context dependency of IGF-1 neuroprotection in relation to excitotoxicity.

A role for excitotoxicity in many neurodegenerative diseases is gaining increasing acceptance (Zheng et al., 2014). Aberrant production of p-tau in the central nervous system is linked to neurodegenerative diseases such as Alzheimer Disease dementia, Parkinson's disease, traumatic brain injury, epilepsy or stroke, all of them associated to aging and neurodegeneration (Zheng et al., 2014). However, as already commented, the role of excitotoxicity in brain aging is still unclear (Shultz et al., 2015). An attempt to explain these apparently opposing observations is that increased hyperphosphorylated tau levels may activate neurodegenerative pathways (Liu et al., 2016). The present findings confirm this proposal. Thus, doses of KA up to 100 M do not elicit astrocyte death probably because IGF-1 helps maintain their anti-degenerative capacity and at the same time their neuroprotective action. In this regard, our results show that astrocytes in response to KA activate IGF-1-PI3K-Akt signaling including upregulation of IGF-1 coupled to downregulation of phosphorylated GSK-3β at Tyr216. GSK-3β has several phosphorylation sites and the phosphorylated tyrosine kinase 216 can increase its activity to hyperphosphorylated tau proteins, which is a hallmark in AD, and the p-tau plays an important role in different brain diseases in which excitotoxicity is implicated. The fact that co-localization shows p-tau mostly expressed in neurons reinforces results that have been proved by a series of studies previously (Padmanabhan et al., 2006; Quintanilla et al., 2009, 2012). Tau proteins can be phosphorylated at different sites with a profile of phosphorylation, which indicated the extent of p-tau. In our study, we found KA induced p-tau in 198,262 and 396 sites and the increased IGF-1 in astrocytes could reduce these p-tau at separate sites.

Adeno associated virus has been used in clinical session to treat patients. rhIGF-1 has a neuroprotective function, however, it can cause the hyperexcitability and post-traumatic epilepsy. The injection of the AAV: GFAP-IGF-1-GFP could infect the GFAP positive neurons and increase the expression of IGF-1 in astrocytes (shown by **Figure 6**). In this study, we applied gene transfer of hIGF-1 with a GFAP promoter to transfect astrocytes *in vivo* and found hIGF-1 could prevent the motor and cognitive dysfunction in KA mice. And this might be translated to clinical studies for patients with neurodegenerative-like behavior.

According to the PCR array and WB result, co-cultured astrocyte secreted IGF-1, which can further decrease the GSK-3β activity indicated by the decreased ratio of p-GSK-3β (Tyr 216)/Total-GSK-3β. And this effect was blocked with astrocytic IGF-1 inhibitor (AG1024 added in the astrocyte medium). IGF is found to stimulate glycogen synthase mainly mediated via the signaling cascade PI(3)K/Akt/GSK-3β that leads to the inhibited GSK3 kinase activity. However, total GSK-3β expression did not change following KA or co-culture. In this case, we thought inconsistent expression of decreased GSK-3β RNA and normal total GSK-3β protein expression might be due to the GSK-3β alternative splicing which was affected by IGF-1, according to a previous report (Wang et al., 2012). Nevertheless, the alternative splicing of GSK-3β needs further study to confirm it. This might explain the fact that the cell-specific IGF-1 has different roles in neuroprotection after excitotoxic injury. Our findings show that specific increased IGF-1 in astrocytes can prevent neurological degeneration after TBI. However, exogenous IGF-1 treatment following TBI would result in neuronal hyperactivity, and the exogenous IGF-1 might change the original context such as astrocytes function and characteristics in brains and this needs further exploration with electrophysiological studies. Here, we found that neuronal IGF-1 increased after KA treatment with a dose-response effect, while astrocytes can also release IGF-1 after KA but with a consistent concentration, which further decreased p-tau expression via decreasing the GSK-3β activity. The intracellular mechanisms mediating neuroprotection of the endogenous cell-specific IGF-1 against excitotoxicity involve GSK-3β, a kinase phosphorylates tau proteins. These findings indicate astrocytic but not neuronal IGF-1 has a neuroprotective effect in excitotoxicity. Neuronal and astrocytic IGF-1 seem to have different effects in excitotoxicity. When neurons alone are treated with KA, they are prone to be hyperphosphorylated with increased GSK-3β activity; by contrast, while neurons are co-cultured with astrocytes, they are resilient to KA and

become less phosphorylated due to secretion of astrocytic IGF-1 (**Figure 8**). And it would be useful to investigate the structural difference of IGF-1 secreted from neurons or astrocytes, due to the fact that IGF-1 has different isoforms in neurons and astrocytes, respectively, and these isoforms may have different biological effects. In summary, cell specific IGF-1 in brain responses to excitotoxicity challenge underscores the need to design therapeutic strategies that consider all aspects of biological organization, leading, for example, to cell-specific targeting of anti-aging drugs.

CONCLUSION

Taken together, our data demonstrate that astrocyte-derived IGF-1 has a beneficial effect on function and pathology following an excitotoxic injury. Importantly, we show that the IGF-1 reduces not only neuronal apoptosis but also expression of phosphorylated tau via regulating GSK-3β. Thus, the action of the IGF-1 is multifold and impacts a number of cell survival and regulatory pathways both *in vitro* and *in vivo*. As cell-specific IGF-1 shows tremendous promise as therapeutics, understanding the downstream and upstream pathways is critical for understanding their mechanism of action as well as determining how to apply the cell-specific IGF-1 with the most potential to benefit.

DATA AVAILABILITY

The datasets supporting the conclusions of this article are available from the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the local ethics committee in Shanghai Pudong New area People's Hospital, Shanghai University of Medicine and Health Sciences (20170223-001).

AUTHOR CONTRIBUTIONS

PZ and WC conceived the study, designed the experiments, and performed the cell cultures. WT and JZ ran the molecular tests. PZ and BH wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2019.00298/full#supplementary-material

FIGURE S1 | Neuronal culture images show the neural outgrowth in primary cortical neurons.

FIGURE S2 | PCR-array results for PI3k-Akt pathway in co-culture system and neuron only after KA.

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REVIEW
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Brain-Derived Neurotrophic Factor: A Key Molecule for Memory in the Healthy and the Pathological Brain

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Brain Derived Neurotrophic Factor (BDNF) is a key molecule involved in plastic changes related to learning and memory. The expression of BDNF is highly regulated, and can lead to great variability in BDNF levels in healthy subjects. Changes in BDNF expression are associated with both normal and pathological aging and also psychiatric disease, in particular in structures important for memory processes such as the hippocampus and parahippocampal areas. Some interventions like exercise or antidepressant administration enhance the expression of BDNF in normal and pathological conditions. In this review, we will describe studies from rodents and humans to bring together research on how BDNF expression is regulated, how this expression changes in the pathological brain and also exciting work on how interventions known to enhance this neurotrophin could have clinical relevance. We propose that, although BDNF may not be a valid biomarker for neurodegenerative/neuropsychiatric diseases because of its disregulation common to many pathological conditions, it could be thought of as a marker that specifically relates to the occurrence and/or progression of the mnemonic symptoms that are common to many pathological conditions.

Keywords: BDNF, hippocampus, perirhinal cortex, Alzheimer disease, memory, depression, stress

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BDNF: A DYNAMICALLY REGULATED PLAYER IN SYNAPTIC PLASTICITY AND MEMORY

The brain derived neurotrophic factor (BDNF) belongs to a family of neurotrophins that have a crucial role in survival and differentiation of neuronal populations during development (Huang and Reichardt, 2001). In the adult brain, BDNF also maintains high expression levels and regulates both excitatory and inhibitory synaptic transmission and activity-dependent plasticity (Tyler et al., 2002; Wardle and Poo, 2003).

The expression of BDNF is regulated during transcription and translation, and also by post-translational modifications. The presence of a complex multi-level regulation demonstrates the importance and diversity of BDNF functions. Transcription is controlled by multiple promoters that determine activity-dependent and tissue specific expression (Timmusk et al., 1993; Chen et al., 2003). There have been identified at least four BDNF promoters in the rat (Timmusk et al., 1993), each one driving the transcription of mRNAs that contain one of the 8 non-coding exons spliced to the common 30 coding exons, which produce an heterogeneous population of BDNF

transcripts. BDNF splicing has been described for several species, including humans (Liu et al., 2005), mice (Hayes et al., 1997), and rats (Timmusk et al., 1993). Additionally, the expression of specific BDNF exons can be regulated by epigenetic mechanisms (Lubin et al., 2008), suggesting that environmental experiences dynamically influence mature BDNF levels.

Regarding the pattern of expression of BDNF in the brain, high levels of this molecule have been detected in the hippocampus, amygdala, cerebellum and cerebral cortex in both rodents and humans, with the highest levels found in hippocampal neurons (Hofer et al., 1990; Timmusk et al., 1993). Lower levels of BDNF have been detected in organs such as the liver, heart, lung, among others (Ernfors et al., 1990; Maisonpierre et al., 1991). The regulation of each transcript is controlled and/or modulated by factors like neuronal activity (Metsis et al., 1993), exercise (Oliff et al., 1998), antidepressants (Russo-Neustadt et al., 2004), stress (Lauterborn et al., 1998), and hormones such as estrogens (Singh et al., 1995).

Brain derived neurotrophic factor is synthesized as the precursor proBDNF, that can be stored in either dendrites or axons (Lessmann et al., 2003), and undergoes cleavage intra or extracellularly (Lee et al., 2001; Mowla et al., 2001) to produce a mature BDNF protein. BDNF is released in an activity dependent manner as a mixture of pro and mature BDNF (Pang et al., 2004). Interestingly, BDNF and proBDNF are associated with opposing effects on cellular function, which gives BDNF protein function an additional level of complexity. The proBDNF form is secreted under both pathological and non-pathological conditions (Barker, 2009). ProBDNF preferentially binds p75 NTR receptor, which facilitates LTD (Woo et al., 2005) and induces apoptosis (Friedman, 2010). On the other hand, BDNF in its mature form binds specifically to tyrosine kinase receptors (TrkB) and promotes cell survival (Volosin et al., 2006), facilitates LTP and increases spine complexity (McAllister et al., 1999; Zagrebelsky et al., 2005). When $p75_{NTR}$ is co-expressed with TrkB receptor it increases neurotrophins binding affinity thereby facilitating ligand discrimination (Bibel et al., 1999). In this way, proBDNF can be thought as part of a regulatory mechanism of BDNF activity in non-pathological conditions. In addition, the truncated forms of TrkB receptor can act as dominant negative inhibitors of BDNF signaling by internalizing and clearing BDNF from the synapse (Haapasalo et al., 2002; **Figure 1**).

Many studies have shown the critical role of BDNF for the regulation of plastic changes in the adult brain, including regulation of the trafficking (Caldeira et al., 2007), phosphorylation (Lin et al., 1998) and expression levels of NMDARs (Suen et al., 1997) associated with augmented synaptic strength. Due to its critical role in LTP, BDNF has been postulated to be an essential part of the cellular mechanism supporting memory formation and maintenance by promoting synaptic consolidation (Bramham and Messaoudi, 2005). According to this hypothesis, BDNF increases memory storage by favoring changes in spine morphology leading to the stabilization of LTP. BDNF can also increase the number, size and complexity of dendritic spines (Horch and Katz, 2002; Alonso et al., 2004), probably through unpregulated actin polymerization (Rex et al., 2007). Furthermore, BDNF increases neurogenesis through changes in cell survival (Lee et al., 2007) and proliferation (Katoh-Semba et al., 2002).

Changes in synaptic connections are thought to support memory storage. There are several lines of evidence that directly link BDNF with learning and memory. For example, BDNF could be a mediator of the plastic changes underlying both spatial and recognition memory processes (Kesslak et al., 1998; Mizuno et al., 2000; Cirulli et al., 2004; Bekinschtein et al., 2007; Heldt et al., 2007).

In this review, we will focus on the role of BDNF in cognitive function in the adult brain under normal and pathological conditions, and evaluate the potential therapeutic actions of BDNF for the treatment of cognitive alterations associated with aging, neuropsychiatric and neurodegenerative diseases. We will particularly focus on the effects of these treatments on mnemonic function.

THE BDNF Val66Met POLYMORPHISM

In the human BDNF gene, a common single nucleotide polymorphism identified with a Met to Val substitution at codon 66 in the pro-domain of BDNF, called rs6265 or Val66Met polymorphism, affects synaptic targeting of BDNF-containing vesicles and activity-dependent neuronal release of BDNF (Egan et al., 2003). Met66 homozygous knock-in mice showed a selective impairment in activity-dependent synaptic plasticity *in vitro* (Ninan et al., 2010). Additionally, exogenous application of proBDNF in Val-carriers facilitated LTD and inhibited LTP, while not in Met carriers (Kailainathan et al., 2016). Because of its frequency in the human population [up to 30% Met carriers in a European sample (Egan et al., 2003)] and its association with lower serum levels of BDNF (Ozan et al., 2010), this single nucleotide polymorphism has been a matter of great interest. This polymorphism has been associated with structural (Pezawas et al., 2004) and functional differences in the brain, such as synaptic plasticity (Kleim et al., 2006) and memory performance (Hariri et al., 2003). Some of these structural changes include volumetric decreases in specific regions -such as the hippocampus (Szeszko et al., 2005), the parahippocampal gyrus, the prefrontal cortex and the amygdala (Matsuo et al., 2009; Montag et al., 2009)-. The presence of the Met allele is, in turn, associated with poor mnemonic performance on verbal tasks both at short and long delays and also deficits in working and spatial memory tasks (Dempster et al., 2005; Hansell et al., 2007; Goldberg et al., 2008). Since most studies relayed mainly on tasks that evaluate item memory, and particularly, verbal memory, the effects over other more complex cognitive functions remain to be tested (Mandelman and Grigorenko, 2012). Yogeetha et al. (2013) did evaluate multiple types of memories, finding only an influence on visuospatial memory, although Raz et al. (2009) describes an additional effect of associative memory.

Besides these correlational studies of Met allele dosage, hippocampal volume/activation and memory performance in healthy subjects, evidence for a role of Val66Met polymorphism

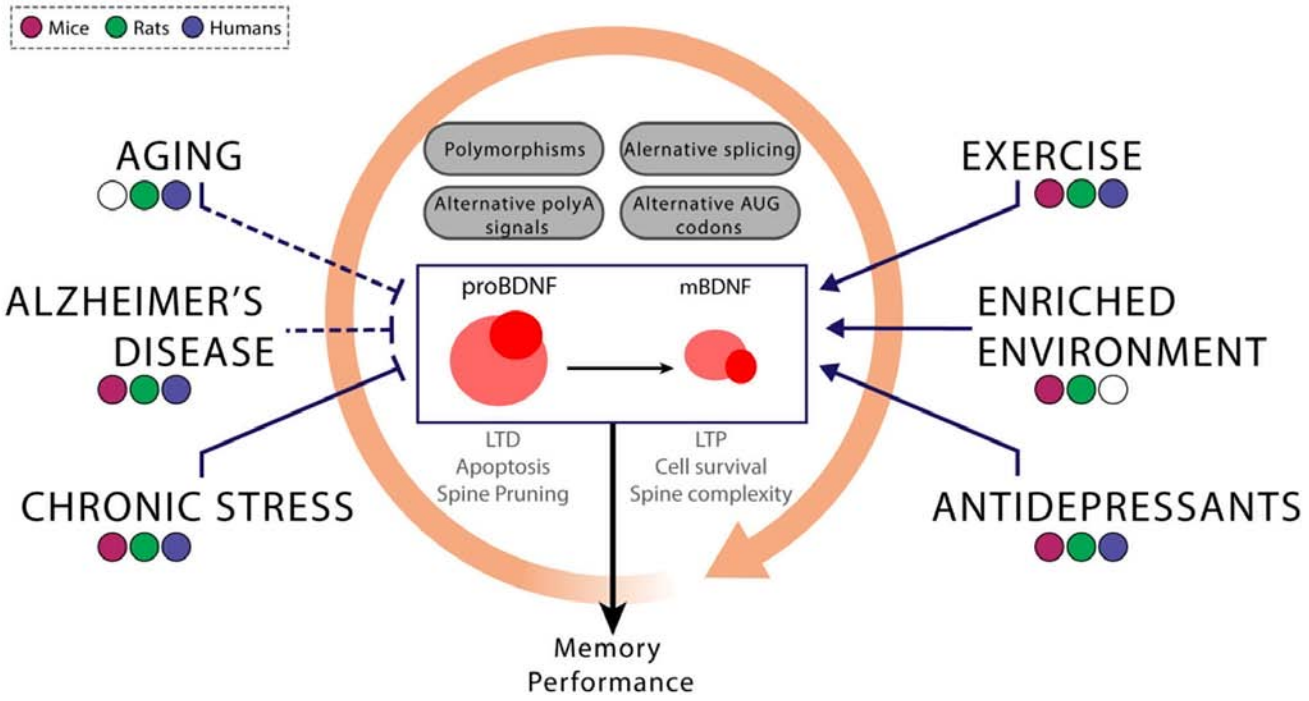

FIGURE 1 | The interplay between genetic and environmental factors modulates the expression of the BDNF variants. BDNF gene expression is controlled at many levels. The inclusion of particular exons and the differential use of polyadenylation sites and/or starting codons modify not only the BDNF variant but also the temporal dynamics of its expression through the modification, for instance, of the stability of the BDNF mRNA. Variability at any of these levels of regulation can lead to differential levels of mature BDNF in healthy or diseased subjects. These differences could be related to genetic (i.e., Val66Met and other polymorphisms) or environmental factors (chronic stress, exercise, and amongst others). In addition to correlational studies performed in humans, the use of non-human animal models, particularly rodent models, can lead to establish certain relationships schematized above between these factors. Blunt arrows indicate that a decrease in BDNF expression and the sharped ones symbolize the opposite pattern. Although the consensus agrees that aging, the development of Alzheimer's disease and the exposure to chronic stress are related to reductions in BDNF levels, some studies could not find those associations or found the opposite pattern (cases represented with dashed lines). On the other hand, some external interventions are able to enhance BDNF expression, such as exercise, enriched environment and antidepressants. For this reason, the aforementioned interventions could be potential treatments for cognitive impairments related to low BDNF expression. Since these external factors can interact with each other (represented by the orange circular arrow), it is important to take into account all of these potential interactions at the time of determining causal links between the effects of any of these variables on BDNF expression.

in brain structure and function is not conclusive and, even meta-analysis studies show patterns of conflicting results. Kambeitz et al. (2012) conducted three separate meta-analyses to determine the effect of the Val66Met polymorphism on declarative memory performance, hippocampal volume and hippocampal activation in humans. They reported that all these measures are reduced in carriers of the Met allele compared to Val homozygotes and this effect cannot be explained by random variables such as age, gender or diagnosis criteria. However, Dodds et al. (2013) argued that the effect sizes of fMRI data are susceptible to the method used to select the voxels and that the one used in Kambeitz et al. may have led to an inflated estimate of the effect size. In fact, the meta-analysis conducted by Mandelman and Grigorenko (2012) did not detect a significant association between the BDNF Val66Met polymorphism and several phenotypes including general cognitive ability, memory, executive function, visual processing skills, and cognitive fluency. They hypothesized alternative scenarios to explain this incongruence in the literature and proposed that instead of being grouped by their behavioral similarity, cognitive phenotypes should be categorized depending on the brain activation pathways engaged. Although an important group of studies could not found an association between Val66Met genotype and memory performance (Tsai et al., 2008; Houlihan et al., 2009; Karnik et al., 2010), the results linking BDNF polymorphism and memory are not likely spurious. It is possible that differences in the effect that the BDNF gene exerts across the lifespan and the uncontrolled effects of variables such as gender, stress and physical exercise, known to affect BDNF levels, may have led to a dilution of the effect in certain samples. Furthermore, Met carriers might be able to compensate for the deficiency in BDNF levels. In fact fMRI studies suggest that they present increased medial temporal lobe activity during the engagement of an episodic memory task when compared with non-carriers, and this might "hide" the potential deficits (Dennis et al., 2011). In conclusion, these studies suggest that presence of the Met allele may confer a disadvantage in cognitive performance, and particularly episodic memory (for a review, see Bath and Lee, 2006), but that the effects of BDNF polymorphism may be too complex to be analyzed under the idea of a simple "risk allele."

BDNF IN THE AGING BRAIN

Aging is a major risk for the development of cognitive disorders (Horn and Cattell, 1966). Cognitive performance gradually declines with age, probably as a result of cellular and metabolic changes that lead to a progressive reduction in synaptic plasticity in brain regions crucial for cognitive functions (Barnes, 1994; Smith et al., 2000; Erickson and Barnes, 2003). Aging is related to a significant shrinkage of gray matter (Driscoll et al., 2003, 2009; Raz et al., 2005), an important reduction in the number of synapses (Burke and Barnes, 2006) and also changes in neuroplasticity-related proteins (Assuncao et al., 2010; Erickson et al., 2010). The hippocampus is a brain region with high levels of plasticity-related molecules (Neves et al., 2008) and is particularly sensitive to aging-related cellular alterations that lead to volume reductions (Greene and Naranjo, 1987; Lee et al., 1994; Rosenzweig and Barnes, 2003). Particularly numerous changes were reported in the dentate gyrus local inhibitory and excitatory circuitry (Patrylo and Williamson, 2007). Furthermore, decreases in adult neurogenesis in the dentate gyrus start emerging at middle age and continue throughout the aging process (Drapeau et al., 2003). Other structures also linked to episodic memory formation, such as the frontal and entorhinal cortices, also show volume reduction and may contribute to the deterioration of cognitive function (Driscoll et al., 2009).

At the cellular level, changes in hippocampal LTP have also been reported in aged animals, with deficits either related to induction, maintenance and/or expression of LTP depending on the stimulation pattern used for induction (Erickson and Barnes, 2003; Gooney et al., 2004). Given that alterations in the thresholds for LTP and LTD in the MTL region have been associated with impairments in long-term memories (for review, see Kumar, 2011), age-related cognitive impairment could also be linked to altered LTP function.

