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RhoA is dispensable for axon guidance of sensory neurons in the mouse dorsal root ganglia

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RhoA, a member of the Rho family small GTPases, has been shown to play important roles in axon guidance. However, to date, the physiological function of RhoA in axon guidance events *in vivo* has not been determined genetically in animals. Here we show that *RhoA* mRNA is strongly expressed by sensory neurons in the developing mouse dorsal root ganglia (DRG). We have deleted *RhoA* in sensory neurons of the DRG using *RhoA*-floxed mice under the *Wnt1-Cre* driver in which Cre is strongly expressed in sensory neurons. Peripheral projections of sensory neurons appear normal and there are no detectable defects in the central projections of either cutaneous or proprioceptive sensory neurons in $RhoA^{f/f}$; *Wnt1-Cre* mice. Furthermore, a co-culture assay using DRG explants from $RhoA^{f/f}$; *Wnt1-Cre* embryos, and 293T cells expressing semaphorin3A (Sema3A) reveals that RhoA is not required for Sema3A-mediated axonal repulsion of sensory neurons. Expression of RhoC, a closely related family member, is increased in *RhoA*-deficient sensory neurons and may play a compensatory role in this context. Taken together, these genetic studies demonstrate that RhoA is dispensable for peripheral and central projections of sensory neurons in the DRG.

Keywords: RhoA, axon guidance, semaphorin, dorsal root ganglia, cutaneous sensory neurons, proprioceptive sensory neurons, spinal cord

INTRODUCTION

RhoA, a member of the small Rho GTPase family that regulates the cytoskeleton, has been implicated in various processes during the nervous system development, including the formation of adherens junctions, neuronal migration, and axon guidance (Giniger, 2002; Guan and Rao, 2003; Gallo and Letourneau, 2004; Heasman and Ridley, 2008; Hall and Lalli, 2010). The functions of RhoA in the mammalian nervous system have mainly been discerned from studies using a dominant negative or a knockdown approach. The physiological roles and functions of RhoA in the mammalian nervous system have just begun to be elucidated by loss-of-function studies using conditional gene-targeting strategies (Herzog et al., 2011; Katayama et al., 2011; Cappello et al., 2012). These recent studies demonstrate that RhoA is essential for proper formation of adherens junctions and proliferation of neural progenitor cells in the mouse nervous system (Herzog et al., 2011; Katayama et al., 2011; Cappello et al., 2012), which is consistent with previous *in vitro* and invertebrate studies (Fukata and Kaibuchi, 2001; Bloor and Kiehart, 2002; Magie et al., 2002; Yamada and Nelson, 2007). However, it remained unclear whether RhoA is required for other functions, including axon guidance, in the mammalian nervous system.

Extensive studies using *in vitro* culture experiments have shown that activation of RhoA induces growth cone collapse and axonal repulsion by increasing actomyosin contractility (Giniger, 2002; Guan and Rao, 2003; Gallo and Letourneau, 2004; Heasman and Ridley, 2008; Hall and Lalli, 2010). For example, *in vitro* experiments have implicated RhoA in Sema3A-mediated growth cone collapse of sensory neurons in the DRG (Dontchev and Letourneau, 2002; Wu et al., 2005; Hengst et al., 2006). Suppression of ROCK, a RhoA effector, by pharmacological inhibitors reduces Sema3A-induced growth cone collapse (Dontchev and Letourneau, 2002). In addition, Sema3A induces local translation of RhoA, and a knockdown approach reveals that RhoA is necessary for Sema3A-mediated growth cone collapse of DRG sensory neurons (Wu et al., 2005; Hengst et al., 2006). Despite these previous studies, the requirement of RhoA in Sema3A-dependent or -independent axonal repulsion *in vivo* during mammalian nervous system development remains unanswered.

To determine the physiological roles of RhoA in axon guidance, we have taken a loss-of-function approach. Since *RhoA* is strongly expressed by DRG neurons during development, *RhoA* was deleted from the DRG using *RhoA*-floxed mice together with *Wnt1-Cre* or *Advillin-Cre* mice in which Cre is expressed in the DRG. Surprisingly, loss of RhoA does not cause any obvious defects in the peripheral or central projections of DRG sensory neurons. In addition, RhoA is not required for Sema3A-mediated DRG axonal repulsion. Importantly, the protein level of RhoC, a related family member, is up-regulated in DRGs from $RhoA^{f/f}$; *Wnt1-Cre* embryos. Taken together, these findings suggest that RhoA itself is not essential for axon guidance of DRG sensory

neurons and that RhoC may compensate for RhoA function in the DRG *in vivo*.