In fact, one of the most consistent plasticity-related deficits associated with aging is the reduction in neurotrophic signaling. BDNF-LTP in the dentate gyrus is impaired in the aged brain, and may be reverted by endogenous BDNF induction by manipulations such as ampakine administration (Rex et al., 2006), which can also ameliorate age-associated memory deficits in rodents (Granger et al., 1996). Since dysfunction of synaptic plasticity and changes in neuronal activity are associated with worse performance in different cognitive tasks (Lynch et al., 2006), efforts have been done to link changes in BDNF levels to aging-dependent cognitive decline and to the related alterations in structural and functional integrity of neuronal networks (for review, see Tapia-Arancibia et al., 2008). Mattson et al. (2004) suggested that age-dependent impairment in cognitive function could be associated with decreases in BDNF expression in the primary regions of the brain affected by aging. Consistent with this idea, the circulating concentrations of BDNF are reduced in aged primates and humans (Hayashi et al., 2001; Shimada et al., 2014) and brain concentration reduced in rats (Silhol et al., 2005). In aged rodents, the BDNF system is affected at different levels, including reduced transcription, protein synthesis and processing (Calabrese et al., 2013), however, other publications could not find an association between age-related plastic changes and BDNF (Lapchak et al., 1993; Driscoll et al., 2012). The found reductions correlate with hippocampal shrinkage (Erickson et al., 2010), spatial memory decline (von Bohlen und Halbach, 2010) and neuronal atrophy (Murer et al., 2001). For example, higher BDNF mRNA induction levels were reported after Water Maze task on unimpaired rats in comparison with aged animals (Schaaf et al., 2001). The decrease in BDNF levels observed in aged individuals, was accompanied by a reduction in the expression and/or activation of TrkB receptor and a concomitant increase in the levels of proBDNF and $p75_{NTR}$, suggesting the presence of additional age-related deficits in BDNF signaling pathway and in the processing of proBDNF to mature BDNF. Additionally, these changes were negatively correlated with performance on the Water Radial Maze (Buhusi et al., 2017). Furthermore, the induction of specific BDNF transcripts after fear conditioning is altered in aged rats (Chapman et al., 2012), suggesting that aging is not only associated with reduced neurotrophin expression under resting conditions, but might lead to a functional impairment of BDNF in response to a specific task. This is consistent with the aforementioned role of BDNF in activity and experience-dependent structural and functional connectivity changes.

However, some researchers found no change or even an increase in BDNF (Lapchak et al., 1993; Narisawa-Saito and Nawa, 1996; Newton et al., 2005) associated with aging. This could suggest that the loss of hippocampal BDNF is not part of the mechanisms involved in age-related cognitive decline. However, considering that neuronal cell death is an undoubtedly important element of age-related cognitive impairment (Morrison and Hof, 1997), a transient BDNF-related response to neuronal degeneration could be the underlying reason for the increasing levels of hippocampal BDNF reported in some studies. However, since *in vivo* measurements of BDNF in the brain are not possible, studies in humans rely on inferences about the levels of BDNF on central nervous system. Given that there is evidence that BDNF can cross the blood-brain barrier (Pan et al., 1998), these studies assume that serum BDNF is a proxy of BDNF levels in the brain. Consistently, measures of BDNF in the central nervous system (brain BDNF, b-BDNF) correlate with measures of BDNF from the periphery (serum BDNF, sBDNF) (Sartorius et al., 2009; Klein et al., 2011) with as much as 75% of brain origin BDNF (Rasmussen et al., 2009). However, the functional significance of BDNF in the serum is a matter of debate. BDNF is also secreted in several peripheral sites such as platelets, lymphocytes, and skeletal and smooth muscle cells, and also recent studies have questioned the idea of BDNF being able to cross the blood-brain barrier (Pardridge et al., 1998; Di Lazzaro et al., 2007). Another important consideration in these measurements is whether pro and mature forms of BDNF are being measured, because only some assays can differentiate mature BDNF and, considering that they can have opposing effects, these should be taken into account (Polacchini et al., 2015). For this reason, the results of serum studies should be interpreted cautiously, as this could be a potential cause for inconsistencies reported in sBDNF levels between studies. The use of enriched extracellular vesicles of neuronal origin from peripheral blood could provide a novel way to bypass these issues since they could more closely

reflect brain changes compared to plasma (Mustapic et al., 2017; Suire et al., 2017).

There are several reasons to think that a decrease in BDNF levels could be detrimental to normal brain functioning, like its role in synaptic plasticity, as described above. Another motive is that BDNF exerts various trophic effects on hippocampal neurons that could help counteract the noxious effects of neuronal cell death (Almeida et al., 2005). In fact, high levels of BDNF in the hippocampus are related to both survival and differentiation of dentate gyrus progenitor cells in the adult (Pencea et al., 2001; Shetty et al., 2004), and low levels of BDNF have been linked to deficient neurogenesis in aged animals (Apple et al., 2017). Moreover, BDNF is known to increase with oxidative stress as part of an antioxidant defense during aging (Mattson et al., 2002). Neuronal loss is an important component of normal aging, however, it does not contribute significantly to learning and memory impairments (West, 1993; Rapp and Gallagher, 1996; Rasmussen et al., 1996), suggesting that memory deficits associated with aging are more likely related to alterations in synaptic physiology and aberrant cell signaling, that might contribute to an altered connectivity (Lister and Barnes, 2009).

While aging-related neuroanatomical changes are evident, there is an enormous variability amongst healthy individuals in the pattern of cognitive decline (Morris and Price, 2001). Memory performance is also partly under genetic control (Payton, 2006), probably because of age-sensitivity in many cognitive processes (Kremen et al., 2007; Lessov-Schlaggar et al., 2007). Genetic variance could explain these individual differences in cognitive capacity, especially since heritability of cognitive function increases over the lifespan as brain resources decrease (Haworth et al., 2010), and can account for as much as one third of the variance in cognitive decline (Finkel et al., 2005). Both apolipoprotein E (APOE) (Wisdom et al., 2011) and BDNF (Miyajima et al., 2008) have been associated with variance in cognitive performance in healthy individuals more frequently than other genes, although some results have not been reliably replicated (Harris and Deary, 2011).

Since BDNF decreases throughout life (Erickson et al., 2010), it would be interesting to assess the possible associations between the Val/Met polymorphism and age-related cognitive decline. For example, Miyajima et al. (2008) reported poor verbal recall in a sample of healthy elderly Met homozygotes, and Sambataro et al. (2010) found that Met carriers showed greater age-related decline in hippocampal activation during both encoding and retrieval, while other studies also limited to older adults found no impact of age on cognitive tests evaluating learning and memory (Houlihan et al., 2009; Laing et al., 2012). Considering these results, this polymorphism could help understand individual differences in cognitive function by genetic-dependent changes in neurotransmitter and neurotrophic factor levels, amongst other factors (Raz and Lustig, 2014). However, since reports indicate that both APOE and BDNF polymorphisms accounts for less than 2.3% of the variance, it is fundamental to take into account the complex interplay between associations with other genes and interactions with environmental factors to interpret these results. Many environmental and hormonal factors such as physical exercise (Cotman and Berchtold, 2007), caloric restriction (Mattson et al., 2003), estrogen levels (Scharfman and Maclusky, 2005), and environmental enrichment (van Praag et al., 2000) can influence BDNF levels, making it challenging to link BDNF to age-related memory impairment and hippocampal atrophy. For example, BDNF genotype can modulate the effect of physical exercise on episodic memory performance and brain volume. This is evident by the fact that only Val homozygous benefited from physical exercise with larger MTL volume and hippocampal gray matter, whereas in Met carriers the contrary effect was found (Brown et al., 2014). In another study, in a cohort aged 65 or older the strength of the association between incidence of cognitive decline and physical activity increased with the number of Met alleles, suggesting that the Met allele may confer vulnerability to dementia in elders with less physical activity (Kim et al., 2011). On the other hand, there are studies reporting a reduced vulnerability of Met carriers to age-related decline in executive function (Harrisberger et al., 2014), pointing toward a differential effect of BDNF on cognitive function related to the areas supporting the task.

The influence of BDNF on cognitive function may change across the lifespan. In fact, the effects of the BDNF Val66Met polymorphism on brain structure and cognitive function were found to differ in an age-dependent manner: while Met carriers showed a reduction in episodic memory performance and hippocampal/parahippocampal volume in samples of around 65 years in comparison to Val carriers (Egan et al., 2003; Pezawas et al., 2004), in the elderly (mainly samples of around 75 years) Val/Val individuals had diminished entorhinal cortex thickness, white matter tract integrity, and episodic memory performance (Harris et al., 2006; Erickson et al., 2008; Voineskos et al., 2011). It has been hypothesized that this effect could be related to changes in the level of cleavage, since cleavage molecules such as tPA are known to decline with age (Cacquevel et al., 2007) which could create a paradoxical effect where greater BDNF secretion would, in fact, lead to cognitive decline (Pang et al., 2004). Another explanation could be a decrease in the penetrance of the BDNF genotype across the lifespan, as other factors such as the independent incidence of age-related diseases increase their influence on brain structure and cognition (Lindenberger et al., 2008). In fact, there are results that suggest that Met carriers have more preserved frontostrial functions than Val/Val subjects (Gajewski et al., 2012), what may lead to an increase in the use of stratium-dependent mnemonic strategies, that could give a potential advantage on Met carriers that might hinder the original deficits reported in young individuals. This might help explain conflicting results in this regard, since there are studies showing that aged Met-carriers still have deficits in mnemonic performance and they experience a steeper impairment in memory tasks as they age (Kennedy et al., 2015) and have diminished performance in remembering neutral faces when compared with Val/Val individuals (Mascetti et al., 2013).

Aging is normally accompanied by a loss of memory function (Erickson and Barnes, 2003). Episodic memories are particularly more sensitive to the aging process (Verhaeghen et al., 1993) than procedural or non-declarative memories (Light, 1991). Within the limits of the episodic memory domain, some aspects can be more vulnerable to aging than others. For example,

associative memory tasks that require binding of multiple pieces of information can be more sensitive to the aging process (Naveh-Benjamin, 2000; Old and Naveh-Benjamin, 2008). This deficit is mostly related to spatial (Tanila et al., 1997; Oler and Markus, 1998) and recognition memory loss (Moss et al., 1988; Danckert and Craik, 2013). The vulnerability of particular mnemonic processes to aging is probably due each of these functions being supported by distinct brain regions showing differential rates of functional decline with age (Buckner, 2004). Diminished input from the EC to the DG could contribute to deprive the HP from sensory information crucial for discrimination of novel versus familiar stimuli when this stimuli are similar (Wilson et al., 2005; Holden and Gilbert, 2012), and in fact reductions in DG-CA3 connectivity are associated with spatial learning decline in old animals (Smith et al., 2000). The medial temporal lobe region (MTL), that is thought to support episodic memory function, is particularly vulnerable to cellular alterations that happen during aging and/or pathological dysfunction (Jobst et al., 1994). Since episodic memory decline is correlated to decreases in hippocampal volume (Charlton et al., 2010), these changes could explain age related mnemonic deficits. Another important structure for episodic memory in the MTL is the perirhinal cortex (Prh), a region involved in the discrimination of novel and familiar stimuli (Malkova et al., 2001; McTighe et al., 2010) that is particularly crucial to solve tasks involving ambiguous features (Bartko et al., 2007). Aging alters discrimination in both in rats and humans increasing a propensity to identify novel stimuli as familiar (Plancher et al., 2009; Burke et al., 2010). This effect was, in some cases, interpreted as a deficit in the ability to bind features of an object, so that decisions are made based on the familiarity of a single component. The consequence is the incapacity to detect novel compositions of familiar features (Jones and Jacoby, 2005). In fact, complexity and ambiguity of the features was proposed as a determining variable in recognition memory deficits in aged rats (Burke et al., 2011; Gamiz and Gallo, 2012), as well as in Prh-lesioned animals using a configural task with complex objects (Norman and Eacott, 2004). Several molecular and biochemical alterations have been reported in the Prh of aged animals that could contribute to the associated cognitive deficits (Liu et al., 2008; Moyer et al., 2011). Since exposure to novel objects is related to an increase in BDNF levels in the Prh (Romero-Granados et al., 2010), and familiarity discrimination in the presence of ambiguous stimuli (and not clearly distinguishable ones) is impaired with BDNF antisense ODNs infusions during a restricted time window after the task (Seoane et al., 2011; Miranda et al., 2017), BDNF is an interesting molecular candidate that could help to establish a link between molecular and biochemical alterations and the pattern of both spatial and recognition memory deficits associated with aging. In particular, because optimal cognitive function is linked to efficient neuronal plasticity, these memory deficits might be coupled to alterations in the expression and regulation of plasticity-related proteins such as BDNF, a protein whose expression is both affected in the aging brain and is crucial for memory consolidation and particularly for discrimination of similar memories.

In correspondence with this idea, decrease in BDNF expression has been associated with neuronal atrophy and death occurring in some neurological disorders (Murer et al., 2001). Administration of exogenous BDNF can prevent pathological changes in the nervous system associated with aging (Nagahara et al., 2009) [but see Fischer et al. (1994) for inconsistent results; for review, Fumagalli et al. (2006)], and can rescue both BDNF-induced LTP and spatial memory performance in aged animals (Rex et al., 2006). Since BDNF has been linked to synaptic plasticity, neurogenesis, neuronal survival and protection against brain insults (Bath and Lee, 2006), the above results imply the possibility that BDNF could act as a synaptic repair molecule. There are a few evidence that support this idea, for example, acute application of the TrkB agonist 7,8-dihydroxyflavone rescues synaptic plasticity in the hippocampus of aged rats *in vitro* (Zeng et al., 2011). Additionally, chronic treatment also prevents age-related impairments in contextual and cued fear conditioning with a simultaneous normalization of the spine levels that normally decrease with age (Zeng et al., 2012). Furthermore, the Lou/C rat, an animal model of successful aging that presents a preserved cognitive performance across its longer lifespan (Kollen et al., 2010), showed higher hippocampal BDNF than Wistar rats and a decrease in proBDNF with age. This contrasts with the increase in proBDNF seen in aged Wistar rats (Tapia-Arancibia et al., 2008). However, the beneficial effect of BDNF on neuroprotection and mnemonic performance in rats decrease as age increases (Sohrabji and Bake, 2006), probably due to additional changes in the processing and signaling pathway. Consistent with the role of BDNF on synaptic plasticity and memory, elderly Lou/C rats never showed short- or long-term memory decline in recognition memory tasks or impaired LTP (Kollen et al., 2010). However, Silhol et al. (2007) found that learning-associated cognitive training could increase TrkB receptor expression in aged animals and also increased proBDNF processing both in aged and young rats, indicating that learning leads to a strengthening of BDNF pathway, especially in aged animals where this pathway is affected.

BDNF AND ALZHEIMER'S DISEASE

Reduced levels of BDNF have been reported not only under normal aging conditions but also in pathological conditions including Huntington (HT), Alzheimer's disease (AD), and Parkinson's disease. However, the profile of cognitive deficits greatly differs between these pathologies according to the brain regions affected by degeneration. For example, the most profound BDNF deficits are reported in the hippocampus, parietal, entorhinal and frontal cortex for AD (Hock et al., 2000) and in the striatum and motor cortex for HT (Zuccato et al., 2008). In this section we will focus on AD because it starts mainly as impairment in declarative memories, without affecting other neurological functions (Walsh and Selkoe, 2004). It has been proposed that this feature is related to the degenerative profile of the disease that starts in the hippocampus, parahipocampal cortices and amygdala, but not in primary sensory and motor cortices (Selkoe, 2001).

There is a substantial amount of studies supporting the idea that neurotrophic factors are crucial for the etiology of AD, in particular BDNF. BDNF protein and mRNA levels (Hock et al., 2000) as well as proBDNF (Peng et al., 2005) are reduced in the post-mortem brain of AD patients compared with age-matched controls, with no changes in TrkB levels (Savaskan et al., 2000). This reduction was also reported in Mild Cognitive Impairment (MCI) (Shimada et al., 2014), a potentially prodromal stage of AD (Flicker et al., 1991). Furthermore, reduced circulating levels of BDNF were also found in MCI (Forlenza et al., 2010). BDNF levels are correlated to the severity of the disease and with episodic memory performance in patients (Peng et al., 2005), suggesting that these decreases could be related to the pathogenesis of the disease. In conclusion, downregulation of BDNF and proBDNF are thought to be an underlying mechanism related to early AD (Peng et al., 2005). However, Laske et al. (2006) found that patients in the early stages of AD had significantly higher sBDNF levels than patients in the late stages and also than age-matched controls. This highlights that it is difficult to establish a causal link between BNDF downregulation and the development of this neurodegenerative disease because the pathology is accompanied by a loss of cell density and dendritic spines that could secondarily affect BDNF levels. In this regard, there are also post-mortem and serum level studies that report an increase in BDNF and TrkB concentrations in the hippocampus and parietal cortex of AD patients (Durany et al., 2000; O'Bryant et al., 2009). This increase may be related to compensatory mechanisms that could contribute to the repair by degradation of β-amyloid. In addition, other potential moderators could contribute to differences and heterogeneity seen in these studies. Differences in diagnostic criteria, stages of the disease, sex and education and the use of pharmacological treatments such as acetylcholinesterase inhibitors or psychotropic medication that are known to raise BDNF levels (Leyhe et al., 2008), or could come from other potential sources outside the CNS such as immune cells (Kerschensteiner et al., 1999).

Given that synaptic loss is the major correlate of cognitive impairment, much stronger than the presence of plaques or tangles (Terry et al., 1991), there is a recent view of AD as a "synaptic pathology" (Lippa et al., 1992; Heffernan et al., 1998). Aβ monomers are normally generated and secreted at firing synapses, and are not toxic but neuroprotective as they have an active role in synaptic regulation (Giuffrida et al., 2009) and are crucial for neuronal function (Abramov et al., 2009). Aβ monomers are one of the many factors that regulate synaptic function and they can activate CREB via the PI3K/AKT pathway, leading to a sustained CREB-regulated transcription and release of BDNF (Giuffrida et al., 2018; Zimbone et al., 2018). In this way, BDNF can act as a converging point of many synaptic regulators. In Alzheimer's disease (AD), neurotoxic β-amyloid (Aβ) oligomers are formed from the self-association of Aβ monomers. These oligomers can promote neurotoxicity through different ways (Pearson-Leary and McNay, 2012). Arshavsky (2006) suggested that the selective vulnerability of memory related areas could be, in fact, a result of specific cellular modifications required for the process of memory consolidation. An important event in AD is the pathogenic Aβ-mediated alterations in the levels of neurotrophic factors (NTFs) (Budni et al., 2015). Since pathogenic Aβ oligomers cannot can activate PI3K/AKT pathway and induce CREB activation, the increase in the levels of Aβ-oligomers can lead to an impairment in CREB activation in the brain of patients with AD and mouse models of AD (Bartolotti et al., 2016). Soluble Aβ oligomers are known to alter signal transduction pathways crucial for learning and memory processes such as CREB-regulated transcription (Caccamo et al., 2010) and trafficking of NMDA type of glutamate receptors (Snyder et al., 2005). Thus, alterations in those pathways could play an important role in the etiology of the disease. Altered levels of BDNF in AD are downstream of Aβ-accumulation and could be related to Aβ–induced dysregulation of CREB transcription (Caccamo et al., 2010; Pugazhenthi et al., 2011). Even if BDNF does not modify Aβ accumulation, it could have an important function in moderating the effects of Aβ on cognitive and structural aspects (Nagahara et al., 2009). BDNF protects against Aβ-mediated toxicity by contributing to its degradation and preventing tau hyperphosphorylation (Elliott et al., 2005; Tapia-Arancibia et al., 2008). In this sense, BDNF is expressed by microglial and astroglial cells in the plaque vicinity and seems to protect from neuroinflammation, thereby supporting neuronal survival (Lindvall et al., 1994; Kerschensteiner et al., 1999) and preventing apoptosis (Tamatani et al., 1998). On the other side, Aβ down-regulates BDNF mRNA *in vitro* via reduction of CREB (Rosa and Fahnestock, 2015) and disrupts retrograde axonal transport of BDNF (Poon et al., 2011) and conversion of pro-BDNF to mature BDNF (Zheng et al., 2010). It also interferes with synaptic plasticity mediated by BDNF even at concentrations that do not kill the cells (Wang et al., 2006). This downregulation occurs before the appearance of plaques and is linked to memory deficits in AD animal models (Francis et al., 2012) and in MCI (Peng et al., 2005). Tau, a mediator of Aβ-induced toxicity, can significantly downregulate BDNF via transcript IV both *in vitro* and *in vivo* by itself (Rosa et al., 2016). As mentioned before, many studies found that decreases in serum BDNF levels can be detected in individuals with MCI, so it is tempting to speculate that BDNF loss could be involved as an early event in this synaptic dysfunctions. However, the presence of some inconsistencies between studies with MCI patients warns us to be cautious with these speculations. Nevertheless, these results suggest a critical role of BDNF in the regulation of Aβ-amyloid toxicity, suggesting that BDNF dysregulation could contribute to synaptic dysfunction and mnemonic impairment related to AD. This data implies that, although central to the development of AD, changes in BDNF expression could be an effect of earlier functional modifications in other synaptic related proteins. In particular, one of these proteins could be Aβ, that in its monomeric form has a normal physiological role in synaptic plasticity and neuronal survival in the brain and can actually have an active role in these BDNF changes by regulating BDNF transcription and release (Parihar and Brewer, 2010). In any case, the beneficial effects of BDNF on memory and cognition could reflect its synapse repair features.

Changes in the cell microenvironment, where a lack of trophic support can lead to a decrease in neuronal survival and proliferative activity (Drapeau and Nora Abrous, 2008), could contribute to the degeneration of specific neuronal subpopulations in pathological conditions. During this period, changes in BDNF levels contribute to age-related hippocampal volume changes, and atrophy associated with pathological conditions (Erickson et al., 2012). There is evidence of as much as 1–2% annual hippocampal atrophy in the elderly without signs of dementia, while in patients with AD this deterioration goes up to 3–5% per year (Jack et al., 1998). In patients with MCI, hippocampal volume is predictive of rapid conversion to dementia (Jack et al., 1998), evidencing its importance in the progress of the disease.

Most studies report that BDNF genotype is not related to the risk of developing AD (Combarros et al., 2004; Nishimura et al., 2004; Li et al., 2005) [but see for evidences of effects present only in women (Fukumoto et al., 2010)], and Genome Wide Association Studies could not find a relationship between BDNF Val66Met polymorphism and risk of AD (Lambert et al., 2013). However, some studies do report an increase in the risk for AD in Val carriers (Ventriglia et al., 2002; Matsushita et al., 2005; Voineskos et al., 2011). Other studies found an association between Met carriers and greater rates of decline in episodic memory and hippocampal atrophy in patients with MCI (Forlenza et al., 2010; Lim et al., 2013, 2016), leaving Aβ accumulation unaffected (Lim et al., 2013). Although there are certain inconsistencies among the literature, BDNF role in the development of AD seen with Val66Met has been replicated with other BDNF polymorphisms (Kunugi et al., 2001; Riemenschneider et al., 2002). The lack of consistency between studies could be related to differential effects of BDNF during distinct stages of the disease, with more circulating BDNF in MCI patients, and less in AD patients (Yu et al., 2008; Forlenza et al., 2010). Lower BDNF levels may be linked to neuronal death in AD, concealing any effect of the BDNF gene. Since the complexity of the pathological changes stemming from the disease increases as the severity progresses, associations between BDNF Val66Met polymorphism and AD should be more obvious in preclinical stages in which the disease presents almost exclusively subtle alterations in mnemonic performance (Fahnestock, 2011).

Neurotrophic factors not only moderate neuronal and synaptic dysfunction but also cognitive decline in AD (Fahnestock, 2011). Higher sBDNF is associated with a protection against future occurrence of dementia and AD (Weinstein et al., 2014) and predictive of slower rates of decline (Laske et al., 2011). In the same manner, changes in BDNF levels induced pharmacologically or by aerobic exercise are related to better cognitive function and diminished synaptic dysfunction both in humans at risk of developing AD and in animal models of AD (Baker et al., 2010; Intlekofer and Cotman, 2013). These effects could be related to the ability of BDNF to prevent lesion-induced neuronal degeneration (Morse et al., 1993; Kiprianova et al., 1999). According to this idea, post-lesion gene transfer of BDNF partially restored the deficits in learning capacity and synaptic plasticity in an AD model in which BoNTx-induced damage to the entorhinal cortex was used to mimic AD pathology (Ando et al., 2002). Neural stem cell transplants or CREB binding protein gene transfers reversed spatial memory deficit via BDNF in AD mouse models, despite widespread Aβ plaque and tau pathology (Blurton-Jones et al., 2009; Caccamo et al., 2010). In a recent study, delivery of BDNF to the entorhinal cortex in amyloid transgenic mice reversed neuronal atrophy and synaptic loss, regulated neuronal signaling, and diminished the related mnemonic deficits without changes in the amyloid plaque load (Nagahara et al., 2009) indicating that BDNF can act through amyloid-independent mechanisms to exert its protective effect. Furthermore, 7,8-dihydroflavone (7,8-DHF), Neotrofin (a hypoxanthine derivative that stimulates neurotrophic factor production) and Neuropep-1 (a BDNF modulating peptide) have shown to reverse memory deficits in animal models of AD or even in preclinical trials (Glasky et al., 1994; Devi and Ohno, 2012; Shin et al., 2014). In this way, BDNF could mediate the protective effect of exercise and caloric restriction on neurodegeneration (Vaynman et al., 2004b). This strengthens the need to develop behavioral interventions that could prevent the risk of developing dementia or slow the progression to dementia in patients with MCI, a path that is currently in progress. Many of these new paths point to lifestyle changes that range from antioxidant diet, environmental enrichment and social interaction to physical or cognitive exercise as potential interventions (Fahnestock et al., 2012).

THE EFFECT OF CHRONIC STRESS ON BDNF AND THE LINK TO PSYCHIATRIC DISORDERS

Chronic stress is a known factor involved in the incidence of AD and cognitive impairment (Wilson et al., 2007c). Structures involved in the control of the physiological status of an organism are susceptible to modulation by chronic stress. In particular, the hippocampus is altered by prolonged exposure to aversive situations (Kim et al., 2015). These abnormalities are reflected in deficits in spatial memory tasks and novel object recognition (Luine et al., 1994; Vedhara et al., 2000; Baker and Kim, 2002), but also in altered synaptic plasticity processes (Shors et al., 1989; Kim and Yoon, 1998) like suppression of LTP (Artola et al., 2006). Chronic stress typically decreases BDNF hippocampal expression (Smith et al., 1995; Murakami et al., 2005), however, when the cause of the stress disappears, the hippocampus shows amelioration of the cognitive and synaptic deficits (Sousa et al., 2000; Hoffman et al., 2011).

To this date, a wide variety of strategies have been assessed to reduce the deleterious effects caused by chronic stress. Infusions of BDNF in the rat hippocampus before a chronic restraint stress protocol can protect against the deficits in learning and memory in the MWM and in LTP (Radecki et al., 2005) and shRNA against BDNF before a stress protocol can revert the spatial reference memory deficits during the post-stress-rest period.

Exercise, is a well-known strategy to increase BDNF brain levels, so it has been proposed as a non-invasive way to mimic the effects of direct BDNF administration over chronic stress. Radahmadi et al. (2016) found that the hippocampal BDNF

increases in response to exercise after a chronic stress protocol. On the other hand, Dief et al. (2015) showed that animals that followed a 30 days swimming training program improved their performance in the T maze after being exposed to chronic stress and this enhancement correlated with upregulation of hippocampal BDNF. Also, Kwon et al. (2013) described a BDNF-mediated improvement in MWM performance in chronically stressed mice that started treadmill running 12 weeks before the beginning of the stress protocol and continued throughout it.

Shafia et al. (2017) investigated the palliative effects of exercise (alone or combined with fluoxetine) on a rat model of post-traumatic stress disorder. This model shows impairments in fear conditioning and extinction, inhibitory avoidance task and location recognition memory. Interestingly, in most tests, the effects of the combined treatment were similar to the ones obtained with exercise alone. They also found, in agreement with the work of Garza et al. (2004), that exercise alone and exercise plus antidepressant enhanced hippocampal BDNF expression, but not antidepressant alone.

Enriched environment (EE) has been shown to increase BDNF levels in the hippocampus in comparison with standard housing conditions (Novkovic et al., 2015). Thus, EE could be an easy way to promote the systemic and neural recovery from the effects of chronic stress. Shilpa et al. (2017) showed that exposure to EE following 10 days of immobilization (2 h/day) ameliorates spatial memory deficits in a version of the radial arm maze and depressive-like behavior. Recovery seems to be achieved through the modulation of several signaling cascades, including BDNF's.

Seong et al. (2018) suggested that EE is as effective as Fluoxetine when it occurs after exposure of the animals to a chronic stress protocol, but additional measures of the effectiveness of the stress protocol would be needed to establish the success of the treatment. Interestingly, BDNF levels were increased in the hippocampus of rats that receive either EE or Fluoxetine in comparison with the control group (stressed but without posterior treatment). Considering that chronic stress is linked to depressive-like symptoms (Garcia, 2002; Calabrese et al., 2009), the results obtained using antidepressants do not seem surprising. The mechanisms of antidepressant actions over chronic stress and the putative involvement of BDNF have also been extensively studied but with no consistent results yet. Larsen et al. (2010) showed that chronic antidepressant treatment reversed depressive-like behavior caused by chronic unpredictable stress-induced and increased BDNF mRNA expression in the granular cell layer of the dorsal hippocampus (independently of exposure to stressors). Using a different stress model, Tsankova et al. (2006) were able to normalize behavioral alterations in mice exposed to a social defeat stress protocol followed by chronic (but not acute) administration of imipramine They proposed a model in which chronic stress induces repression and chronic imipramine induces de-repression of the *bdnf* gene in the hippocampus through changes in the chromatin structure.

Some studies are focusing in compounds that have been originally used to treat other diseases but have shown some antidepressant effects in animal models, such as resveratrol. Resveratrol and curcumin, when chronically administrated, prevent the behavioral and biochemical alterations induced by chronic restraint and unpredictable stress, respectively, and those effects seem to be mediated by an increment in the expression of BDNF (Xu et al., 2006; Zhang et al., 2017). Zhou et al. (2017) show that biperiden alleviates depression-like symptoms induced by chronic unpredictable stress, increasing performance in the sucrose preference, novelty suppression feeding and forced swimming tests. Importantly, these effects were inhibited by pretreatment with the TrkB antagonist K252a.

Since the evidence suggests that BDNF may drive the recovery from stress-induced effects on the hippocampus, an interesting question emerges: Is BDNF capable of reversing the effects of chronic stress in the presence of the stressor?

Radahmadi et al. (2016) tested the effect of exercise during the exposure to stressors ("protective exercise"). Unlike preventive and therapeutic exercise, no increment on BDNF hippocampal levels was found. On the other hand, Miller et al. (2018) explored, in mice, the potential palliative effects of running on chronic stress-related impairments when exercise and stress are co-occurring. They found that the TrkB receptor had higher expression levels in both exercised groups (stressed and non-stressed) compared with both sedentary groups, supporting the hypothesis that the mitigation of the negative consequences of stress by exercise could be mediated by BDNF.

Depending on the chronic stress protocol (duration and type of stressor), different and even contrasting results have been found (Vasquez et al., 2014). BDNF appears to be an important underlying molecule behind the restitution of a normal cognitive phenotype in animal models of chronic stress. The fact that BDNF could be increased with non-invasive protocols and/or drugs -some of which are used in clinical trials- makes it attractive for human therapies.

The link between stress, specific genes and the development of psychiatric disorders has been extensively studied (Abbott et al., 2018), and a causal role has been ascribed of gene-environment interactions in the etiology of many of them (Rogers et al., 2019). In fact, psychiatric disorders can be defined as clinical entities emerging from the genetic-environmental interaction (for review, Gallo et al., 2018).

PSYCHIATRIC DISORDERS AND BDNF

In the last few years, evidence from animal models and clinical studies strongly suggest that dysregulation of neurotrophic factors could play an important role in the etiology of the bipolar disorder (BD), major depressive disorder (MDD), and schizophrenia (SZ) (Duman and Monteggia, 2006; Autry and Monteggia, 2012; Nieto et al., 2013). Due to the role of BDNF in neural plasticity, there could be a link between BDNF expression and the cognitive symptoms associated with memory impairments (Autry and Monteggia, 2012).

The *mnemonic* domain is commonly affected in different psychiatric disorders, such as BD (Zhou et al., 2018; Lin et al., 2019), MDD (Roca et al., 2015; Ahern and Semkovska, 2017), or SZ (Ricarte et al., 2017). Moreover, studies of post-mortem brain tissue of patients with BD and MDD reported that BDNF

levels are decreased in structures involved in memory processes, such as the hippocampus (Reinhart et al., 2015) and the prefrontal cortex (Dwivedi et al., 2003). In the case of SZ, post-mortem brain tissue analyses have shown more controversial results. While some studies observed an increase in BDNF expression in the prefrontal cortex (Takahashi et al., 2000) and the hippocampus (Iritani et al., 2003), others have shown a decrease in both structures (Weickert et al., 2003; Issa et al., 2010). Although BDNF was originally thought as a viable indicator of pathological brain functioning for early detection of BD, MDD, SZ, or AD, the discriminative power of BDNF as a biomarker is highly limited, since it seems to be a non-specific marker of many neuropsychiatric disorders.

The BD is a neuropsychiatric disorder that emerges from the interaction between genetic and environmental factors and is characterized by the switching between maniac and depressive episodes (for review, Harrison et al., 2018). It has been proposed that BDNF signaling participates in the physiological effects produced by some pharmacological treatments used for BD (Shaltiel et al., 2007). It has been shown that sBDNF decreases in the first episode of unmedicated BD patients and that, after 1-year of pharmacological intervention, sBDNF concentration increases (Palomino et al., 2006). in addition, there was a a negative correlation between the number of episodes and sBDNF levels (Kauer-Sant'Anna et al., 2009). It has been reported that sBDNF positively correlates with the duration of the maniac and depressive episodes (Dias et al., 2009). This evidence suggests that episode-related changes in the structure of the brain could be linked to peripheral BDNF concentration. Cao et al. (2016) have shown that hippocampal volume is reduced in patients with BD that present Val66Met BDNF polymorphism compared with controls and patients with MDD. Moreover, they proposed a link between the hippocampal volume and the performance in an episodic memory task. Another work has shown that the peripheral BDNF correlates with the performance in episodic memory task in BD patients with the BDNF Val66Met polymorphism (Chang et al., 2018). In this line, a recent study has shown that high levels of sBDNF are associated with good cognitive performance, including verbal memory (Mora et al., 2019). This evidence suggests that changes in BDNF expression in BD patients could produce structural modifications in the hippocampal formation related to episodic memory impairments. Despite this, most of the studies report alterations in other cognitive domains that are important for a good performance in episodic memory tasks, such as attention and working memory (for review, Sole et al., 2018). Thus, BDNF dysregulations could be related with the emergence of more complex symptom's profiles. For this reason, the relationship between BDNF and episodic memory in BD remains unclear.

Major depressive disorder is one of the most common mood disorders worldwide and is characterized by the absence of pursuit of pleasurable activities and the presence of negative thoughts (Kim and Moore, 2019). Since most common drugs used as antidepressant block the serotonin transporter (SERT), increasing extracellular serotonin in the raphe's nucleus post-synapses (for review, Teissier et al., 2017), it has been proposed that a misbalance in the serotonergic release could be related to the etiology of the depressive symptoms (for review, Liu et al., 2018). BDNF regulates the growth and reconstruction of 5-HT containing neuronal terminals in the cortex (Mamounas et al., 1995), and administration of BDNF in the raphe nucleus reduces behaviors related to depressive symptoms in rats (Siuciak et al., 1997). In addition, MDD patients present cognitive decline in different domains (Zuckerman et al., 2018), including episodic memory (Jayaweera et al., 2016) but only recently these deficits have been studied in detail. A large amount of work shows that sBDNF is decreased in MDD (Molendijk et al., 2011). Oral et al. (2012) found that patients that recurrently present depressive episodes show lower levels of sBDNF compared with those patients that were cursing their first episode. Interestingly, antidepressant treatment increases sBDNF concentration (Molendijk et al., 2011), but there is no consensus on whether lower sBDNF correlate with poor performance in memory tasks observed in this pathology (Oral et al., 2012).

In the case of the SZ, different studies have shown that the level of sBDNF correlates with cognitive performance in different domains (Carlino et al., 2011). Despite the lack of consensus on whether basal sBDNF is increased or decreased in SZ patients (Fernandes et al., 2015), some studies have indicated a correlation between memory performance and sBDNF levels (Zhang et al., 2012; Hori et al., 2017). Interestingly, there are evidences that pro-cognitive effects of pharmacological interventions in SZ could be mediated by BDNF (Einoch et al., 2017). For example, Zhang et al. (2018) have found that a 12-week chronic treatment with olanzapine produced an increase in BDNF plasma concentration. Moreover, BDNF concentration positively correlated with cognitive performance in a RBANS scale of memory. Not only the pharmacological interventions were effective on the reduction of mnemonic symptoms, different cognitive training protocols were also designed to enhance specific cognitive domains, especially memory (Guimond et al., 2018; O'Reilly et al., 2019). Fisher et al. (2016) conducted a computerized cognitive training in SZ patients and the patients exposed to this program present higher levels of sBDNF compared to the control group. They observed an enhancement in memory, but a causal link between sBDNF and memory remains unclear (Heitz et al., 2018).

BDNF AS A POTENTIAL MEDIATOR UNDERLYING THE BENEFITS OF THERAPEUTIC STRATEGIES

Considering all the results mentioned above, it would be tempting to suggest the use of BDNF as a therapeutic target for both age-related and neuropsychiatric-related cognitive dysfunction. This idea has found many difficulties to be put to practice because of the poor brain barrier penetration of BDNF and short half-life on plasma. Moreover, gene therapy and BDNF mimetic strategies came across many negative side effects that led to their abandonment (Thoenen and Sendtner, 2002). Clinically plausible alternative approaches could include a natural increase the production of endogenous BDNF (Balkowiec and Katz, 2000). In this sense, epidemiological studies

have suggested that a number of lifestyle factors such as physical exercise, diet and social activity and education may reduce the long-term risk of cognitive impairment and dementia (Larson et al., 2006; Wilson et al., 2007b; Verburgh, 2015), and animal studies are consistent with this idea (Adlard et al., 2005).

In particular, the risk of developing AD is highly increased in a lonely person (Wilson et al., 2007a), indicating that social interaction could delay the onset of the disease. Physical activity is another lifestyle factor that could influence the progress of the disease. Recent reports from both epidemiological and interventional studies reinforce the idea of using physical activity as a strategy to increase neuroplasticity in pathological conditions (Gregory et al., 2012). The influence of behaviors such as exercise and social interaction on learning and memory processes has been thoroughly studied. Researchers have found a relationship between frequent social activity and improved cognitive function (Stern, 2006). In the same direction, the cognitive improvement due to physical exercise has also been well documented (Smith et al., 2010). Physical exercise has shown not only to ameliorate structural changes in the brain, but also to protect against aging-related cognitive decline (Voss et al., 2013; Duzel et al., 2016).

Considering that lifestyle implementations have the ability to impact in the brain, a central question is how these changes in energy metabolism and social stimuli can impact on the brain structure and interact with synaptic plasticity and molecular systems to improve cognitive function.

A current model explains the effects of these lifestyle factors in terms of changes in vasculature and neurotrophic and neurotransmitter-support system (Vivar et al., 2013). Of all these changes, BDNF is the only one that is present in all the aforementioned lifestyle manipulations. BDNF is increased by social interaction with conspecifics in APP/PS1 mice, leading to the reversal of memory deficits (Hsiao et al., 2014). Also, BDNF could be important for the regulation of energy homeostasis, since diminished BDNF levels are associated with disorders of energy metabolism such as obesity and hyperglycemia (Rios et al., 2001). In fact, a high fat diet was shown to decrease BDNF levels in the hippocampus and impair learning and memory (Molteni et al., 2002a). Additionally, the increase in BDNF is one of the most consistent changes reported following exercise, as recently discussed in a meta-review (Szuhany et al., 2015). The most robust experiments supporting the fundamental role of BDNF in exercise-induced improvement in cognitive function are the ones in which blockade of BDNF impaired the cognitive improvements induced by exercise (Vaynman et al., 2004a; Garcia-Mesa et al., 2014; Kim and Leem, 2016). Vaynman et al. (2004a) showed that the exercise-induced enhancement of learning in the MWM task was blocked by TrkB-IgG administrated during the exercise period. Furthermore, exercise enabled the acquisition of sub threshold experiences (object location memory task) and this effect was dependent on BDNF. A similar effect was reported by Intlekofer and Cotman (2013) using BDNF siRNA to diminish BDNF function.

For its practicality, physical activity is the lifestyle change with more potential as a therapeutic/prevention strategy. A bulk of studies have focused on the idea of aerobic exercise as a potential non-pharmacological and low cost treatment to maintain and improve neurocognitive function (Hillman et al., 2008). A meta-analysis of several longitudinal training studies showed that exercise improved cognitive function regardless of the task type (Colcombe and Kramer, 2003). Recent studies confirmed this effect showing that not only spatial or contextual hippocampal dependent- memory tasks improve with exercise (Albeck et al., 2006; Luo et al., 2007), but also non-spatial memories such as object recognition that are thought to rely more heavily on the Prh than the HP (Hopkins and Bucci, 2010). A single session of cardiovascular exercise benefits long-term memory but does not influence short-term memory (Roig et al., 2013). Moreover, exercise can improve memory in aged animals specifically during a restricted time window after the experience, reinforcing the specific role of exercise over the memory consolidation process (Snigdha et al., 2014). Interestingly, many studies found that sex may be an important variable when evaluating the effectiveness of exercise interventions (Barha et al., 2017) and this is consistent with the sex-specific mechanisms of action of BDNF (Chan and Ye, 2017). The timing of the intervention could also be relevant. In several models of traumatic neurological injury, when interventions are given prior to the damage, induction of BDNF reduced neuronal degeneration and improved cognitive outcome (Bruce-Keller et al., 1999; Zhang et al., 2011). Although the effects of exercise are somewhat short-lived (Alaei et al., 2007; Hopkins and Bucci, 2010), some interventions can improve the outcome even when given after the damage (Griesbach et al., 2004). However, the duration of the benefits depends on the age of the subject during the exercise exposure. While adolescent exercise training did not affect BDNF levels immediately after an object recognition task, it did lead to greater BDNF levels in the Prh if the task was done 2 weeks after. In adulthood, exercise increased BDNF levels immediately after the task but this effect was short-lived and lasted less than 2 weeks (Hopkins et al., 2011). These data suggest that exercise could modulate learning related plastic changes in an age-dependent manner. Although the benefits of exercise are related to many growth factors, BDNF is the only one consistently elevated after a few weeks of continuous exercise (Molteni et al., 2002b). This neurotrophin is rapidly induced in the hippocampus and cortical regions (Cotman and Berchtold, 2002), and remains elevated for several days after exercise (Erickson et al., 2010). In addition, BDNF levels can be rapidly re-induced up to peak levels by a subsequent sub threshold exposure to exercise, even several days after the end of the exercise program (Berchtold et al., 2005).

Exercise does not influence brain regions uniformly, but affects them in a more selective way, which suggests location-specificity of the molecular pathways involved in exercise-induced plasticity. Interestingly, the effects of exercise on BDNF expression occur in regions related to mnemonic functions such as the anterior hippocampus, cerebellum and frontal cortex, but not others such as the striatum (Neeper et al., 1996). This is in accordance with previous reports indicating that exercise-induced increases in sBDNF levels are associated with changes in hippocampal volume, which, in turn, correlate with spatial memory performance (Erickson et al., 2011).

The positive effects of exercise on plasticity are particularly relevant for the aging population, in which BDNF levels are

decreased (Erickson et al., 2010). Considering that the aging brain is still capable of plasticity, lifestyle related experiences could be a way to recruit plastic processes and counteract the detrimental effects of aging (Churchill et al., 2002). In aged animals, hippocampal neurogenesis and BDNF levels can increase with exercise (Marlatt et al., 2012). Although these effects are not as robust as those seen in younger animals (van Praag et al., 2005), the increase in BDNF seems to ameliorate mental deterioration and improve memory function (Erickson et al., 2012). In fact, long-term exercise programs are able to rescue these cognitive deficits even after the first signs of mnemonic impairment (Tsai et al., 2018). A recent clinical trial examined the impact of aerobic cardiorespiratory training versus stretching on MCI patients and reported sex-dependent cognitive improvements related with trophic factor and Ab-40 and Ab-42 circulating levels (Baker et al., 2010).

Physical activity is also associated with a lower risk of developing dementia (Friedland et al., 2001). Many clinical trials point toward improved cognition and reduced incidence of psychiatric symptoms when patients with mild AD received a physical training protocol (Hoffmann et al., 2016; Cammisuli et al., 2018). However, some studies suggest that environmental enrichment could be more beneficial for cognition than physical exercise alone (Wolf et al., 2006; Cracchiolo et al., 2007).

Coordinative exercise (Niemann et al., 2014) and cognitive training (Basak et al., 2008; Hall et al., 2009) can also induce gray matter plasticity and enhance cognitive functions in older adults. However, the improvement generated by prior training is usually domain-restrictive and only acts over memory systems affected by the previous experience (Markowska and Savonenko, 2002; Green and Bavelier, 2008). Nonetheless, there are some reports that show a generalized benefit of prior experience to different tasks and contexts (Buschkuehl et al., 2008). A combination of physical and cognitive training with control of nutritional and cardiovascular risk factors during a 2-year period led to improved cognitive performance in old adults at risk of developing dementia (Ngandu et al., 2015). In animal studies, environmental enrichment could be seen as a multidomain intervention. It consists of social enrichment, physical exercise and environmental changes and has been shown to increase BDNF levels and enhance learning and memory in different domains such as object recognition, spatial learning and motor abilities (Greenough et al., 1972; Frick and Benoit, 2010). The combination of both sensory enrichment and physical activity has more impact on neuronal plasticity than these elements given independently. This motivated original therapeutical proposals in human research. For example, a novel dancing program with higher cognitive and coordinative demands than previous physical activity programs induced more gray matter increases in an aged group than a traditional sport program with comparable cardiovascular demands (Muller et al., 2017; Rehfeld et al., 2018).

One important question regarding the effects of exercise on cognitive function is to establish the mechanisms responsible for the cross talk between cardiovascular/muscle activity and the central nervous system. The muscle higher metabolic rates could lead to the secretion of signaling molecules, that could subsequently upregulate plasticity related gene expression and protect the brain from damage. In this way, elevated plasticity molecules such as BDNF could prime the brain to be better prepared for subsequent changes related to learning or could be selectively secreted in an activity-dependent manner during learning experiences. As a result, exercise could enhance the activity of a general molecular machinery important for learning and memory. In accordance with this view, molecules such as CREB, NMDARs subunits and BDNF are particularly induced following exercise (Molteni et al., 2002b) and brain regions important for memory formation such as the hippocampus are selectively influenced by physical activity (Vaynman et al., 2004b).