MATERIALS AND METHODS

MICE

The following mouse strains were used in this study: *RhoA*-floxed (Chauhan et al., 2011; Katayama et al., 2011; Melendez et al., 2011), *Wnt1-Cre* (Danielian et al., 1998), and *Advillin-Cre* (da Silva et al., 2011). We used $RhoA^{f/w}$; *Wnt1-Cre* or $RhoA^{f/w}$; *Advillin-Cre* mice as controls.

TISSUE PREPARATION

Spinal cords and their surrounding tissues were dissected from embryos at embryonic day (E) 10.5, E13.5, E15.5, E16.5, E17.5, and postnatal day (P) 1. They were then fixed with 4% paraformaldehyde (PFA) on ice for 2 h for immunofluorescence staining or overnight for *in situ* hybridization. Afterwards, they were cryoprotected in 30% sucrose, embedded in OCT compound, and sectioned at 16 μm.

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For immunofluorescence, cryosections were stained with the following antibodies: rabbit anti-parvalbumine (PV) (Swant), rabbit anti-TrkA (R&D systems), goat anti-TrkC (R&D systems), rabbit anti-CGRP (Peninsula Lab), and guinea pig anti-vGlut1 (Chemicon). Alexa 488 and Cy3-conjugated secondary antibodies were purchased from Invitrogen and Jackson Immuno Research. Immunohistochemistry was performed as described (Leslie et al., 2011). Images were obtained using a LSM510 confocal microscope (Zeiss).

In situ HYBRIDIZATION

Digoxigenin (DIG)-labeled cRNA probes were used for *in situ* hybridization as described Schaeren-Wiemers and Gerfin-Moser (1993).

DRG REPULSION ASSAY

DRG explants from E12.5 embryos were co-cultured with 293T cell aggregates expressing Sema3A and/or GFP in collagen gel matrices for 48 h in the presence of NGF, then fixed with 4% PFA, and immunostained with mouse anti-Tuj1 antibody (Covance), and Cy3-conjugated secondary antibody. Images were obtained on an Axioplan microscope (Zeiss).

WHOLE-MOUNT IMMUNOSTAINING

Whole-mount immunostaining was performed on E10.5 embryos for neurofilament staining or E13.5 embyos for peripherin staining according the method described by Huber et al. (2005) and Mandai et al. (2009), respectively. Briefly, the embryos were fixed with 4% PFA overnight, after which they were rinsed with phosphate buffered saline (PBS) three times and then fixed in Dent's fix (20% DMSO, 80% Methanol) overnight. The embryos were washed in PBS three times. The primary antibody, anti-neurofilament 2H3 (Developmental Studies Hybridoma Bank) or rabbit anti-peripherin (Millipore) was added in blocking solution (5% normal goat serum, 75% PBS, 20% DMSO) and incubated at room temperature for 3–4 days. The embryos were washed with PBS five times for 1 h each. Then, an Alexa 488-conjugated secondary antibody (Invitrogen) was added in blocking solution and kept in the dark for 1–2 days. The embryos were washed five times in PBS before being viewed.

IMMUNOBLOTTING

Samples were lysed using RIPA buffer (Cell Signaling Technology) according to manufactory's suggestion. Supernatants were collected for SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories). Specific protein expression was detected using the following antibodies: anti-RhoA (Cell Signaling Technology), anti-RhoC (Cell Signaling Technology), anti-Cdc42 (Cell Signaling Technology), anti-Lamin B (Santa cruz), and anti-Rac1 (BD Transduction Laboratories).