A potential mechanism for the exercise-related neuroprotective effects of BDNF is via modulation of synaptic and structural plasticity. Plastic changes induced by exercise include increased neurogenesis (van Praag et al., 1999b; Merkley et al., 2014), greater arborization of neuronal dendrites and synaptogenesis (Eadie et al., 2005; Dietrich et al., 2008), as well as increased amplitude and reduced threshold for LTP (van Praag et al., 1999a). Since these effects are accompanied by a concurrent increase in BDNF levels (Ding et al., 2006; Ferris et al., 2007), BDNF could be a potential mediator. In addition, increased vascularization (Morland et al., 2017) accompanied by greater dendritic complexity and neurogenesis could explain the increase in hippocampal volume following exercise (Erickson et al., 2011). The progressive age-related decline in neurogenesis has been associated with a non-permissive microenvironment with low levels of neurogenesis-promoting factors. However, this microenvironment is still responsive to environmental changes and can be stimulated even at late stages to provide molecular cues for proliferation (van Praag et al., 2005; Kronenberg et al., 2006; Lugert et al., 2010; Silva-Vargas et al., 2013; Smith et al., 2018). Changes in growth factor levels such as BDNF might underlie the decrease in neurogenesis seen as a consequence of disease or aging, and the aged brain retains the capacity to respond to the neurogenesis-stimulating effects of growth factors.

Exogenous application of BDNF can restore the levels of hippocampal neurogenesis in aged animals (Scharfman and Maclusky, 2005). In the same way, exercise-induced increases in neurogenesis are necessary for the physical activity-dependent enhancement in learning and memory (Clark et al., 2008). This led to the idea that neurogenesis could be the substrate of this cognitive enhancements mediated by BDNF (Bekinschtein et al., 2011).

Another potential beneficial effect of BDNF is its ability to protect neurons from oxidative damage or excitotoxic stress (Cheng and Mattson, 1994; Wu et al., 2004) and from Aβ-induced degeneration (Counts and Mufson, 2010) in animal models of normal and pathological aging. In fact, BDNF is upregulated in response to different kinds of insults to the nervous system (Hsu et al., 1993; Yang et al., 1996; Hayashi et al., 2000). An exercise regime can lessen the accumulation of oxidative cell damage and the dysfunction characteristic of aged animals (Radak et al., 2001), and selective suppression of BDNF increases the vulnerability of neurons to excitotoxicity (Jiang et al., 2005) and increases amyloidogenesis (Matrone et al., 2008).

Although there should be a clear excitement to establish a systematic exercise program to ameliorate or even prevent symptoms of memory deficits related to aging, psychiatric disorders or diseases, many limitations still exist for this therapeutic line. One of the most important drawbacks of this approach is that the high prevalence of chronic diseases in the aged population affects exercise performance and feasibility, increasing the potential risks of the treatment, especially for high intensity protocols (Hundley et al., 2001). This directly impacts on the motivation to follow these kinds of treatments, which are known to have low adherence (Kosse et al., 2013). Programs with lower intensities could be a better choice, since they are still able to impact positively on cognition and neurophysiology in aged subjects without affecting adherence to the treatment (van der Bij et al., 2002). It has been reported that the lack of time is also one of the main reasons for avoiding regular practice of aerobic exercise (Gillen and Gibala, 2014). Thus, there is interest in developing more efficient training programs involving less time demand but inducing a similar BDNF response. Pietrelli et al. (2018) demonstrated, in animals, that the practice of low/moderate intensity aerobic exercise through 2 to 18 months of life increased BDNF in various brain structures like the prefrontal cortex and the hippocampus and reduces the normal decline due aging. Moreover, this protocol improved both novel object recognition and context discrimination capacity. Szuhany et al. (2015) conducted a meta-analysis to determine the impact of acute and regular exercise on BDNF levels in humans. They found that the moderate effect from a single session of exercise was intensified if it was executed after a regular program of exercise. They posit that each episode of exercise results in a "dose" of BDNF activity and that the magnitude of this "dose" can be enhanced over time by regular exercise.

On the other side, attempts to directly use recombinant BDNF as a therapy have found many methodological limitations. One of the main problems for translating BDNF-based therapies into the clinic is problem of delivery to the brain and the challenge of sustaining the expression for longer intervals since since the recombinant protein has a very short half-life. In this sense, some preclinical studies were oriented to using BDNF fused to cell-penetrating peptides and packed in AAV-constructs and intranasal delivery of these AAV constructs to central nervous system (Arregui et al., 2011; Ma et al., 2016). A10-day-AAV treatment could alleviate depression-like behaviors in mice (Ma et al., 2016), and AAV delivery of BDNF striatal neurons induced neurogenesis and increased the lifespan of an animal model of HD (Benraiss et al., 2012). Although beneficial, the use of AAV has been limited by difficulties in biodistribution and the immunogenicity to the virus. An alternative approach is the use of stem cell transplants that can express BDNF and other beneficial factors and can migrate into damaged areas of the brain by their selective tropism to inflammation and apoptosis sites, although they are not permanently integrated into the organism (Kidd et al., 2009; Joyce et al., 2010; Fink et al., 2015; Deng et al., 2016; Pollock et al., 2016). Mesenchymal stem cell transplantations have shown improvements in behavioral deficits in a murine model of HD, have also slowed the neurodegenerative processes by a diminished atrophy and an increased neurogenesis (Dey et al., 2010; Benraiss et al., 2013; Pollock et al., 2016).

An alternative possibility to the use of exercise as a 'natural' and non-invasive way of increasing BDNF signaling in neuronal networks, is the administration of drugs already available for clinical use (Stranahan et al., 2009). Many medications are capable of impacting on BDNF levels. Memantine and donepezil are a pharmaceutical compound used to alleviate the symptoms of AD that markedly increases BDNF levels in a dose-dependent manner (Marvanova et al., 2001; Leyhe et al., 2008; Meisner et al., 2008).

Ampakines are good candidates because they can increase excitatory transmission affecting BDNF levels. In rat hippocampal slice cultures, a very brief clinically tested ampakine treatment produced elevated BDNF protein levels that lasted several days after the exposure (Lauterborn et al., 2003). Another therapeutic possibility that has emerged recently is the use of transcranial magnetic stimulation (TMS) to increase BDNF levels. TMS restored the levels of BDNF and TrkB that were normally reduced in aged mice and improved spatial memory (Zhang et al., 2015).

In conclusion, several environmental and lifestyle interventions that reduce age-dependent cognitive decline and pathological degeneration can also increase BDNF production, suggesting that BDNF is neuroprotective (**Figure 1**). Given that cognitive training is a focused approach that selectively acts on sets of memory domains and that drugs are invasive, exercise is still a 'favorite' when thinking of potential therapeutic approaches.

CONCLUSION

Although BDNF is a key player in synaptic plasticity and memory process, its role in the etiology of cognitive symptoms in pathological conditions remains unclear. BDNF-mediated plastic changes have been proposed as one of the underlying neurobiological substrates of memory consolidation, and changes in BDNF were shown to directly affect memory performance in animal models of neurodegenerative/neuropsychiatric diseases and in normal conditions. In human post-mortem brain tissue, BDNF expression is higher in memory related structures, such as the hippocampus and the amygdala. However, in humans, the relationship between memory and BDNF still remains correlational, mainly because the available techniques do not allow the control of BDNF expression in humans. Despite this difficulty, numerous studies attempted to establish causal relationships between these two factors by analyzing memory performance under conditions that up-regulate or down-regulate BDNF expression. Since brain BDNF expression correlates with sBDNF concentration, this association has been extensively used to study the implication of BDNF in mnemonic functions in humans under normal and pathological conditions. In fact, the concentration of this blood-measurable protein is correlated with the memory impairment in different disorders. Additionally, changes in the trafficking and release of BDNF due to Val66Met polymorphism have also been used to correlate BDNF levels

with mnemonic performance and structural changes in memory-related regions in healthy and diseased individuals. It has been shown that interventions such as exercise, chronic administration of fluoxetine, and cognitive training can enhance the sBDNF concentration and correlates with a better performance in memory tasks. The initial idea of using BDNF as a biomarker for neurodegenerative/neuropsychiatric diseases was discarded because changes in BDNF levels are common to many pathological conditions, which underscores its discriminative value and potency. However, considering the data reviewed here, we suggest that BDNF can be thought of as a marker that specifically relates to the occurrence and/or progression of the mnemonic symptoms that are common to many pathological conditions that share deficits in this cognitive domain. Moreover, BDNF was shown to be a shared factor in which converge most of the therapies that have been able to fight these mnemonic symptoms.

AUTHOR CONTRIBUTIONS

MM was responsible for drafting the manuscript and revising its content. JM and MZ contributed to writing the manuscript and figure design. PB was responsible for the general idea, writing and critically revising, and correcting the manuscript. All authors read and approved the final manuscript, and contributed to the conception of the work.

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Differential Hippocampal Expression of BDNF Isoforms and Their Receptors Under Diverse Configurations of the Serotonergic System in a Mice Model of Increased Neuronal Survival

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Neurotrophic factors are relevant regulators of the neurogenic process at different levels. In particular, the brain-derived neurotrophic factor, BDNF, is highly expressed in the hippocampus (HC) of rodents and participates in the control of neuronal proliferation, and survival in the dentate gyrus (DG). Likewise, serotonin is also involved in the regulation of neurogenesis, though its role is apparently more complex. Indeed, both enhancement of serotonin neurotransmission as well as serotonin depletion, paradoxically increase neuronal survival in the HC of mice. In this study, we analyzed the protein expression of the BDNF isoforms, i.e., pro- and mature-BDNF, and their respective receptors p75 and TrkB, in the HC of mice chronically treated with para-chloro-phenyl-alanine (PCPA), an inhibitor of serotonin synthesis. The same analysis was conducted in hyposerotonergic mice with concomitant administration of the 5-HT_{1A} receptor agonist, 8-Hydroxy-2-(di-n- propylamino) tetralin (8-OH-DPAT). Increased expression of p75 receptor with decreased expression of pro-BDNF was observed after chronic PCPA. Seven-day treatment with 8-OH-DPAT reestablished the expression of pro-BDNF modified by PCPA, and induced an increase in the expression of p75 receptor. It has been demonstrated that PCPA-treated mice have higher number of immature neurons in the HC. Given that immature neurons participate in the pattern separation process, the object pattern separation test was conducted. A better performance of hyposerotonergic mice was not confirmed in this assay. Altogether, our results show that molecules in the BDNF signaling pathway are differentially expressed under diverse configurations of the serotonergic system, allowing for fine-tuning of the neurogenic process.

Keywords: BDNF signaling pathway, serotonin depletion, 5-HT_{1A} receptor, mice model, newborn neurons survival, pattern separation

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INTRODUCTION

With the extension of life expectancy, several pathologies affecting the central nervous system have gained greater visibility. In this context, disentangling the etiopathogenesis of neurodegenerative diseases and seeking for more effective therapies represent key challenges. Strategies to replace lost neurons because of neurodegenerative processes are focused on two types of interventions: on the one hand, transplantation of exogenous nerve cells and, on the other, the use of the endogenous neural niches still active in the adult nervous system (Boda et al., 2017). Indeed, neurogenic potential has been specifically characterized in the hippocampus (HC) and the subventricular zone of adult mammal brains (Gage, 2000). However, a thorough knowledge of the molecular mechanisms controlling neurogenesis and its possible manipulation in pathological conditions is required in view of its therapeutic use. In this sense, neurotrophic factors have recently emerged as regulators of the neurogenic process at different levels (see review in Vilar and Mira, 2016). In particular, the brain-derived neurotrophic factor, BDNF, is highly expressed in the HC of rodents (Aid et al., 2007), and its role in the regulation of the proliferation and survival of newborn neurons in the dentate gyrus (DG) has been extensively studied (see review in Foltran and Diaz, 2016). BDNF is secreted as pro-BDNF, a proneurotrophin which can be converted to mature BDNF (mBDNF). Pro-BDNF acting through the p75 receptor is responsible for pro-apoptotic actions, whereas mBDNF-TrkB receptors complex mediates pro-neurogenic effects. Globally, the actions of both BDNF isoforms contribute to the regulation of neuronal homeostasis.

At the beginning of this century, an original report showed that chronic administration of serotonin selective reuptake inhibitors antidepressants is able to induce neurogenesis in the HC of adult rats (Malberg et al., 2000). Since this pivotal discovery, the neurogenic actions of serotonergic drugs were extensively proved in the adult HC of mice and even in humans (see review in Kempermann et al., 2018). However, the specific role of serotonin is still unclear, since increased neuronal survival is also described in the HC of diverse mice models of constitutive (Diaz et al., 2013; Karabeg et al., 2013; Sachs et al., 2013; Song et al., 2017) as well as induced (Jia et al., 2014; Song et al., 2016) serotonin depletion. Particularly, we have previously reported increased survival of 1 week- and 4 week- old newborn neurons in the DG of mice chronically treated with PCPA during 5 and 8 weeks, respectively (Diaz et al., 2013). These paradoxical effects, i.e., supernumerary newborn neurons in the DG of mice with either increased or decreased serotonergic neurotransmission, could be partially explained by the participation of one or several of the serotonin receptors subtypes. Indeed, stimulation of 5-HT_{1A} receptor for 7 days was enough to reestablish the increased survival of 1-week-old neurons shown in hyposerotonergic mice, induced either by genetic, or pharmacological ablation (Diaz et al., 2013).

Immature neurons in the DG appear to be responsible for enhanced pattern separation, i.e., the ability to discriminate between two very similar but different spatial contexts (Sahay et al., 2011; Nakashiba et al., 2012). Nevertheless, we have shown that hyposerotonergic Pet1$^{-/-}$ mice, displaying increased number of immature neurons in the DG, and have a normal performance in contextual fear-discrimination learning (Diaz et al., 2013). A recently developed test, called object pattern separation (van Hagen et al., 2015), provides the opportunity to measure both deterioration, and improvement in the ability of pattern separation. It is based on the natural tendency of rodents to explore an object that is new to them, so they do not have to be trained for a special skill and it is not highly stressful to the animals. Likewise, its highest resolution allows finding subtle differences, which may shed light in the search for the specific role of immature neurons in hyposerotonergic mice models.

We analyze here the protein expression of the BDNF signaling pathway in the HC of hyposerotonergic mice, revealing changes in the expression of p75 receptor and pro-BDNF. Likewise, 7-day treatment with a 5-HT_{1A} receptor agonist to these mice partially reversed changes induced by serotonin depletion. Finally, hyposerotonergic mice did not performed better than control mice in the objet pattern separation test.

MATERIALS AND METHODS

Animals

Studies were performed on 102 male, C57BL/6 elite mice purchased at the Instituto de Medicina Experimental, Academia Nacional de Medicina, Buenos Aires, Argentina. Experiments on animals were conducted according to local regulations and were approved by the Institutional Ethical Committee (UBA-FMED, Resol. 2016/1637 and 2019/297). Three-four week-old mice, bred in barrier-conditions to maintain an SPF status, were transported in environmentally controlled conditions to our institute's animal facility. After arrival, mice were housed in 1284L Eurostandard Type II Long (365 mm × 207 mm × 140 mm) Tecniplast microisolator cages with filter tops (five to seven animals per cage), with autoclavated aspen shavings as bedding and tissue paper as nesting material. Mice were maintained under controlled conditions, i.e., 22 ± 2°C room temperature, 60% relative humidity, 12–12 h light–dark cycle (lights on at 8 a.m.), pelleted food for rodents (Cooperación) and water *ad libitum*. Cages were changed twice a week. A period of acclimation of 2 weeks was left before the beginning of experiments, and therefore, mice were 5–6 week-old when treatments begun.

Serotonin Depletion Protocol

Serotonin depletion in mice was induced by oral administration of para-chloro-phenyl-alanine (PCPA), an inhibitor of the rate limiting enzime tryptophane hydroxylase, during 8 weeks, as previously described (Foltran et al., 2019). Briefly, PCPA was suspended in a 0,5% carboxymethyl cellulose solution and mixed in gelatin palatable cubes. Mice were divided into two groups. One group received cubes with PCPA at an estimated dose of 500 mg/kg on days 1 and 2, and 250 mg/kg PCPA for the rest of the treatment (69 mg/cube, and 34.5 mg/cube respectively, per cage with 6 mice). The control group received similar cubes with vehicle. A PCPA or vehicle containing cube was given per cage,

every day between 2 p.m. and 5 p.m. Serotonin depletion in the HC (75%) and cortex (60%) of mice was confirmed by HPLC as soon as 10 days after the beginning of PCPA administration (Foltran et al., 2019).

Experiment 1: Expression of the BDNF Signaling Pathway

Chronic PCPA treatment during 8 weeks increases survival of 4 week-old neurons in the HC (Diaz et al., 2013). To study the potential involvement of the BDNF signaling pathway in these neurogenic effects, the protein expression of BDNF isoforms and their receptors was studied by western blotting. After 8 week of PCPA ($n = 6$) or vehicle ($n = 5$) administration, mice were killed by cervical dislocation and both HC were obtained. Tissue was homogenized with 250 μl of RIPA buffer along with protease inhibitors (150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris) and centrifuged at 4°C for 30 min at 13000 (r/min). The supernatants were recovered and proteins levels were quantified by the Bradford protein assay. Samples (50 μgr in 5× loading buffer) were then loaded into SDS-PAGE gels (12 or 15%) and transferred onto nitrocellulose membranes using the Mini-PROTEAN®Tetra System (BIO-RAD) for 1 h.

Western Blotting

Membranes were incubated for 1 h with blocking solution (5% milk in TBST) and then probed overnight at 4°C with mouse anti-BDNF (1:2000; Icosagen; 327-100 clone 3C11), rabbit anti-p75 (1:700; Alomone Labs; ANT-007), rabbit anti-TrkB (1:700; Alomone Labs; ANT-019), and rabbit anti-proBDNF (1:250; Abcam; ab72440) in TBST. β-III Tubulin was used as a loading control (1:2500; R&D Systems). Binding of primary antibodies was visualized with anti-mouse HRP-conjugated secondary antibody (1:3000; BIO-RAD) or anti-rabbit HRP-conjugated secondary antibody (1:3000; BIO-RAD). Membranes were developed using the ECL Plus Western blotting substrate (Thermo Fisher Scientific) for chemifluorescence with the Storm®Molecular Imager. Densitometry was carried out using ImageJ software (Schneider et al., 2012). The signal of each protein is expressed after subtraction of background signal and related to tubulin signal.

Experiment 2: Chronic Treatment With the 5-HT_{1A} Agonist, 8-OH-DPAT

We have previously reported that chronic PCPA induced increased survival of 1 week- and 4 week-old newborn neurons corresponding to 5 and 8 weeks of PCPA administration, respectively (Diaz et al., 2013). Also we have shown that stimulation of the 5-HT_{1A} receptor for 7 days is able to reestablish the basal level of survival of 1 week-old newborn neurons in the HC of PCPA-chronically treated mice (Diaz et al., 2013). To study the role of this serotonin receptor on the BDNF signaling pathway of hyposerotonergic mice, the 5-HT_{1A} receptor agonist 8-Hydroxy-2-(di-n- propylamino)tetralin (8-OH-DPAT) was administered to a group of PCPA- ($n = 11$) or vehicle- ($n = 12$) treated mice. Mice received palatable cubes with PCPA or vehicle for 4 weeks, as explained above. On the 5th week, animals continued receiving PCPA or vehicle, and simultaneously received two daily intraperitoneal (i.p.) injections of 8-OH-DPAT at 0.5 mg/kg or vehicle (NaCl 0.9%) for 7 days as previously described (Diaz et al., 2013). Mice were killed by cervical dislocation on the day of the last injection and HC were collected. Western blotting was performed as already described.

Experiment 3: Object Pattern Separation (OPS) Test in Hyposerotonergic Mice

For an initial set up of the object pattern separation (OPS) test in our experimental conditions, the protocol described by van Hagen et al. (2015), was followed with few modifications. A 27 cm diameter-round open field with red walls was employed (**Figure 3A**). This arena was placed inside a bigger black square open field with visual cues fixed in each wall. Briefly, mice were divided into 5 groups ($n = 8$/experimental group) and habituated for 3 days to the open field without any objects. They were placed in the arena and left for 10 min to explore. Mice were then tested 48 hs after the last habituation session. First, in a pre-test session, they were presented two identical objects placed in two symmetrical spots in the center of the arena. The objects were caramel color bottles of 7 cm of height filled with a dark solution so that mice were not able to move them. Animals were left 4 min to explore. The test session was performed 1 h later, where one of the objects was placed in a novel location inside the arena 2, 4, 6, or 8 cm away from the initial location (positions 2, 3, 4, and 5, respectively). Position 1 meant no change in any object. Each group was assigned a different position. Mice were left again 4 min to explore. Time spent exploring each object in both sessions was recorded. Exploration was defined as directing the nose toward the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered as exploratory behavior. Mice that spent less than 10 s in total exploratory time in the pre-test or test were eliminated from the data, since it has been shown that animals require at least 10 s of object interaction for reliable object discrimination (şik et al., 2003; Akkerman et al., 2012). Exploration time was measured manually in a computer with the Solomon Coder program. A discriminatory index was calculated, as "(time spent exploring the moved object – time spent exploring the stationary object)/total exploratory time." According to the results of the set up, the position 3 was chosen to test treated mice.

To study the effects of PCPA treatment on the process of pattern separation, mice were divided in two groups (n = 13–15), and received a palatable cube containing either PCPA or vehicle during 8 weeks, as described above. PCPA was administered as a tool to increase the survival of newborn neurons in the HC (Diaz et al., 2013). The OPS test was conducted as described above, on the 7th week of PCPA or vehicle treatment. All habituation and test sessions were recorded, and videos were analyzed to measure exploratory activity. Distance moved by mice in the habituation videos were used to quantify locomotor activity, via EthoVision XT 14. Time spent exploring each object in both, the pre-test or test

sessions, was quantified to calculate the discriminatory index as already explained.

Chemical Substances

PCPA (4-Chloro-DL-phenylalanine): Sigma, C6506; 8-OH-DPAT (8-Hydroxy-2-(dipropylamino) tetralin hydrobromide): Sigma, H8520.

Statistical Analysis

All data were checked to verify whether normality and homoscedasticity assumptions were met. Different treatments in Experiment 1 were analyzed using Student's *t*-test. Results in Experiment 2 were analyzed by a two-way analysis of variance (ANOVA), with hyposerotonergic condition, and DPAT treatment as factors. Tukey's test was used for *post hoc* comparisons. Simple effects tests were performed to analyze differences inside each condition. The OPS was analyzed using the one-sample t-statistics in order to assess whether the discrimination index, for each experimental condition separately, differed significantly from 0. In all cases, $P < 0.05$ was considered statistically significant. A summary of tests employed and statistics is presented in **Table 1**.

RESULTS

Experiment 1: Expression of the BDNF Signaling Pathway

Protein-expression of BDNF isoforms and their receptors was analyzed in the HC of 8-week PCPA- or vehicle-treated mice (**Figures 1A–H**; see statistics in **Table 1**). Whereas no significant differences were found between experimental groups for mBDNF, its receptor TrkB, and proBDNF, expression of the proBDNF receptor, p75, was significantly increased ($p = 0.0427$) in PCPA-treated mice.

Experiment 2: Chronic Treatment With the 5-HT$_{1A}$ Agonist, 8-OH-DPAT

Protein expression of the BDNF isoforms and their receptors was analyzed in the HC of PCPA- and vehicle- chronically treated mice receiving the 5-HT$_{1A}$ agonist DPAT or NaCl 0.9% (**Figures 2A–E**). Statistical analysis showed a significant interaction between hyposerotonergic condition and DPAT treatment for TrkB, proBDNF, and p75 receptor (for statistics, see **Table 1**). Five weeks of PCPA administration induced a decreased expression of proBDNF ($p = 0.0085$), and a tendency to increased expression of p75 receptor ($p = 0.0931$), without significantly modifying the expression of mBDNF or TrkB receptor. One week administration of DPAT in vehicle-treated mice induced a significant decrease in the expression of mBDNF ($p = 0.0403$) and TrkB receptor ($p = 0.0004$), without significantly affecting the levels of proBDNF or p75 receptor. Finally, administration of DPAT to PCPA-treated mice induced a significant increase in the expression of proBDNF ($p = 0.0035$) and p75 receptor ($p = 0.0239$), without significantly modifying the levels of mBDNF and TrkB receptor.

Experiment 3: Object Pattern Separation (OPS) Test

In the set up, each mouse was assigned to a different position for the moved object, identified as P1, P2, P3, P4, or P5 (**Figure 3A**). As the objects were more separated from the original position, the discrimination index became higher and increasingly different from 0 (**Figure 3B**). Although results at P4 were unexpected, P5 resulted in an index significantly different from 0 ($p = 0.0088$), whereas P3 showed a tendency for significance ($p = 0.1203$). Altogether, as naïve mice are barely able to discriminate positions in P3, the performance in this position can be improved, which would result in a higher index and possibly, a p value <0.05. If mice were tested in P5, where the discrimination index is high, a difference between treated and control mice would not be seen, as all of them are expected to perform well.

To study the role of newborn cells in the HC of mice treated with PCPA or vehicle, animals were assayed in the OPS at P3, given that subtle differences, i.e., a mild better performance, between experimental groups were expected. Mice were habituated to the open field for 3 consecutive days, for 10 min each day. All mice got habituated to the arena (**Figure 3C**), with less distance traveled on each passing day ($p < 0.05$). No significant difference in locomotor activity was seen between mice treated with PCPA and vehicle. After a resting day, mice were presented with two identical bottles for 4 min at P1. One hour later, one of the bottles was moved to P3 and the animals were left another 4 min to explore. Five mice were eliminated from the data because they did not meet the criteria for minimal exploration time of 10 s. A discrimination index was calculated, as previously explained (**Figure 3D**). Whereas the index in control mice was significantly different from 0 ($p = 0.0435$), PCPA-treated mice showed a high p value ($p = 0.6736$).

DISCUSSION

Previous reports on hyposerotonergic mice demonstrated increased survival of the newborn neurons generated in the adult HC, with unchanged cell proliferation. The results presented herein support the hypothesis about an interplay between the BDNF signaling pathway in the HC and the serotonergic system that could participate in the regulation of the survival of newborn neurons. On the other hand, in our experimental conditions, a facilitating role for supernumerary immature neurons on pattern separation assays could not be confirmed.

Interestingly, a recent study, found increased mBDNF expression in the prefrontal cortex of 1 month-old Tph2-deficient rats (Brivio et al., 2018). Although this result observed in serotonin-deficient rats was not reproduced in our mice model, it is not completely unexpected. Indeed, several studies have analyzed the effects of serotonin depletion on the neurogenic process in rats and mice, revealing a clear dichotomy: whereas lack of serotonin induces decreased survival of newborn neurons in the DG of rats (Brezun and Daszuta, 1999, 2000;

TABLE 1 | *P* values.

Paradigm or assay	Parameter measured	Statistical test	Comparison		Statistics	Degree of freedom	*P* value	Figure
Western blot	mBDNF signal relative to tubulin	Student's *t* test	Treatment		$T = 1.056$	21	0,3032	**1A**
Western blot	TrkB signal relative to tubulin	Student's *t* test	Treatment		$T = 0.9418$	21	0,3570	**1B**
Western blot	proBDNF signal relative to tubulin	Student's *t* test	Treatment		$T = 1.577$	18	0,1304	**1C**
Western blot	P75 signal relative to tubulin	Student's *t* test	Treatment		$T = 2.157$	21	0,0427	**1D**
Western blot	mBDNF signal relative to tubulin	Two-way ANOVA	Interaction		$F = 3,38$	1	0,0817	**2A**
			Factor food		$F = 13,19$	1	0,0018	
			Factor injection		$F = 1,3^{E-03}$	1	0,9714	
		Tukey post-test	Control – DPAT				A	
			Control – NaCl				AB	
			PCPA – NaCl				AB	
			PCPA – DPAT				B	
		Simple effects	Inside food	Ctrl	$F = 5,73$	1	0,0403	
				PCPA	$F = 1,11$	1	0,3161	
			Inside Inject	NaCl	$F = 1,17$	1	0,3039	
				DPAT	$F = 27,71$	1	0,0005	
Western blot	TrkB signal relative to tubulin	Two-way ANOVA	Interaction		$F = 5,34$	1	0,0322	**2B**
			Factor Food		$F = 1,45$	1	0,2433	
			Factor Injection		$F = 19,36$	1	0,0003	
		Tukey post-test	Control – DPAT				A	
			Control – NaCl				A	
			PCPA – NaCl				AB	
			PCPA – DPAT				B	
		Simple effects	Inside food	Ctrl	$F = 29,03$	1	0,0004	
				PCPA	$F = 1,86$	1	0,2027	
			Inside inject	NaCl	$F = 4,23$	1	0,0668	
				DPAT	$F = 1,44$	1	0,2614	
Western blot	proBDNF signal relative to tubulin	Two-way ANOVA	Interaction		$F = 4,63$	1	0,0453	**2C**
			Factor food		$F = 0,43$	1	0,5214	
			Factor injection		$F = 11,59$	1	0,0032	
		Tukey post-test	Control – DPAT				A	
			Control – NaCl				AB	
			PCPA – NaCl				B	
			PCPA – DPAT				B	
		Simple effects	Inside food	Ctrl	$F = 0,79$	1	0,3973	
				PCPA	$F = 15,36$	1	0,0035	
			Inside inject	NaCl	$F = 11,25$	1	0,0085	
				DPAT	F = 0,68	1	0,4314	
Western blot	P75 signal relative to tubulin	Two-way ANOVA	Interaction		$F = 5,63$	1	0,0284	**2D**
			Factor food		$F = 22,70$	1	0,0001	
			Factor Injection		$F = 6,79$	1	0,0174	
		Tukey post-test	Control – DPAT				A	
			Control – NaCl				A	
			PCPA – NaCl				A	
			PCPA – DPAT				B	

(Continued)

TABLE 1 | Continued

Paradigm or assay	Parameter measured	Statistical test	Comparison		Statistics	Degree of freedom	*P* value	Figure
		Simple effects	Inside food	Ctrl	$F = 0,39$	1	0,5469	
				PCPA	$F = 7,07$	1	0,0239	
			Inside inject	NaCl	$F = 3,45$	1	0,0931	
				DPAT	$F = 21,28$	1	0,0013	
Object pattern separation	Discrimination index	One sample *t* test	P1 vs. 0		$T = -0,03$	–	0,9810	**3B**
			P2 vs. 0		$T = 0,30$	–	0,7706	
			P3 vs. 0		$T = 1,77$	–	0,1203	
			P4 vs. 0		$T = 1,03$	–	0,3618	
			P5 vs. 0		$T = 3,82$	–	0,0088	
Object pattern separation	discrimination index	One-way ANOVA	Between positions		$F = 1,81$	4	0,1544	**3B**
Object pattern separation	Habituation	Two-way ANOVA	Interaction		$F = 0,14$	2	0,8694	**3C**
			Factor treatment		$F = 1,05$	1	0,3131	
			Factor Day		$F = 17,98$	2	<0,0001	
		Tukey post-test	Day 1 vs. Day 2				<0,05	
			Day 1 vs. Day 3				<0,05	
			Day 2 vs. Day 3				ns	
Object pattern separation	Discrimination index	One sample *t* test	Control vs. 0		$T = 2,18$	–	0,0435	**3D**
			PCPA vs. 0		$T = 0,43$	–	0,6736	

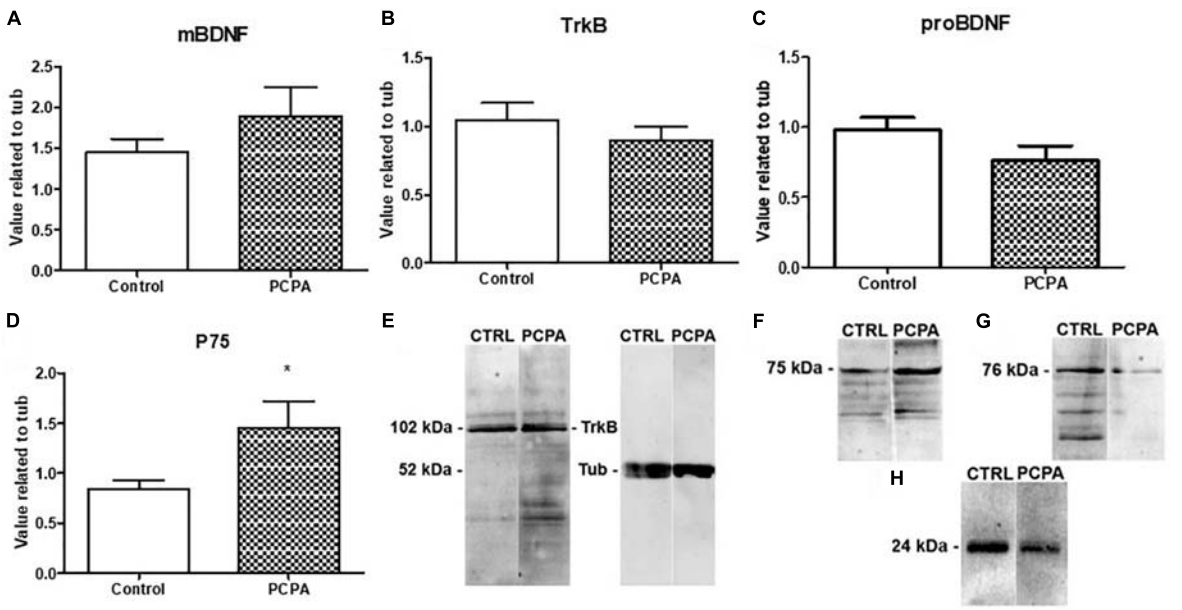

FIGURE 1 | Protein expression of molecules from the BDNF signaling pathway determined by Western Blot and representative membranes. **(A)** mBDNF. **(B)** TrkB. **(C)** proBDNF. **(D)** p75 in hyposerotonergic (PCPA-treated) or control (vehicle-treated) mice for 4 weeks. **(E–H)** Representative membrane showing signal for TrkB, p75, pro-BDNF, and mBDNF in Control and PCPA-treatd mice. Data are expressed as mean ± S.E.M., $n = 11$ (control) and 12 (PCPA). $^*p < 0.05$.

Banasr et al., 2001; Jha et al., 2006; Kohl et al., 2016), the opposite effect has been demonstrated in serotonin-depleted mice (Diaz et al., 2013; Karabeg et al., 2013; Jia et al., 2014; Song et al., 2016, 2017). Therefore, caution is required when comparing neurogenic effects in these two rodent species. Indeed, there are few reports conducted in hyposerotonergic mice. In the present study, a mice model in which serotonin depletion was pharmacologically induced, after inhibition of the Tph rate-limiting enzyme, was employed. This pharmacological model, allows us to specifically target the process of adult neurogenesis,

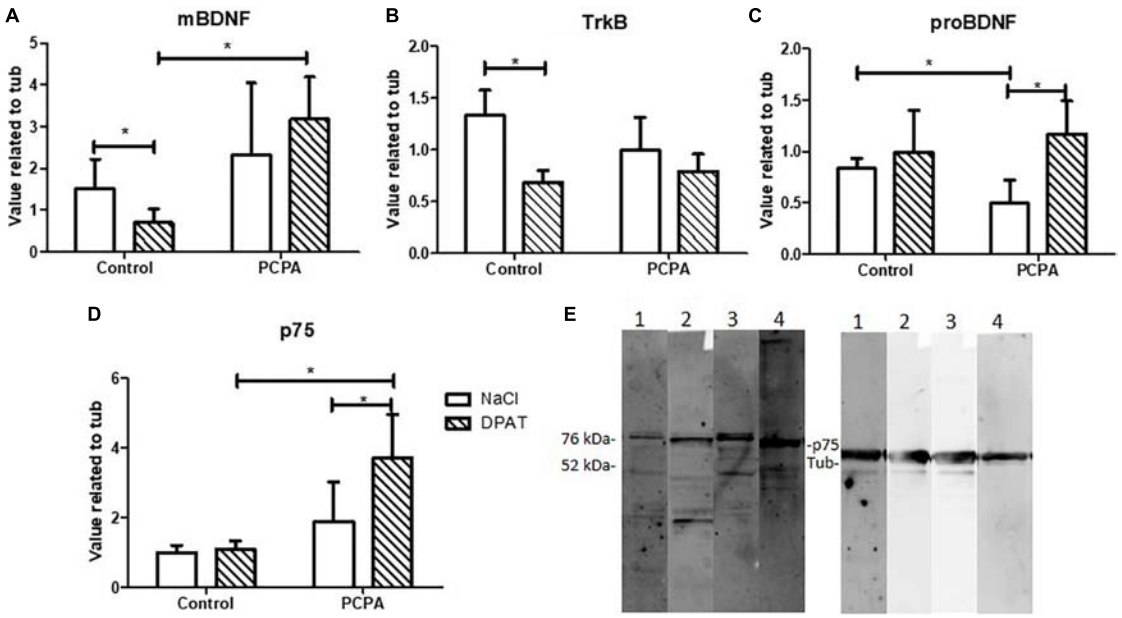

FIGURE 2 | Effect of 8-OH-DPAT on the protein expression of molecules from the BDNF signaling pathway in the HC, determined by Western Blot. **(A)** mBDNF. **(B)**TrkB. **(C)** proBDNF. **(D)** p75 in hyposerotonergic (PCPA-treated) or control (vehicle-treated) mice receiving the 5-HT_{1A} agonist 8-OH-DPAT (striped bars) or NaCl (white bars). **(E)** Representative membrane showing signal for p75 (75 kDa) and tubuline (52 kDa). 1: Control-NaCl. 2: Control-DPAT. 3: PCPA-NaCl. 4: PCPA-DPAT. Data are expressed as mean ± S.E.M., $n = 6$ per experimental group.*$p < 0.05$.

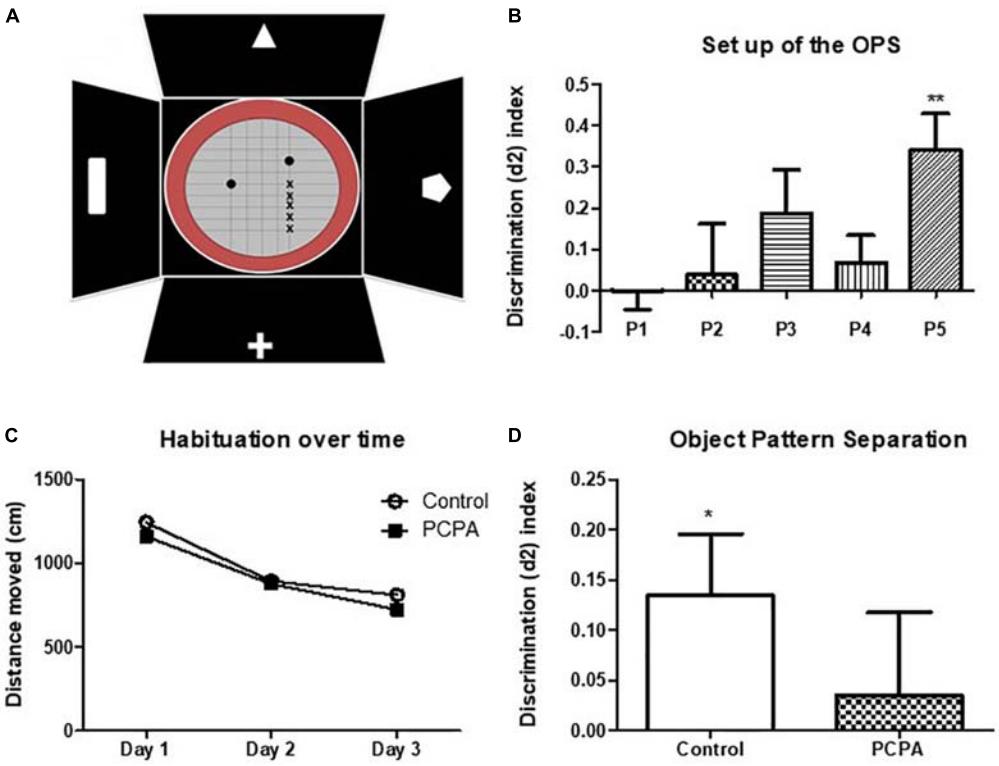

FIGURE 3 | Object pattern separation (OPS) test. **(A)** Scheme of the arena and objects used for the test. The *x* represents the different possible positions for the moved object. Full circles represent the bottles used as objects. **(B)** Discrimination index for the different new positions for the moved object in naive mice. **(C)** Locomotor activity in an open field along 3 days. **(D)** Discrimination index in the OPS of mice chronically treated with PCPA or vehicle (control) for 8 weeks. Data are expressed as mean ± SEM., $n = 13$ (Control) and 15 (PCPA).*$p < 0.05$; **$p < 0.01$.

in opposition to transgenic mice like Pet1$^{-/-}$ (Diaz et al., 2013), Tph2$^{-/-}$, SERT$^{-/-}$ (Kronenberg et al., 2016) mice in which constitutive depletion of serotonin affects as well the process of developmental neurogenesis that occurs during the first 2 weeks of life. In our experimental conditions, an increased protein expression of the p75 receptor was detected in the HC of mice after 4 and 5 weeks of PCPA administration. In addition, after 5 weeks of PCPA treatment, reduced protein expression of the p75 receptor ligand pro-BDNF was also evident. Since pro-BDNF is known to facilitate cell death (Barker, 2004), the decreased levels revealed herein in the HC of hyposerotonergic mice are in line with the increased cell survival previously reported in mice treated with PCPA for 8 weeks (Diaz et al., 2013). On the contrary, we did not observe any change in the expression of mBDNF and its receptor TrkB after chronic PCPA. These observations are in agreement with a recent article conducted in mice in which serotonin synthesis disruption was induced during adulthood (Pratelli et al., 2017). Indeed, Tph2 was specifically knocked-out at post-natal day 60 and hippocampal BDNF and TrkB receptor expression was unchanged compared to wild type mice. On the contrary, studies on the HC of mice with constitutive serotonin depletion showed an increase of BDNF mRNA (Migliarini et al., 2013) as well as of BDNF protein levels (Kronenberg et al., 2016). All in all, these results suggest that lack of serotonin induces dichotomic responses in the BDNF signaling pathway depending on the time of onset of serotonin depletion.

Stimulation of 5-HT$_{1A}$ receptors during 4 weeks was described to increase cell proliferation (Santarelli et al., 2003). However, shorter periods of stimulation, i.e., 1–2 week, did not induced neurogenic effects (Banasr et al., 2004; Huang and Herbert, 2005; Klempin et al., 2010; Diaz et al., 2013). Here, in our study, 1-week administration of the 5-HT$_{1A}$ agonist promoted a decreased protein expression of mBDNF and TrkB receptor in the HC of control mice. To our knowledge, the only data published in mice so far, concerned animals receiving DPAT from postnatal day 1 to 21, yielding a trend to decreased expression of BDNF mRNA in the HC (Ishikawa and Shiga, 2017). Interestingly, in the case of hyposerotonergic mice, 1-week stimulation of the 5-HT$_{1A}$ receptor did not significantly affect the mBDNF/TrkB pathway, but induced an increased protein expression of proBDNF and p75 receptor. Considering the pro-apoptotic properties reported for this complex (Barker, 2004), its enhanced expression could be playing a role in the reestablishment of cell survival in DPAT-treated hyposerotonergic mice (Diaz et al., 2013). All in all, our results suggest that the BDNF signaling pathway is differentially affected according to the serotonergic neurotransmission context.

The object pattern separation task allows the detection of subtle differences in mice performance (van Hagen et al., 2015). Surprisingly, PCPA-treated mice failed to get an index significantly different from 0, as it was the case for control mice. Although PCPA-treated mice have higher number of immature neurons in the HC (Diaz et al., 2013), this result was not completely unexpected, and given that we have previously found no improvement in the performance of hyposerotonergic mice in the contextual fear discrimination learning test (Diaz et al., 2013). As serotonin is involved in several different processes in the brain, reduced serotonin levels may impair cognitive abilities required to solve this kind of tests, independently of the number of immature neurons. It is interesting to mention that when setting up the OPS test, we were able to reproduce the discrimination curve reported in the original work (van Hagen et al., 2015), except for the position 4, where we could not get a discrimination index intermediate between P3 and P5. However, this unforeseen fact do not appear to interfere with the obtained results.

The observations made in our experimental conditions shed light on the role of the BDNF isoforms in the regulation of the neurogenic process that takes place in the HC of adult mice, under different configurations of the serotonergic system. This knowledge is key to propose new therapeutic targets for the development of more efficacious drugs.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

Experiments on animals were conducted according to the local regulations and were approved by the Institutional Ethical Committee (UBA-FMED, Resol. 2016/1637 and 2019/297).

AUTHOR CONTRIBUTIONS

RF designed the study, conducted the experiments, and wrote the manuscript. KS, AB, and AR conducted the experiments. SD designed the study, supervised the experiments, wrote the manuscript, and provided funding.

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MINI REVIEW
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On the Involvement of BDNF Signaling in Memory Reconsolidation

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When retrieval occurs concomitantly with novelty detection, mismatch perception or reactivation of conflicting information, consolidated memories can enter into a labile state, and to persist, must be restabilized through a protein synthesis-dependent reconsolidation process during which their strength and content can be modified. Extensive literature implicates brain-derived neurotrophic factor (BDNF), a key regulator of synaptogenesis and synaptic plasticity, in the acquisition, consolidation and extinction of several memory types. However, the participation of BDNF in memory reconsolidation has been less studied. In this review, we discuss recent reports supporting the involvement of BDNF signaling in reactivation-induced memory updating.