RESULTS

RhoA IS EXPRESSED BY BOTH SENSORY AND MOTOR NEURONS

To examine the expression pattern of *RhoA*, we performed *in situ* hybridization at various time points during development in the lumbar spinal cord and the DRG of wild-type mice. At E10.5, *RhoA* appears to be ubiquitously expressed throughout the spinal cord but showed relatively high expression in motor neurons and neural progenitors (**Figure 1A**). *RhoA* was expressed by most or all DRG sensory neurons at E10.5 (**Figure 1E**). At E13.5, strong expression of *RhoA* was detected in motor neurons (**Figure 1B**). At E16.5 and P1, similar to E13.5, *RhoA* was ubiquitously expressed in the spinal cord with high expression in motor neurons (**Figures 1C,D**). In the DRG, strong expression of *RhoA* was detected throughout development from E10.5 to P1 (**Figures 1E–H**). These expression analyses suggest that RhoA may have a role in spinal neurons including motor neurons and DRG sensory neurons. In this study, we focus on the expression of *RhoA* in the DRG.

NO OBVIOUS DEFECTS IN PERIPHERAL PROJECTIONS OF SENSORY NEURONS IN $RhoA^{f/f}$; *Wnt1-Cre* EMBRYOS

To determine the physiological roles of RhoA in DRG sensory neurons, we deleted *RhoA* in DRG sensory neurons using *RhoA*-floxed mice (Chauhan et al., 2011; Katayama et al., 2011; Melendez et al., 2011) together with *Wnt1-Cre* mice (Danielian et al., 1998; Hsu et al., 2010). Cre is expressed in the DRG and in the dorsal spinal cord of *Wnt1-Cre* mice (Danielian et al., 1998; Hsu et al., 2010). We confirmed that most RhoA expression was indeed deleted from DRG sensory neurons by performing Western blot analysis on DRG tissues from E12.5 control and $RhoA^{f/f}$; *Wnt1-Cre* embryos (**Figure 2**). As shown in **Figure 2**, RhoA was greatly reduced in $RhoA^{f/f}$; *Wnt1-Cre* embryos compared to control embryos. The expression of Cdc42 as well as Rac1 in the DRG was not changed in $RhoA^{f/f}$; *Wnt1-Cre* embryos compared to control embryos (**Figure 2**).

We first examined the peripheral projections of DRG sensory neurons of E10.5 $RhoA^{f/f}$; *Wnt1-Cre* embryos. To do this, we performed whole-mount immunostaining using anti-neurofilament antibody, which visualizes peripheral axonal projections of both sensory and motor neurons. DRG sensory neurons projected axons to the peripheral tissues of E10.5 $RhoA^{f/f}$; *Wnt1-Cre*