Keywords: neurotrophin, memory reconsolidation, TrkB, BDNF, retrieval

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INTRODUCTION

Memory consolidation is the time and protein synthesis-dependent stabilization process that takes place after learning to convert short-term memory into long-term memory. Consolidated memories are stable but mutable and can return to a labile state when activated during retrieval, requiring a restabilization phase known as reconsolidation to endure. Consolidation and reconsolidation share several molecular properties and result in persistent synaptic changes for memory storage. However, they are distinguishable processes that serve different biological purposes.

Brain-derived neurotrophic factor (BDNF) regulates neurogenesis, neuronal differentiation, maturation and survival during development (Park and Poo, 2013). BDNF is enriched in the adult's brain hippocampus and cerebral cortex (Conner et al., 1997), where it exerts neuroprotective effect, enhances synaptogenesis and neurotransmission, and mediates activity-dependent synaptic plasticity (Poo, 2001; Panja and Bramham, 2014).

BDNF is one of the most commonly studied proteins in memory research. In fact, in the last two decades plenty of studies described the participation of this neurotrophin in the acquisition, consolidation and long-lasting storage of different memory types (Ou et al., 2010; Martínez-Moreno et al., 2011; Bekinschtein et al., 2014). In particular, the role of BDNF in memory extinction, a process induced by repeated non-reinforced reactivation resulting in an inhibitory memory that opposes the original learned response, is well documented (Peters et al., 2010; Andero and Ressler, 2012; Xin et al., 2014). Maybe because of that, BDNF involvement in memory reconsolidation has been less investigated, although recent studies indicate that BDNF does play an essential function in this process, too.

MEMORIES ARE ADAPTABLE: THE RECONSOLIDATION PROCESS

Müller and Pilzecker (1900) postulated that learning does not generate permanent memories instantly but these are initially vulnerable to disruption and become stable only after a period of

consolidation. Almost 50 years later, Hebb (1949) proposed that recently acquired information persists during a short period as reverberant activity in local circuits. This resonance would induce structural changes in synapses of that network, allowing permanent memory storage. The idea that structural modifications underlie long-term memory gave rise to the synaptic consolidation hypothesis, which postulates that newly learned information is stored in the brain through a consolidation process that depends on gene expression and *de novo* protein synthesis for developing new synaptic connections and/or remodeling existing ones to support lasting memory storage (McGaugh, 1966).

The consolidation hypothesis posits that memories are immutable representations of the events that originated them. However, literature on experimental amnesia from the late 1960s conflicted with this view, pointing that memories can be altered upon reactivation. In those years, Donald Lewis and coworkers found that well-established fear memories could be impaired by electroconvulsive shock treatment given after a brief re-exposition to the conditioned stimulus (CS) that originated the conditioned fear response. Importantly, the same treatment was unable to affect retention when the reminder was omitted. The fear response also persisted in animals re-exposed to the CS alone, excluding the possibility that memory extinction could account for these results (Misanin et al., 1968). Based on these findings, it was suggested that retrieval induces the transition of memory from an inactive to an active state, and that interfering with this process could lead to memory loss (Lewis and Bregman, 1973; Lewis, 1979). Schneider and Sherman (1968) found similar results for avoidance memories, and it was later reported that administration of strychnine after aversive memory reactivation facilitated retention in rats (Gordon and Spear, 1973). Nevertheless, some studies failed to replicate Lewis' group findings (Dawson and McGaugh, 1969; Squire et al., 1976), and hence, the consolidation hypothesis continued dominating the field of learning and memory for decades. This conceptual framework excluded the possibility that established memories were actively reprocessed during retrieval. Indeed, it took almost 25 years until Przybyslawski and Sara (1997) successfully reinstated the idea that memories are susceptible to updating by ongoing experiences as a mainstream hypothesis showing that NMDA receptors blockade following consolidated spatial memory reactivation induces persistent amnesia. These findings led Przybyslawski and Sara (1997) to propose that some biochemical pathways activated during consolidation are also necessary to reconsolidate the active trace destabilized upon retrieval, a hypothesis confirmed by Nader et al. (2000) using auditory fear conditioning as a learning paradigm. Since then, memory reconsolidation has been observed in numerous animal species, using experimental paradigms evaluating distinct memory types, and employing a plethora of pharmacological treatments and behavioral challenges able to modulate it (Nader, 2015). However, memory retrieval does not always trigger reconsolidation but several boundary conditions constrain the occurrence of this process. For example, depending on the learning paradigm, memory labilization upon retrieval can be contingent on novelty detection (Morris et al., 2006; Rossato et al., 2007), mismatch perception (Pedreira et al., 2004) or reactivation of conflicting information (Radiske et al., 2017a). In addition, it has been reported that old as well as strong memories are usually more resistant to reconsolidation blockers than new and/or weak ones (Milekic and Alberini, 2002; Eisenberg and Dudai, 2004; Suzuki et al., 2004), suggesting that different reactivation protocols might be required to destabilize deep-rooted, robust memories. Extinction induction can also restrain memory reconsolidation (Pedreira and Maldonado, 2003; Suzuki et al., 2004) and although reconsolidation and extinction are dissociable and reciprocally exclusive processes (Merlo et al., 2014) they share several neurotransmitter systems and intracellular signaling pathways (Cahill and Milton, 2019) and also can influence each other. Indeed, phenomena involving extinction within the reconsolidation window, as well as reconsolidation of reactivated extinction memory, have been described (Monfils et al., 2009; Rossato et al., 2010).

It has been proposed that memory reconsolidation would mediate incorporation of new information into previously stored representations to support mental schema reorganization (Sara, 2000; Hupbach et al., 2007; Rossato et al., 2007) or would maintain memory relevance by preventing forgetting and supporting the lingering systems consolidation process that gradually stabilizes memories (Dudai and Eisenberg, 2004; Alberini, 2011). These two hypotheses are not mutually exclusive but the former requires an initial memory destabilization stage while the latter does not necessarily do so. Then, it could be expected that the molecular mechanisms responsible for restabilizing an updated memory differ from those involved in an ongoing consolidation process that evolves over time to strengthen the trace. However, our knowledge about the neurochemical bases of reconsolidation is still incipient. Difficulties do not rely only on identifying brain regions and intracellular pathways that might be differentially required for additional learning or memory modification through reconsolidation but on the fact that several neurotransmitter systems and signaling cascades that seem to be involved in reconsolidation are also required for other retrieval-induced cognitive processes, such as extinction (revised in Cahill and Milton, 2019). For example, extinction and reconsolidation are NMDA-dependent processes (Suzuki et al., 2004) modulated by dopaminergic and endocannabinoid neurotransmission (Marsicano et al., 2002; Hikind and Maroun, 2008; Lee and Flavell, 2014; Rossato et al., 2015) that involve AMPA receptor trafficking (Kim et al., 2007; Rao-Ruiz et al., 2011), all of which directly or indirectly control synaptic plasticity.

BDNF AND MEMORY RECONSOLIDATION

Neurotrophins are key regulators of long-term synaptic modifications. They are synthetized and secreted in an activity-dependent manner, acting locally at active synapses to enhance neurotransmission efficacy (Canossa et al., 1997; Poo, 2001). In particular, BDNF synthesized at dendrites is critical for LTP, a form of long-term plasticity and a putative cellular

mechanism for memory storage (Morris et al., 1986), mediating post-translational modifications at pre- and post-synaptic terminals and regulating local translation. BDNF contributes also to structural changes in synaptic spines (Tanaka et al., 2008) and sustains LTP even when protein synthesis is inhibited (Pang et al., 2004). Reactivation of potentiated synapses can render LTP sensitive to protein synthesis inhibition once again, indicating that LTP stability is a function of neuronal activity level (Fonseca et al., 2006) and suggesting that molecular mechanisms involved in LTP might also be important for reconsolidation. It is not surprising then that BDNF can also mediate reconsolidation-induced plasticity helping to remodel synapses activated by retrieval without affecting other circuits. In fact, memory reconsolidation depends on several molecules involved in LTP maintenance, such as Zif-268 and PKMζ (Lee et al., 2004; Rossato et al., 2019). In this respect, Samartgis et al. (2012) found that BDNF administration following a reminder session facilitates avoidance memory in chickens, showing for the first time that BDNF is indeed necessary for memory reconsolidation. In agreement with these results, reactivation-induced fear conditioning memory enhancement requires hippocampal BDNF expression in stressed rats (Giachero et al., 2013). Also, post-retrieval intra-CA1 spermidine administration lengthens contextual fear memory duration through a mechanism that depends on hippocampal BDNF maturation as well as on the interaction between this neurotrophin and its main receptor, tropomyosin-related receptor kinase B (TrkB; Signor et al., 2017). Further, increased BDNF mRNA and protein levels as well as TrkB activation in the insular cortex accompany hippocampus-independent conditioned taste aversion (CTA) memory retrieval, and interfering with BDNF synthesis in this cortex after reactivation causes amnesia. Notably, post-retrieval intra-insular cortex BDNF administration reverses CTA impairment and enhances weak CTA memory retention (Wang et al., 2012). Reactivation of fear extinction memory also increases BDNF levels and TrkB phosphorylation in the rat hippocampus while intra-CA1 administration of function-blocking anti-BDNF antibodies after extinction memory retrieval hampers extinction memory reconsolidation causing reinstatement of the extinguished fear. Importantly, hippocampus BDNF signaling activation preserves the learned extinction response when extinction memory reconsolidation is blocked (Radiske et al., 2015). This suggests that the mnemonic representation that controls behavior during retrieval is the one that gets weaken, as proposed by the trace dominance theory (Eisenberg et al., 2003), and also that BDNF signaling is sufficient to reconsolidate the prevailing memory. In line with these results, BDNF Val66met polymorphism, which is associated with hippocampus plasticity and BDNF trafficking (Egan et al., 2003), impairs conditioned fear memory storage when a brief fear reactivation session is followed by extended extinction training in humans (Asthana et al., 2016).

The participation of BNDF in memory reconsolidation is not restricted to distressing memories. Recognition memory, a major component of declarative memories, provides the ability to identify previously encountered events, objects and individuals. In rats, object recognition memory (ORM) maintenance requires *de novo* hippocampal protein synthesis after retrieval, but only when novelty is perceived during reactivation, suggesting that reconsolidation recruits the hippocampus to incorporate new information into the active recognition trace (Rossato et al., 2007). In neurons, BDNF is synthetized as a precursor peptide, proBDNF, which is stored or further cleaved to produce mature BDNF (Pang et al., 2004; Hwang et al., 2005). In the hippocampus, pro and mature forms of BDNF are abundant in presynaptic terminals of glutamatergic neurons and after release they act locally through their binding to p75 neurotrophin receptor (p75R) or TrkB, respectively. Activation of p75R by proBDNF facilitates LTD at CA1 synapses (Woo et al., 2005), and proBDNF extracellular conversion to BDNF by the tissue plasminogen activator (tPA)/plasmin system is essential for sustaining LTP (Pang et al., 2004). In accordance with these observations, ORM reconsolidation modifies hippocampal synaptic efficacy in rats, inducing a rapid depotentiation phase that occurs around 1.5 h after retrieval and is followed by a synaptic potentiation stage taking place ∼4.5 h thereafter (Clarke et al., 2010). Consistent with these findings, ORM reconsolidation is also accompanied by post-retrieval proteolysis of proBDNF, which augments BDNF levels and promotes functional BDNF/TrkB interaction in the hippocampus to restabilize the reactivated representation and incorporate new declarative information concurrently (Radiske et al., 2017b). PKMζ is a constitutively active PKC isoform highly expressed in the hippocampus. that would be responsible for sustaining long-term memory storage (Sacktor, 2008). BDNF modulates PKMζ turnover (Kelly et al., 2007) and maintains PKMζ-dependent late-LTP in the hippocampus even in the absence of protein synthesis (Mei et al., 2011). Interestingly, we recently demonstrated that BDNF mediates ORM reconsolidation-induced plasticity through PKMζ, which, in turn, regulates AMPAR trafficking at postsynaptic densities in the dorsal hippocampus to update the reactivated memory trace (Rossato et al., 2019). **Figure 1** shows a model of the molecular mechanism that might be mediating ORM reconsolidation. The study of Rossato et al. (2019) also provides behavioral, pharmacological and electrophysiological evidence supporting the idea that disrupting the reconsolidation process causes memory erasure. Importantly, blocking BDNF maturation as well as inhibition of BDNF downstream effectors after retrieval delete the reactivated recognition memory trace but leave dormant ORM intact, suggesting that memory destabilization specifically affects reactivated synapses and that BDNF modulates local synaptic remodeling to restabilize the updated trace. Notwithstanding this, other studies found that BDNF involvement in memory processing is restricted to memory consolidation and plays no role in reconsolidation (Lee et al., 2004; Barnes and Thomas, 2008; Lee and Hynds, 2013). This discrepancy must be due to the fact that most of these studies employed pre-training or pre-reactivation infusions of BDNF antisense oligodeoxynucleotides to hinder BDNF expression by knocking down proBDNF mRNA translation but were unable to affect the conversion of already available proBDNF to mature BDNF, which is essential for memory reconsolidation (Radiske et al., 2015).

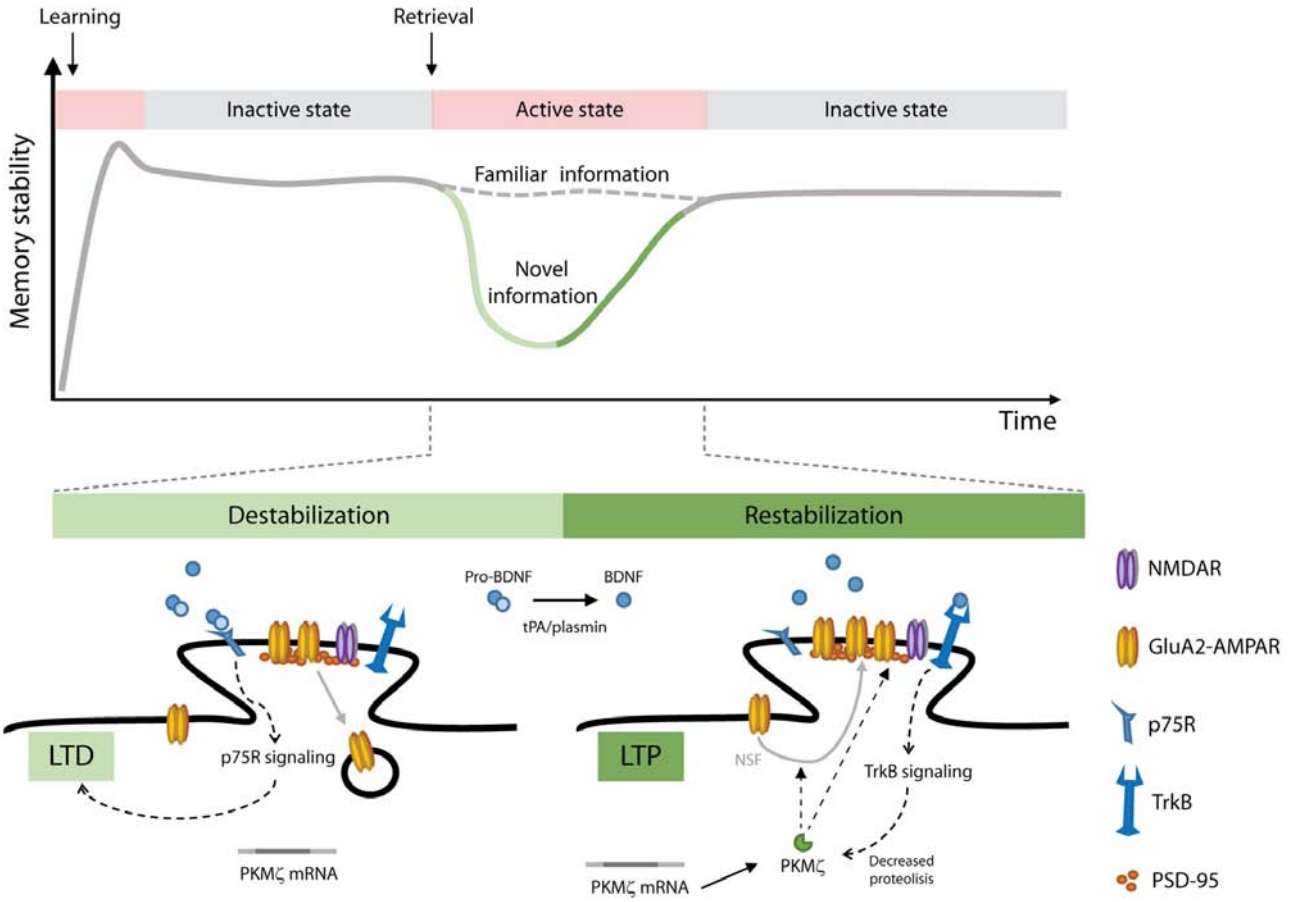

FIGURE 1 | A model of object recognition memory (ORM) reconsolidation. After learning, memories are gradually stabilized through consolidation. Consolidated memories are activated upon retrieval and depending on the conditions prevailing during this process (i.e., perception of novel information) they can be destabilized allowing their modification. To persist, updated memories must be restabilized through reconsolidation. We propose that ORM destabilization involves pro-BDNF/p75R signaling and LTD induction in the hippocampus. Restabilization of the updated memory trace involves pro-BDNF proteolysis, BDNF/TrkB signaling, PKMζ upregulation and insertion of GluA2-containing AMPAR into hippocampal postsynaptic densities. NSF, *N*-ethylmaleimide sensitive fusion protein; PSD-95, post synaptic density 95 protein; tPA/plasmin, tissue plasminogen activator/plasmin system.

IMPLICATIONS OF BDNF SIGNALING MANIPULATION DURING MEMORY RECONSOLIDATION

Determining the molecular basis of retrieval-induced cognitive processes is necessary not only to understand the dynamics of the memory storage process but also to prevent memory decline and treat disorders associated with the persistent reenactment of maladaptive recollections (Kida, 2019). The studies reviewed here suggest that targeting BDNF is a promising adjuvant to help patients recontextualize disturbing memories during reconsolidation-based therapies.

Memories about emotionally arousing events are usually persistently stored which, in some cases, lead to intrusive and distressing recollections that may result in anxiety, phobia or other types of disarrays such as post-traumatic stress disorder (PTSD; Ehlers, 2010). A potential tool to treat the exacerbated avoidance responses caused by the expression of fear memories at the core of some phobic behaviors is to disrupt its reconsolidation (Beckers and Kindt, 2017). However, this intervention presents limitations because, as mentioned above, retrieval does not always induce memory destabilization. Extinction-based psychotherapies are an alternative strategy to reduce traumatic memory expression, but their effects are not persistent and the current challenge is to maintain the extinction memory over time (Vervliet et al., 2013). In this respect, it was recently proposed that enhancing reconsolidation of extinction memory could be a viable strategy to avoid its decay (Radiske et al., 2015; Rosas-Vidal et al., 2015). Findings from the last decade show that BDNF modulates reconsolidation of both aversive and extinction memories (Wang et al., 2012; Giachero et al., 2013; Radiske et al., 2015; Signor et al., 2017). Overall, these studies suggest that drugs interfering with BDNF signaling during reconsolidation of aversive memories could help to impair its retention, while approaches that activate BDNF/TrkB pathways after extinction memory retrieval may promote its persistence, preventing reappearance of the fear response.

Antidepressants can influence BDNF levels bidirectionally. For example, a single dose of the serotonin re-uptake inhibitor fluoxetine decreases BDNF expression (Coppell et al., 2003) and attenuates aversive memory persistence in rats (Slipczuk et al., 2013), but chronic administration of the same agent upregulates BDNF mRNA levels (Coppell et al., 2003). These observations suggest that the patient's medication history can deeply influence the outcome of therapies based on memory reconsolidation. Acute interventions with antidepressants after traumatic memory reactivation may disrupt its reconsolidation, helping to reduce the disturbing symptoms. On the other hand, reconsolidation interference should be avoided in patients that are being treated for depression with drugs able to augment BDNF function. In this case, extinction-based treatments could be more effective, since enhanced BDNF signaling promotes fear memory extinction (Peters et al., 2010) as well as its persistence through memory reconsolidation (Radiske et al., 2017a).

An alternative strategy that is being study to enhance PTSD exposure therapy efficacy consist of coupling extinction sessions with physical exercise, which increases peripheral BDNF levels (Powers et al., 2015). In this respect, it has been recently shown that lactate mediates the facilitatory effect of physical exercise on cognition by upregulating hippocampal BDNF expression (El Hayek et al., 2019). Because the healthy human brain can uptake systemically administered lactate (van Hall et al., 2009), it would be interesting to evaluate lactate as a putative therapeutic molecule to reduce fear relapse by potentiating extinction through the enhancement of extinction memory reconsolidation.

Alzheimer's disease (AD) progression has also been associated with impaired reconsolidation and reduced BDNF signaling (Hock et al., 2000; Ohno, 2009), suggesting that increasing BDNF function during reconsolidation could partially counteract declarative memory deficits in AD patients. In this respect, transcranial direct current stimulation, which activates signaling downstream BDNF and elicits LTP-like mechanisms in rats, improves episodic and semantic memories in AD patients (Cocco et al., 2018) a result in line with earlier findings showing that the blood-brain barrier permeable TrkB agonist 7,8-dihydroxyflavone (7,8-DHF) ameliorates cognitive decline in AD animal models (Devi and Ohno, 2012).

CONCLUDING REMARKS

The studies reviewed in this article suggest that BDNF mediates enduring synaptic changes required for memory strengthening and updating upon retrieval. Several pharmacological and non-pharmacological approaches to modulate BDNF signaling and expression are currently under consideration, and exposure-based psychotherapy could take advantage of those findings. However, future research should also consider BDNF signaling interaction with other mediators of memory reconsolidation. An important issue that limits the effectiveness of reconsolidation-based treatments is that retrieval does not always induce memory destabilization. However, it was recently proposed that memory destabilization can be enhanced pharmacologically (Lee and Flavell, 2014), offering the opportunity to improve familiar declarative memories or treat old traumas that are usually resistant to reconsolidation.

AUTHOR CONTRIBUTIONS

MG and AR conceptualized the review, performed the literature research, interpreted the data, and wrote the manuscript. MC contributed to the data interpretation, edited parts of the manuscript, and critically revised and approved its final version.

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Reversal of Cognitive Impairment in gp120 Transgenic Mice by the Removal of the p75 Neurotrophin Receptor

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Activation of the p75 neurotrophin receptor (p75NTR), by the proneurotrophin brain-derived neurotrophic factor (proBDNF), triggers loss of synapses and promotes neuronal death. These pathological features are also caused by the human immunodeficiency virus-1 (HIV) envelope protein gp120, which increases the levels of proBDNF. To establish whether p75NTR plays a role in gp120-mediated neurite pruning, we exposed primary cultures of cortical neurons from *p75NTR*$^{-/-}$ mice to gp120. We found that the lack of *p75NTR* expression significantly reduced gp120-mediated neuronal cell death. To determine whether knocking down *p75NTR* is neuroprotective *in vivo*, we intercrossed gp120 transgenic (tg) mice with *p75NTR* heterozygous mice to obtain gp120tg mice lacking one or two *p75NTR* alleles. The removal of *p75NTR* alleles inhibited gp120-mediated decrease of excitatory synapses in the hippocampus, as measured by the levels of PSD95 and subunits of the *N*-methyl-D-Aspartate receptor in synaptosomes. Moreover, the deletion of only one copy of the *p75NTR* gene was sufficient to restore the cognitive impairment observed in gp120tg mice. Our data suggest that activation of p75NTR is one of the mechanisms crucial for the neurotoxic effect of gp120. These data indicate that p75NTR antagonists could provide an adjunct therapy against synaptic simplification caused by HIV.

Keywords: HIV, Morris water maze, NMDA receptors, p75NTR, passive avoidance, proBDNF, PSD95

INTRODUCTION

Despite the use of combination antiretroviral therapy (cART) (Ellis et al., 2007; Everall et al., 2009), approximately half of HIV-positive individuals are at a high risk for developing mild to severe cognitive impairments, termed HIV-associated neurocognitive disorders (HANDs) (Clifford and Ances, 2013; Saylor et al., 2016). Cognitive alterations seen in HAND subjects correlate with loss of synapses (Masliah et al., 1997; Albright et al., 2003; McArthur, 2004; Everall et al., 2005; Crews et al., 2009). However, our understanding of the mechanisms of HIV-mediated synaptic

degeneration is incomplete. A better understanding of the molecular mechanisms underlying HIV neurotoxicity could lead to a new adjunct therapy for HIV positive individuals.

The brain serves as a reservoir for ongoing HIV replication (Fois and Brew, 2015); in fact, HAND subjects have detectable levels of HIV RNA in their cerebrospinal fluid (CSF) even when the virus is undetectable in the blood (Di Carlofelice et al., 2018). However, HIV does not infect neurons and thus HAND must result from mechanisms other than neuronal infection. HIV may evoke neuronal injury through indirect mechanisms such as neurotoxins released by infected or immune-stimulated, inflammatory microglia and macrophages (Kaul et al., 2001). Neuronal injury may also result from neurotoxic action of viral proteins such as the activator of transcription Tat (Nath and Steiner, 2013) or the envelope protein gp120 (Meucci and Miller, 1996). The molecular mechanisms whereby gp120 promotes synaptic simplification are still under investigation. The loss of neurons, simplification of neuronal branching, and reduction in dendritic spines can also be triggered by the p75 neurotrophin receptor (p75NTR) (reviewed in Ibanez and Simi, 2012), a member of the tumor necrosis factor receptor family which contains a death domain (Feinstein et al., 1995; Liepinsh et al., 1997). Indeed, activation of p75NTR induces neuronal cell death (Teng et al., 2005) as well as axonal and dendritic spine pruning both during development (Singh et al., 2008) as well as in the adult nervous system (Park et al., 2010; Kraemer et al., 2014).

There are many ligands for the p75NTR. These include mature as well as unprocessed neurotrophins or proneurotrophins (Chao, 2003), myelin-associated glycoproteins (Wong et al., 2002) and beta amyloid peptide (Perini et al., 2002; Knowles et al., 2013). A p75NTR ligand that promotes neuronal apoptosis and synaptic pruning is the proneurotrophin brain-derived neurotrophic factor (proBDNF) (Pang et al., 2004; Teng et al., 2005; Yang et al., 2014; Guo et al., 2016). Previous work from our laboratory has shown that gp120 increases the levels and release of proBDNF in primary neuronal cultures (Bachis et al., 2012). In these cultures, p75NTR inhibitors block gp120-mediated synaptic simplification (Bachis et al., 2012), suggesting that activation of p75NTR by proBDNF may be a crucial mechanism to underlying the synaptic simplification seen in HAND. This suggestion is supported by evidence showing that postmortem brains of HAND subjects exhibit higher levels of proBDNF than HIV positive subjects without cognitive alterations (Bachis et al., 2012). Consistently with this suggestion, recent data have shown that increased hippocampal proBDNF contributes to memory impairment in aged mice (Buhusi et al., 2017).

This present study was undertaken to provide molecular and behavioral evidence of the role that p75NTR plays in gp120-mediated loss of synaptic contacts. We utilized gp120 transgenic (gp120tg) mice intercrossed with *p75NTR* null mice. The gp120tg mice display a multitude of altered neuron-specific processes, including synaptic simplifications (Toggas et al., 1994; Bachis et al., 2016b) and impaired neurogenesis (Lee et al., 2011), as well as cognitive deficits (D'Hooge et al., 1999) and sensorimotor gating impairments (Henry et al., 2014), suggesting that these animals are a suitable model to study HAND (Thaney et al., 2018). We report that the reduction of *p75NTR* expression significantly decreases the neurotoxic effect of gp120 as well as impairment in memory evoked by gp120.

MATERIALS AND METHODS

Reagents

Human T-lymphotropic virus (HTLV)-IIIB ($HIV1_{IIIB}$) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). Gp120IIIB was obtained for Immunodiagnostics, Inc. (Woburn, MA, United States). Uncleavable proBDNF was purchased from Alomone labs (Jerusalem, Israel).

Cortical Neurons

Primary mouse cortical neurons were prepared from the cortex of embryonic wild type after days 17–18 (WT) and $p75NTR^{-/-}$ mice following an established protocol (Avdoshina et al., 2016a,b). Cells were seeded (0.5×10^6/ml) onto poly-L-lysine (Sigma Aldrich) pre-coated plates or glass coverslips in Neurobasal Medium containing 2% B27 supplement, 25 nM glutamate, 0.5 mM L-glutamine, and 1% antibiotic-antimycotic solution (Thermo Fisher Scientific). Cultures were grown at 37°C in 5% CO_2/95% air for 7 days prior to the experiments. At day 7 *in vitro*, cell cultures contained 95% neurons as characterized by an antibody against tubulin β III (TUBB3), as previously described (Avdoshina et al., 2016a,b).

Cell Viability

The viability of primary cortical neurons was estimated by Hoechst 33258 and propidium iodide (Hoechst/PI; Sigma-Adrich) co-staining and visualized using a fluorescence microscope Olympus IX71, as previously described (Avdoshina et al., 2016a). Hoechst/PI-positive cells were then counted using ImageJ (National Institutes of Health, Bethesda, MD, United States) and expressed as a percentage of the total number of neurons.

Animals

Gp120tg breeding mice were obtained from Dr. E. Masliah (University of California, San Diego, San Diego, CA, United States). The characterization of these mice is provided elsewhere (Toggas et al., 1994). Female gp120tg mice were intercrossed with C57BL/6J male $p75NTR^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME, United States) to generate males and female $p75^{-/-}$ and $p75^{+/-}$gp120tg mice, as previously described (Bachis et al., 2016b). Wild type (WT) littermates (gp120 null/$p75^{+/+}$) were generated from these colonies and used as controls for our biochemical, behavioral, and histological studies. Animals were housed under standard conditions with food and water *ad libitum* and maintained on a 12-h light/dark cycle. Mice were maintained in our facility for up to 10 months. 8–10 month old mice (of both sexes) were used for these studies. An animal's genotype was confirmed through

an outsourced genotyping service (Transnetyx, Inc., Cordova, TN, United States) from tail snips taken at time of weaning and at sacrifice. All studies were carried out following the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and approved by the Georgetown University Animal Care and Use Committee.

Behavioral Analysis

All rodents in this study were tested during their dark (active) period. For each behavioral test, mice were brought to the testing room and allowed to habituate to the testing conditions for at least 1 h. White noise (50 dB) was played to obscure noises from outside the testing room. After the conclusion of each test, mice were returned to their home cages in the animal facility. The assays were scheduled in an order to minimize the impact of repeated testing on performance and occurred in the same order as they appear below within section "Materials and Methods."

Open Field Measures

The open field apparatus (Med Associates, Inc., Saint Albans City, VT, United States) measured 27 cm × 27 cm and had transparent walls of 20 cm. The apparatus also contained 16-beam IR arrays on both the X and Y axes for positional tracking within the apparatus and on the Z axis for rearing detection. In order to encourage exploration, the open field was dimly lit by overhead room lights at 75 lux. The apparatus was cleaned with a 70% ethanol solution between trials. Mice were placed in the center of the field and exploration was recorded over a single trial of 60 min. Behavior was tracked through the IR beam array and analyzed by the Med Associates Activity Monitor software. The rodents' behavior in this apparatus was analyzed using IR beam breaks for locomotor activity throughout the trial. The center zone was defined as the zone greater than 6 cm from any of the walls.

Passive Avoidance

The modular passive avoidance chamber (Coulbourn Instruments, Holliston, MA, United States) had two enclosed chambers of equal dimensions separated by a wall. This center wall had a 6 cm by 6 cm guillotine door linked to a computer-controlled AMi-2 interface device (Stoelting, Co., Wood Dale, IL, United States). Each chamber in the apparatus measured 17.0 cm by 17.7 cm and had a height of 30.5 cm. One side of the chamber had opaque walls and provided a dark environment for rodents inside this compartment. The other compartment was brightly illuminated by an overhead light at 300 lux. The chamber was placed in the center of the room with indirect overhead lighting and a side-mounted remote USB camera for viewing mice within the apparatus.

The passive avoidance task was conducted over three consecutive days with a single trial on each day (Day 1: habituation, Day 2: acquisition, Day 3: retention probe trial). In the habituation trial, mice were placed in the lighted chamber with the door closed and allowed to explore for 180 s. For the acquisition trial, mice were again placed in the lit compartment at the beginning of the test. After 30 s, the door lifted and mice were given access to the dark compartment. When a mouse had entered the dark compartment, the experimenter closed the door with a remote switch, and a computer program (Anymaze, Stoelting, Co., Wood Dale, IL, United States) initiated a 2 s foot shock at 0.2–0.4 mA. After five additional seconds in the dark compartment, the test was ended and the mouse was retrieved. The probe trial followed an identical procedure to the acquisition trial, but the door was closed and the mouse was not shocked when it entered the dark zone. The probe trial was limited to 300 s. If the mouse had not entered within 300 s, the mouse was removed from the apparatus and its probe latency was recorded as 300 s. The latency to enter the dark zone on the acquisition and probe trials was recorded by the Anymaze software via a keystroke from the experimenter. A mouse was judged to have entered the dark compartment when all four paws were completely inside the darkened chamber.

Morris Water Maze

The Morris water maze (MWM) apparatus consisted of a circular pool (120 cm in diameter), which was filled to a depth of 50 cm with 26°C water. Habituation, acquisition, and reversal trials included a 6 cm by 6 cm escape platform submerged ∼1 cm below the water's surface. The maze was lit by overhead lights at 75 lux and surrounded by white curtains with large distal cues on each of the four cardinal directions.

We conducted our MWM paradigm over 13 consecutive days. Briefly, rodents were given a single 60 s habituation trial in clear water with a submerged, but visible escape platform before training began. Spatial acquisition trials were performed four times per day and conducted over the next 5 days with the water now made opaque by the addition of white acrylic paint. A single probe trial was performed 24 h after the final spatial acquisition trial with the escape platform now absent from the maze. Reversal trials on the next 5 days were conducted in a manner identical to the spatial acquisition trials, but with the escape platform moved 180° to its initial position within the apparatus. Finally, the reversal probe trial was conducted on the final day in a manner identical to the initial probe trial. The habituation trial and probe trials were limited to 60 s. The acquisition and reversal trials were likewise limited to 60 s, but mice were gently guided to the escape platform if they had not located this platform within 60 s. Animals were allowed to remain on the escape platform at the end of their trial for 15 s in order to examine their location with respect to the distal cues. We used an inter-trial interval of 15 min.

The MWM was virtually divided into four equal quadrants and behavior was analyzed by Anymaze for latency to entry onto the escape platform, duration in target quadrant, duration in the center (non-thigmotaxic) area, passes over the former escape platform location, and average swimming speed. A trial was excluded from MWM analysis if the animal demonstrated non-searching behaviors in the maze, which we defined *a priori* as passive floating for greater than five consecutive seconds or panicked swimming at one location on the maze wall (less than 2% of all trials). An animal was omitted from a testing day if two or more trials were excluded within the same day, but every mouse was allowed to finish the trial and remain on the target platform for each exposure to the MWM.

Preparation of Synaptosomes and Western Blot Analysis

Mice were euthanized by cervical dislocation for the preparation of synaptosomes. Synaptosomes were prepared from brain lysates using Synaptic Protein Extraction Reagent (Thermo Fisher Scientific, Inc., Waltham, MA, United States) according to the manufacturer instructions. Protein content was determined by BCA Protein Assay Reagent Kit (Thermo Fisher Scientific, Inc.) according to the manufacture instructions. Proteins were separated in a NuPAGE 4–12% Bis-Tris Gel and transferred to a nitrocellulose membrane using iBlot device (Thermo Fisher Scientific, Inc.). Membranes were blocked with 5% milk in PBS and 0.1% Tween-20 and probed with antibodies against: PSD95 (1:2000, Thermo Fisher Scientific, Inc.), NMDAR2B (1:1000, Abcam, Inc., Cambridge, United Kingdom), NMDAR2A (1:1000, R&D Systems, Minneapolis, MN, United States), and synaptophysin (1:2000, Sigma-Aldrich, Co., St. Louis, MO, United States). Membranes were stripped with Restore Western Blot Stripping Buffer (Invitrogen) for 30 min at 37°C and re-probed with and anti-β-actin antibody (1:15000, Sigma-Aldrich, Co.) in blocking buffer to serve as a protein loading control. Immune complexes were detected with the corresponding secondary antibody and chemiluminescence reagent (Fisher Scientific). The intensity of immunoreactive bands was quantified using ImageJ and expressed in arbitrary units (AUs) defined as optical densities of synaptic protein relative to β-actin.

Statistical Analysis

Data, expressed as the mean ± SEM, were analyzed using one or two-way analysis of variance (ANOVA) with either Tukey's HSD *post hoc* test for biochemistry, or Kruskal–Wallis for behavior, using GraphPad Prism software v. 7.0 (GraphPad). A p-value < 0.05 was considered statistically significant.

RESULTS

gp120 Is Not Neurotoxic in *p75NTR*$^{-/-}$ Neurons

We have previously demonstrated that gp120, which induces the releases proBDNF, promotes synaptic pruning in rodent primary neurons (Bachis et al., 2012; Avdoshina et al., 2017). The neurotoxic effect of gp120 is prevented by p75NTR antagonists (Bachis et al., 2012). To further support these data, gp120 was applied to primary cultures of cortical neurons obtained from WT or *p75NTR*$^{-/-}$ mice for 6 h and neuronal processes were identified by an antibody against neuron-specific cytoskeleton protein tubulin β III (TUBB3). Consistent with our prior findings (Bachis et al., 2012), exposure of neurons from WT mice to gp120 (5 nM) reduced the overall TUBB3 immunoreactivity, suggesting a decrease in the number of neuronal processes (**Figure 1**). Importantly, we found that neurons lacking *p75NTR* expression have a more complex TUBB3-positive network than neurons from WT animals exposed to gp120 (**Figure 1**).

To provide a quantitative assessment of the neurotoxic effect of gp120, we exposed WT and p75NTR$^{-/-}$ neurons for 24 h to gp120 (5 nM). Hoechst/PI was used to quantify the number of surviving neurons. Furthermore, we examined whether HIV, which shares a similar neurotoxic profile of gp120 in rodent neurons (Bachis et al., 2009), is neurotoxic via p75NTR. As a positive control for p75NTR-mediated loss of neurons, we also exposed both WT and *p75NTR* null neurons to proBDNF for 24 h (10 nM). WT neurons in the presence of gp120, HIV or proBDNF displayed the expected increase in the number of neurons with Hoechst/PI staining, indicating increased apoptosis (**Figure 2**); importantly, the lack of *p75NTR* significantly reduced neuronal loss caused by either gp120, HIV, or proBDNF (**Figure 2**). Overall, a two-way ANOVA for this set of experiments revealed significance for genotype ($F_{(1,60)} = 91.92$; $p < 0.001$), treatment ($F_{(3,60)} = 27.71$; $p < 0.001$), and interaction ($F_{(3,60)} = 12.88$; $p < 0.001$) factors. Taken together, our data suggest that p75NTR mediates the synaptic pruning effect of gp120, most likely shed from the virus.

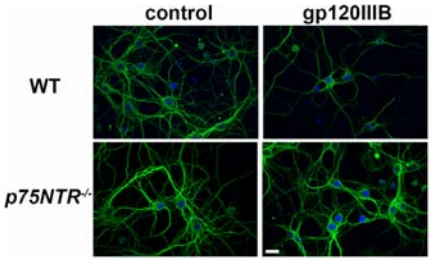

FIGURE 1 | gp120 does not cause neurite pruning in *p75NTR*$^{-/-}$ neurons. Representative images of primary neuronal cultures prepared from E17 cortex of WT or p75NTR$^{-/-}$ mice. At 7 days in culture, neurons were exposed to heat inactivated (boiled) gp120IIIB (control) or 5 nM gp120IIIB for 6 h. Cells were fixed and processes visualized by anti TUBB3 antibody (green). DAPI (blue) was used to stain nuclei. Bar = 20 μm. Note that the neuronal network (TUBB3 positive processes) in p75NTR$^{-/-}$ neurons exposed to gp120 is preserved when compared to WT neurons exposed to gp120. The experiment was repeated twice.

gp120 Decreases PSD95 and NMDA Receptor Subunit Immunoreactivity

We have previously shown that gp120 causes a decrease in the number of dendritic spines in the hippocampus, an effect that is significantly diminished by the removal of one *p75NTR* allele (Bachis et al., 2016b). Dendritic spines form the post-synaptic density of the majority of excitatory synapses. Thus, to determine whether gp120 affects post-synaptic spines, we prepared synaptic fractions from homogenized mouse brains of 8–10 month-old WT and gp120tg mice and measured the levels of post-synaptic and presynaptic proteins. These include post-synaptic density protein 95 (PSD95), an abundant scaffolding protein that determines the functional integrity of excitatory synapses, *N*-methyl-D-aspartate (NMDA) receptor (NR) subunit 2A and 2B (Kornau et al., 1995) and synaptophysin, a transmembrane protein that is involved in synaptic formation and exocytosis. We first verified the appropriateness of the method by determining PSD95 and NR2A and 2B subunits in brain lysates from WT mice containing synaptosomal and cytoplasmic preparation.

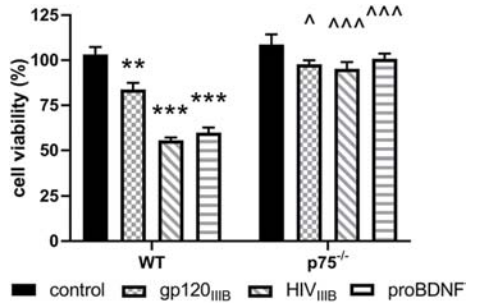

FIGURE 2 | The neurotoxic effect of HIV and gp120 is reduced in *p75NTR*$^{-/-}$ neurons. Cortical neurons from WT and p75NTR$^{-/-}$ mice were exposed to gp120$_{IIIB}$ (5 nM), HIV$_{IIIB}$ (1.5 ng/ml of p24), or proBDNF (10 nM) for 24 h. Boiled inactivated gp120 or HIV (IIIB) were used as controls. Neuronal cell death was determined by counting the number of Hoechst/PI positive cells, as described in Section "Materials and Methods." Data expressed as % of cell viability are the mean ± SEM of eight independent coverslips from four independent culture preparations; coverslip values were averaged from three random fields. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. WT control, $\hat{}p < 0.05$ vs. WT gp120$_{IIIB}$, $\hat{}\hat{}\hat{}p < 0.01$ vs. WT HIV$_{IIIB}$ or proBDNF. Two-way ANOVA and Tukey's HSD.

Data shown in **Supplementary Figure S1** confirm that PSD95, NR2A, and 2B immunoreactivity are only found in synaptosomal preparations. We then examined whether the levels of these synaptic proteins are altered in the hippocampus of gp120tg mice.

When compared to WT, hippocampal synaptosomes from gp120tg mice exhibited a decrease in PSD95, NR2A and 2B subunits (**Figure 3A**). In fact, one-way ANOVAs for synaptosomal contents of PSD95 ($F_{(4,19)} = 3.674$; $p < 0.05$), NR2A ($F_{(4,18)} = 4.101$; $p < 0.05$), and NR2B ($F_{(4,19)} = 6.395$; $p < 0.01$) showed significant differences among means. Interestingly, gp120 did not change the levels of synaptophysin (**Figure 3E**) ($F_{(4,19)} = 0.01391$; $p > 0.9996$). Thus, it appears that gp120 may target mainly post-synaptic densities. Importantly, the removal of *p75NTR* mitigated the effect of gp120 (**Figure 3**). It is important to note that lack of *p75NTR* expression *per se* did not change the levels of these synaptic markers when compared to WT mice (**Figure 3**), supporting a previous study showing that removal of p75NTR *in vivo* does not induce abnormal alteration of synapses (Qian et al., 2018).

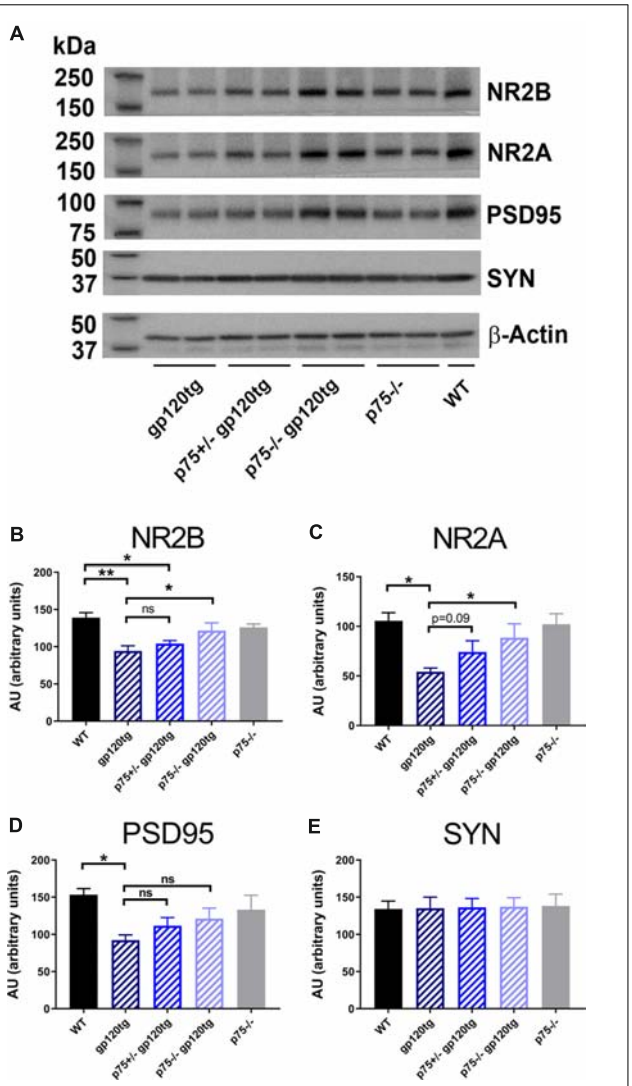

FIGURE 3 | Analysis of synaptosomal preparations from gp120 and *p75NTR* mice. **(A)** Example of a Western blot analysis of synaptosomal preparation from the hippocampus of the indicated mouse genotypes. The blot was probed with antibodies that recognized the indicated synaptic proteins. Beta-actin was used for loading control. Molecular weights of analyzed proteins are listed on the left. **(B–E)** Levels of synaptic proteins, quantified as described in Section "Materials and Methods" and expressed in AU, are the mean ± SEM of five animal per group. $^{*}p < 0.05$, $^{**}p < 0.01$. In all panels, comparisons of means of WT and p75$^{-/-}$ mice reveal no significant differences. ANOVA and Tukey's HSD.

gp120-Mediated Deficits in Performance on a Passive Avoidance Task Is Inhibited by the Removal of *p75NTR* Alleles

Gp120tg mice develop age-related cognitive abnormalities, which correlate with loss of synaptic plasticity and neuronal degeneration (Toggas et al., 1994; Krucker et al., 1998; Lee et al., 2011), as well as an increased in the levels of proBDNF in the hippocampus (Bachis et al., 2016b). These data allowed us to speculate that a reduction of *p75NTR* expression would avert the impaired performance on hippocampal-dependent memory tasks previously described in gp120tg mice (Krucker et al., 1998; D'Hooge et al., 1999).

To examine whether loss of hippocampal spines was associated with impaired long-term avoidance memory, we subjected 8–10 month-old WT, gp120tg, p75$^{+/-}$gp120tg, and p75$^{-/-}$gp120tg mice to a passive avoidance task. Mice of each genotype entered the dark compartment with similar latency on the acquisition trial (one-way ANOVA: $F_{(4,81)} = 0.5615$, $p = 0.6912$) (**Figure 4**), suggesting that the absence of one or both *p75NTR* alleles does not affect exploratory drive in this apparatus. Across all probe trials, gp120tg mice showed a significant difference in latency to enter the dark compartment

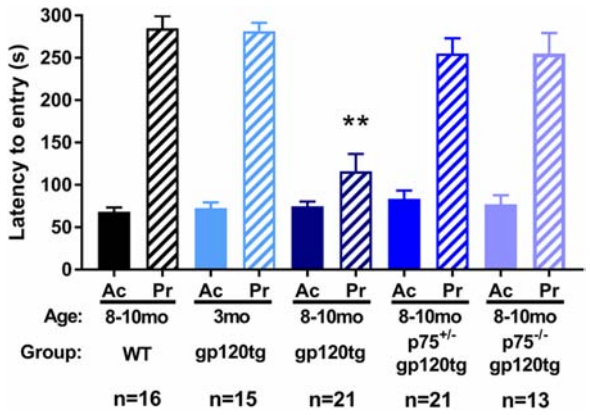

FIGURE 4 | Removal of *p75NTR* rescues gp120-driven impairments on a passive avoidance task in (8–10mo) animals. 3- (3mo) and 8–10 month (8mo)-old mice of the indicated genotypes were subjected to a passive avoidance task to evaluate impairments in avoidance earning. Latency to enter the dark compartment on acquisition (Ac) and probe (Pr) testing days was analyzed. ANOVA with Tukey's HSD for comparisons across genotypes. $^{**}p < 0.001$ vs. WT. Data are expressed as mean ± SEM. *n* = number of animals per group.

compared to WT. The gp120-mediated impairment in passive avoidance was reduced in gp120tg mice with one or both *p75NTR* alleles missing (Kruskal–Wallis with *post hoc* Dunn's test $H = 30.14$, df = 4, $p < 0.001$) (**Figure 4**). Interestingly, 3 month-old (3mo) gp120tg mice performed better than 8–10 month-old (8–10mo) gp120tg mice, supporting previous data that gp120-induced behavioral effects are age dependent (Toggas et al., 1994; D'Hooge et al., 1999; Bachis et al., 2016a). In fact, two-tailed Wilcoxon matched-pairs signed-rank tests performed between the acquisition and probe testing days within each genotype shows significant differences within 8–10mo WT ($p < 0.001$), 3mo gp120tg ($p < 0.001$), 8–10mo p75$^{+/-}$gp120tg ($p < 0.001$), and 8–10mo p75$^{-/-}$gp120tg ($p < 0.001$) groups, but not within 8mo gp120tg ($p = 0.0973$).

The observed effect may be due in part to differential locomotor activity, vigilance, or alertness in a novel environment among experimental groups (i.e., more exploratory animals may enter the dark compartment at a greater rate regardless of a formed association). To address these confounds, we assessed the above behaviors in a single 5-min exposure to an open field. Both the cumulative distance traveled (**Figure 5A**) and the time spent ambulating (**Figure 5B**) in the trial were equivalent between experimental groups (one-way ANOVA: $F_{(3,70)} = 0.0973$, $p = 0.9613$, $F_{(3,69)} = 0.6548$, $p = 0.5827$, respectively). The percentage of total active beam breaks occurring in the center of open field was decreased in 8–10mo gp120tg mice when compared to WT, replicating previous findings that older gp120tg mice display a modest anxious phenotype (one-way ANOVA: $F_{(3,69)} = 3.748$, $p = 0.0148$) (Henry et al., 2014; Bachis et al., 2016a). Interestingly, this effect was rescued in animals by deleting one or both *p75NTR* alleles (**Figure 5C**). However, the total active beam breaks (**Figure 5D**) were equivalent across experimental groups (one-way ANOVA: $F_{(3,71)} = 1.163$, $p = 0.3300$). Thus, although gp120tg mice show modest anxiety-like behaviors in a novel environment, all groups have a comparable exploratory drive and activity level in a novel environment within short passive avoidance timeframes. Based on these data, it is unlikely that the deficits seen in the passive avoidance task arise from anxiousness in an open environment.

Genetic Deletion of *p75NTR* Rescues gp120-Mediated Impairment in Spatial Memory

Previous studies have demonstrated that spatial memory is impaired in gp120tg mice (D'Hooge et al., 1999). We hypothesized that spatial learning and memory, a hippocampal-dependent behavior, would be improved in mice lacking one or both *p75NTR* alleles. To assess impairments in spatial memory, we employed a MWM navigation task over 13 days. WT mice performed significantly better than gp120tg mice in both the acquisition and reversal phase, in which the escape platform was moved 180° from its original location. Indeed, the gp120tg mice showed impairments on the second and third acquisition and reversal days (**Figure 6A**). The removal of one or both *p75NTR* alleles diminished the effect of gp120. **Supplementary Table S1** displays all statistical measures and inter-group comparisons within the two MWM learning phases. A probe trial was administered 24 h after both the final acquisition and reversal trials. These probes revealed differences between gp120tg mice vs. WT controls with respect to the duration of time spent in the target quadrant (**Figure 6B**) and passes over the former target platform's location (**Figure 6C**). Both p75$^{+/-}$gp120tg and p75$^{-/-}$gp120tg groups had non-significant differences in these two probe measures vs. WT controls. Similar results were observed within the reversal probe (**Figures 6D,E**, respectively).

To account for possible confounds due to impairments in swimming, we compared swim speeds during the initial habituation to the water maze. Swim speed did not differ significantly (one-way ANOVA: $F_{(3,71)} = 0.6611$, $p = 0.5787$) between the four genotypes during this single trial (**Figure 6F**). Likewise, the percentage of time spent swimming in the center of the MWM on the first trial following habituation was similarly equivalent across experimental groups (**Figure 6G**; one-way ANOVA: $F_{(3,71)} = 1.964$, $p = 0.1271$), indicating comparable motivation to escape the maze. Taken together, these data indicate that there are differences in spatial learning and memory across genotypes.

DISCUSSION

Dendritic injury and synaptic dysfunction are believed to cause the cognitive decline in HAND and other neurodegenerative diseases. Loss of synapses, similar to what is seen in HAND, is also reproducible in transgenic mice overexpressing gp120 (reviewed in Thaney et al., 2018). In this work we have used this animal model to characterize molecular/cellular mechanisms underlying the neuropathology of HAND. Our previous studies have shown that gp120tg mice exhibit increased levels of proBDNF in the hippocampus (Bachis et al., 2016b). Moreover, gp120 induces the

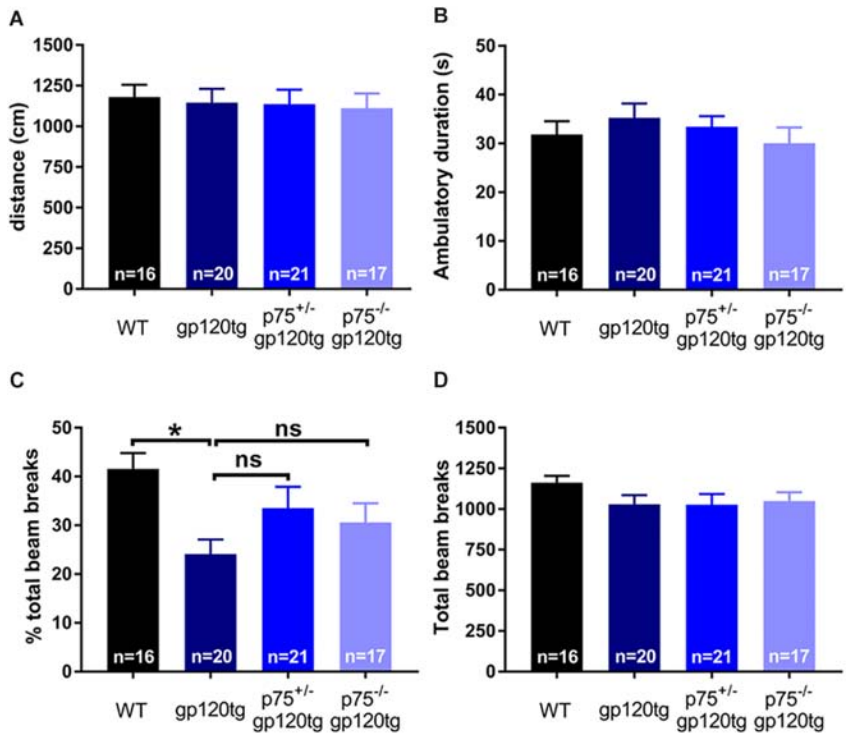

FIGURE 5 | Deletion of one or both *p75NTR* alleles in gp120 mice does not affect locomotion or exploration in a novel environment. 8–10 month-old mice were exposed for 5 min to an open field. **(A)** Cumulative distance traveled in the open field. **(B)** Total time spent ambulating in the open field. **(C)** Percent of total beam breaks occurring in the center of the open field. ANOVA with Tukey's HSD. $^*p < 0.05$ vs. WT. **(D)** Total active beam breaks (ambulatory and stereotypic movements) occurring within the 5-min trial. Data are expressed as mean ± SEM. *n* = number of animals per group.

release of proBDNF from neuronal cultures (Bachis et al., 2012). Here, we show that gp120 neurotoxicity can be attenuated by the removal of p75NTR, a receptor that promotes synaptic pruning (Zagrebelsky et al., 2005; Singh et al., 2008) and neuronal cell death (Bamji et al., 1998; Bhakar et al., 2003). Thus, our results support the suggestion that gp120 promotes synaptodendritic injury by a mechanism that favors the activation of p75NTR.

How can gp120 neurotoxicity be linked to p75NTR activation? ProBDNF, like other proneurotrophins, is cleaved into mature BDNF in the endoplasmic reticulum by the proconvertase furin (Seidah et al., 1996) or extracellularly by proteases such as plasmin and matrix metalloproteases (Pang et al., 2004). Gp120 decreases the level and activity of furin and plasmin, thus reduces the conversion of proBDNF to mature BDNF (Bachis et al., 2012). Consequently, gp120tg mice exhibit higher levels of proBDNF than WT in the hippocampus and other brain areas (Bachis et al., 2016b). Moreover, gp120 promotes the release of proBDNF from cortical neurons and alters the ratio mature BDNF/proBDNF in the synaptic cleft in favor of proBDNF. This release could compromise synaptic connections and neuronal survival as indicated by the increased neuronal loss in cortical neurons exposed to gp120 (Bachis et al., 2012). Our data obtained in p75NTR$^{-/-}$ neurons, in which the neurotoxic effect of gp120 was significantly attenuated, strongly suggest that gp120-mediated synaptodendritic injury and cell loss depend upon an indirect activation of p75NTR.

The number and morphology of dendritic spines have emerged as crucial components underlying synaptic plasticity. Dendritic spines express all ionotropic glutamatergic receptors, which play a central role in long-term potentiation (LTP) (Kasai et al., 2010; Rochefort and Konnerth, 2012), a well-studied form of synaptic plasticity that forms the cellular basis of hippocampal-dependent learning and memory (Herron et al., 1986; O'Dell et al., 1991). Gp120 has been shown to inhibit LTP (Sanchez-Alavez et al., 2000; Dong and Xiong, 2006), which would be consistent with a reduced spine density in the hippocampus described in gp120tg mice (Bachis et al., 2016b). In this study, we have provided preliminary but complimentary data showing that gp120 decreases the levels of NR2A and 2B subunits. This decrease is particularly important because these subunits play a role in glutamate-mediated synaptic plasticity (Liu et al., 2004; von Engelhardt et al., 2008). Moreover, both PSD95 and NR subunits, are considered markers for excitatory post-synaptic sites (Sheng, 2001). Intriguingly, gp120 failed to change the levels of synaptophysin, a synaptic vesicle membrane protein found predominantly presynaptically (Tarsa and Goda, 2002). Thus, it appears that gp120 may target mostly the post-synaptic membrane. This suggestion, although still speculative, is in line with the fact that neurons release proBDNF (Yang et al., 2009), which then acts on post-synaptic p75NTR to decreases spine density of hippocampal pyramidal neurons (Zagrebelsky et al., 2005; Yang et al., 2014).

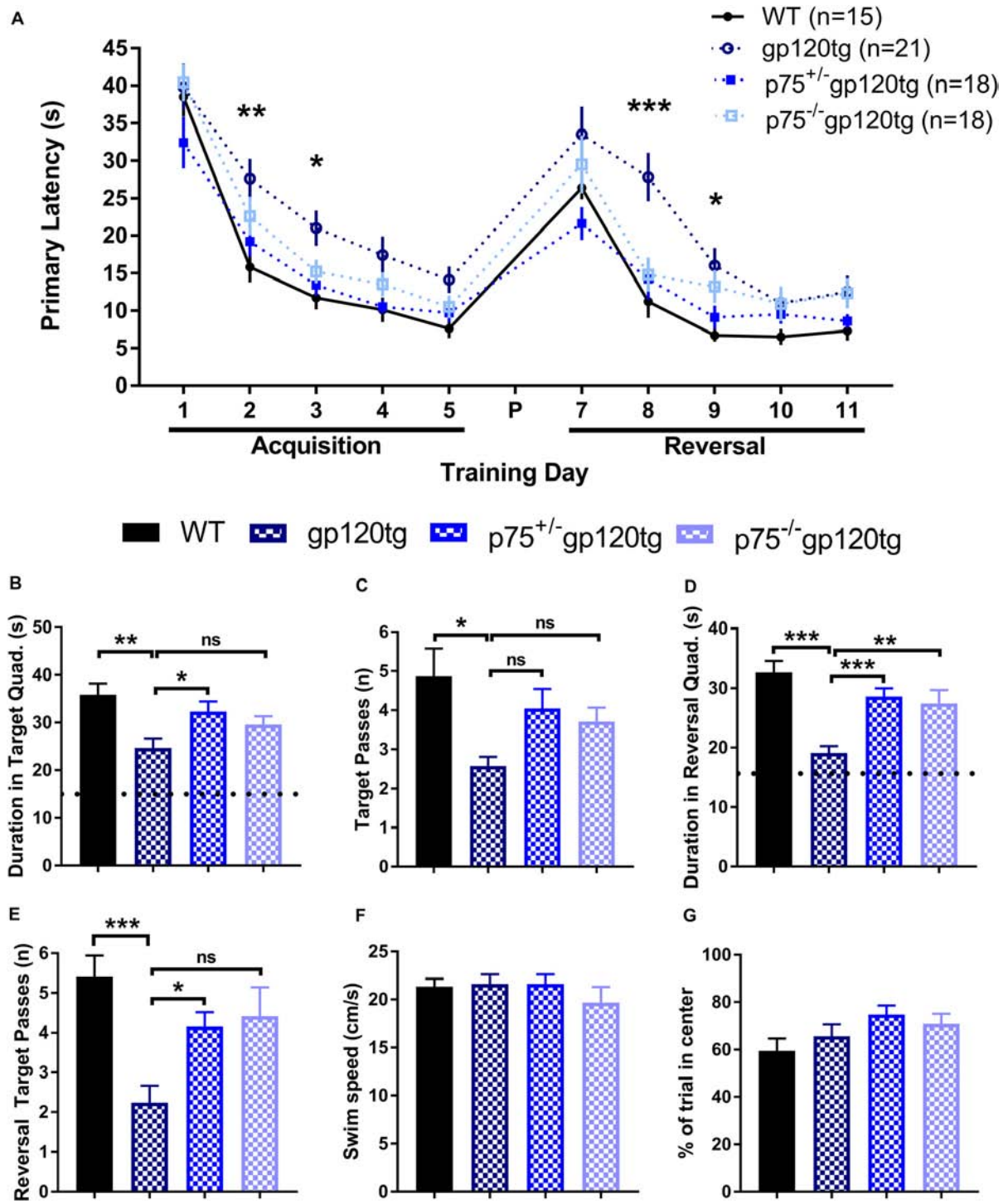

FIGURE 6 | gp120tg mice show impairments in a task of spatial navigation. 8–10 month-old mice were evaluated with the Morris water maze (MWM) navigation task. **(A)** Latency to locate the submerged escape platform in the maze over five training days of acquisition and five days of reversal learning. These two learning phases were separated by a probe (P) on day 6 and a reversal probe on day 12. One-way ANOVA (within each TD) with Tukey's HSD. Each data point represents the genotype mean of the average of an animal's trials on a training day. **(B)** Duration in the target quadrant during the first probe trial. Dotted line indicates chance performance. One-way ANOVA with Tukey's HSD. **(C)** Passes over the former location of the target platform during the probe trial. Kruskal–Wallis with *post hoc* Dunn's. **(D)** Duration in the reversal target quadrant during the first probe trial. Dotted line indicates chance performance. One-way ANOVA with Tukey's HSD. **(E)** Passes over the former reversal escape during the reversal probe trial. Kruskal–Wallis with *post hoc* Dunn's. **(F)** Habituation swim speed and **(G)** percent of first trial in the center of the MWM were taken as control measures for locomotion and motivation to explore the maze, respectively. One-way ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are displayed as mean ± SEM. *n* = number of animals per group.

Cognitive impairment and reduced LTP seen in gp120 mice (Krucker et al., 1998; D'Hooge et al., 1999) correlate with loss of synapses in the hippocampus (Toggas et al., 1994; Lee et al., 2013; Bachis et al., 2016b). These effects appear when mice are at least 6 months old. In the present study, we have used a series of behavioral tests that assess loss of hippocampal connections to determine whether memory impairment in gp120tg mice could be abolished by the removal of *p75NTR* alleles. We have found that the hippocampal-dependent memory deficits observed in 8–10mo gp120tg mice is attenuated when either one or both *p75NTR* alleles are removed. Thus, reduced expression of *p75NTR*, which has been shown to slow down cognitive decline in an animal model of Alzheimer's disease (Qian et al., 2018), not only inhibits the loss of hippocampal spines that we have previously described (Bachis et al., 2016b) but also precludes the impairment in memory observed in 8–10mo gp120tg mice. In addition, we observed that the removal of *p75NTR* alleles reduces the impairments seen in the MWM reversal phase in gp120tg mice. Reversal learning has multiple neural substrates in rodents independent of the hippocampus, including the subnuclei of the basal forebrain and the prefrontal cortex (Ghods-Sharifi et al., 2008; Tait and Brown, 2008). Although we cannot exclude that the ability of gp120 to increase proBDNF in any of these areas may underlie a reversal learning impairment, it is difficult to interpret reversal learning deficits when impairments in general spatial learning are also seen within the MWM test. Therefore, we exert caution in interpretation of this curious finding and recognize that more rigorous assays of reversal learning are needed in future studies with this specific model of HAND.

The mechanism(s) whereby proBDNF activation of the p75NTR reduces spine density remains to be established. p75NTR, after binding to sortilin family member SorCS2, activates several signaling pathways that are crucial for neuronal degeneration. These include c-Jun N-terminal kinase (JNK) (Friedman, 2000; Salehi et al., 2002), the RhoA (Park et al., 2010) and the NF-kB pathways (Carter et al., 1996). JNK is also activated by the HIV protein gp120 (Meucci et al., 1998; Bodner et al., 2004; Singh et al., 2005), suggesting a common neurotoxic mechanism between viral proteins and p75NTR. Experimental studies have also shown that p75NTR, destabilizes actin filaments through inactivation of Rac/fascin interaction (Deinhardt et al., 2011). Actin influences spine morphology and stability (Rust et al., 2010). Moreover, p75NTR has been shown to inhibit neurite outgrowth by interacting with the Nogo receptor complex (Barker, 2004) and Ephrin-A (Lim et al., 2008), important components of synapses and promoters of spine morphogenesis (Lai and Ip, 2009). On the other hand, we need to consider that the hippocampus of gp120tg mice as well as HAND subjects exhibits lower levels of BDNF than controls (Bachis et al., 2012, 2016b). BDNF has been shown to promote maturation and density of dendritic spines (Orefice et al., 2013). Thus, a reduction in BDNF levels in favor to proBDNF levels, as seen in gp120tg mice and HAND subjects, may accelerate synaptic pruning. Moreover, we cannot exclude that gp120-mediated synaptic pruning is linked to the ability of the envelope protein to decrease the levels of BDNF receptor trkB (Bachis et al., 2016b). This receptor modulates synaptic plasticity and spine density in the adult hippocampus (Yacoubian and Lo, 2000; Otal et al., 2005), as well as participates in spine maintenance (Chapleau and Pozzo-Miller, 2012). Higher proBDNF and lower trkB levels have been discovered in the postmortem hippocampus of HAND subjects compared to non-cognitive impaired HIV subjects (Bachis et al., 2012, 2016b). Thus, HIV, through gp120, may promote synaptic pruning by a combination of increased p75NTR activation and a decreased trkB function. More experiments are needed to fully understand these mechanisms.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

All studies were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. The protocol was approved by the Georgetown University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

IM designed the experiments and wrote the manuscript. AS designed and performed the behavioral studies, analyzed the data, and helped with the writing of the manuscript. GA and SS performed the molecular biology experiments and analyzed the data. VA designed, performed, analyzed the *in vitro* experiments, and helped with the writing of the manuscript. PF assisted with the interpretation of the behavioral data. All authors reviewed the results and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2019.00398/full#supplementary-material

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