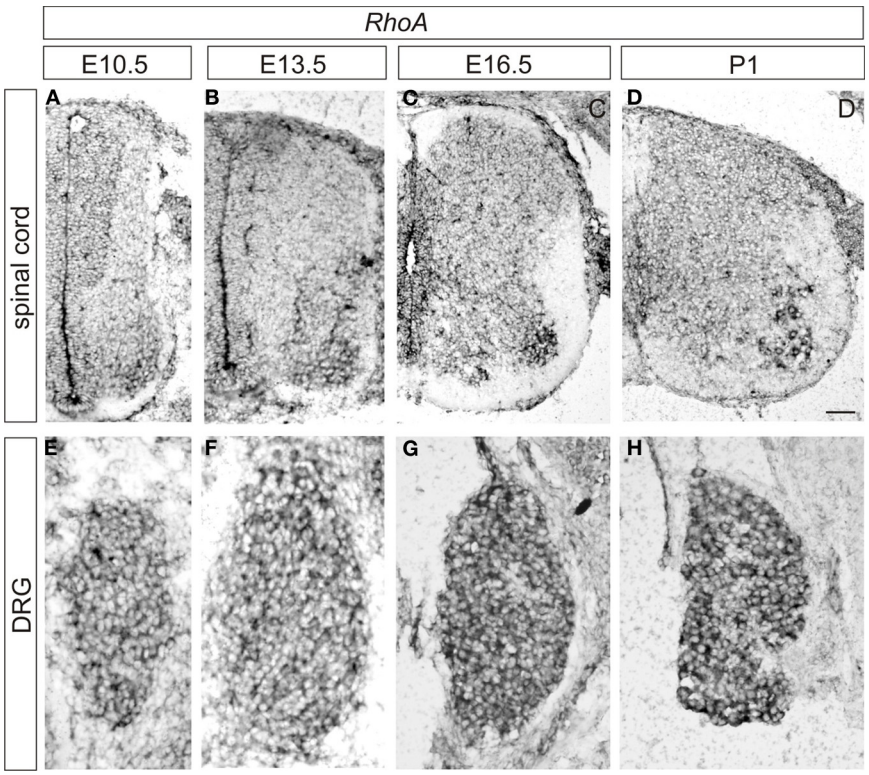

FIGURE 1 | Expression of *RhoA* in the developing mouse DRG and spinal cord. (A–D) Expression of *RhoA* in the spinal cord at E10.5 **(A)**, E13.5 **(B)**, E16.5 **(C)**, and P1 **(D)**. **(E–H)** Expression of *RhoA* in the DRG at E10.5 **(A)**, E13.5 **(B)**, E16.5 **(C)**, and P1 **(D)**. Scale bar, 50 μm.

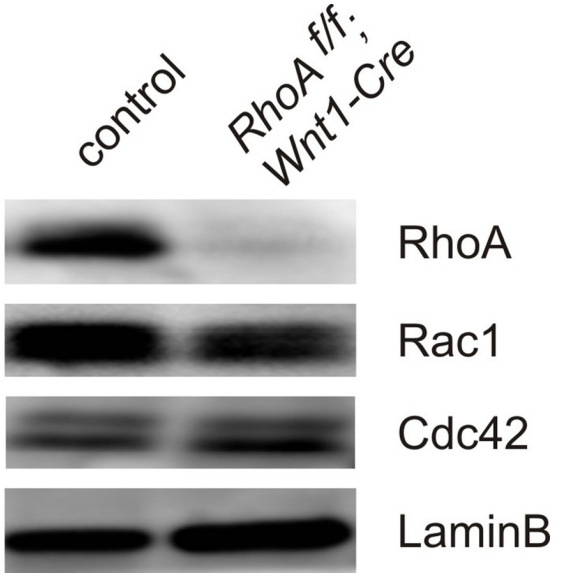

FIGURE 2 | Expression of RhoA, Rac1, and Cdc42 in DRGs from E12.5 control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos. RhoA, Rac, and Cdc42 proteins were evaluated by Western blotting using DRGs from E12.5 control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos. RhoA protein was significantly reduced in DRGs from *RhoA*$^{f/f}$*; Wnt1-Cre* embryos compared to control DRGs. *RhoA* deletion did not affect protein expression of Rac1 and Cdc42. We examined expression of LaminB protein as an internal control.

embryos similar to control embryos (**Figures 3A–D**). To further examine peripheral projections at E13.5, we performed whole-mount anti-peripherin immunostaining to visualize the peripheral axons in the distal limb (Mandai et al., 2009). We did not find any obvious defects in peripherin^{+} peripheral axons in the distal limb of E13.5 *RhoA*$^{f/f}$*; Wnt1-Cre* embryos (**Figures 3E,F**). Although we cannot exclude the subtle defects in peripheral axons in *RhoA*$^{f/f}$*; Wnt1-Cre* embryos, RhoA is unlikely to have a major role in peripheral projections of DRG sensory neurons.

PROPRIOCEPTIVE AXONAL PROJECTIONS SHOW TYPICAL PATTERNING IN *RhoA*$^{f/f}$*; Wnt1-Cre* EMBRYOS

Next, we examined central projections of DRG sensory neurons in the spinal cord. DRG sensory neurons are subdivided into two major groups, proprioceptive and cutaneous sensory neurons (Brown, 1981; Koerber and Mendell, 1992). Proprioceptive neurons convey information about the state of muscle contraction and limb position, whereas cutaneous neurons mediate a wide range of noxious and innocuous stimuli (Brown, 1981; Koerber and Mendell, 1992). Proprioceptive sensory afferents project to the intermediate or ventral spinal cord, while cutaneous sensory neurons project their axons to the superficial dorsal horn (Brown, 1981; Koerber and Mendell, 1992). We analyzed the numbers of proprioceptive sensory neurons and proprioceptive axonal projections using an anti-Pv antibody, which marks all proprioceptive sensory neurons (Honda, 1995; Arber et al., 2000),

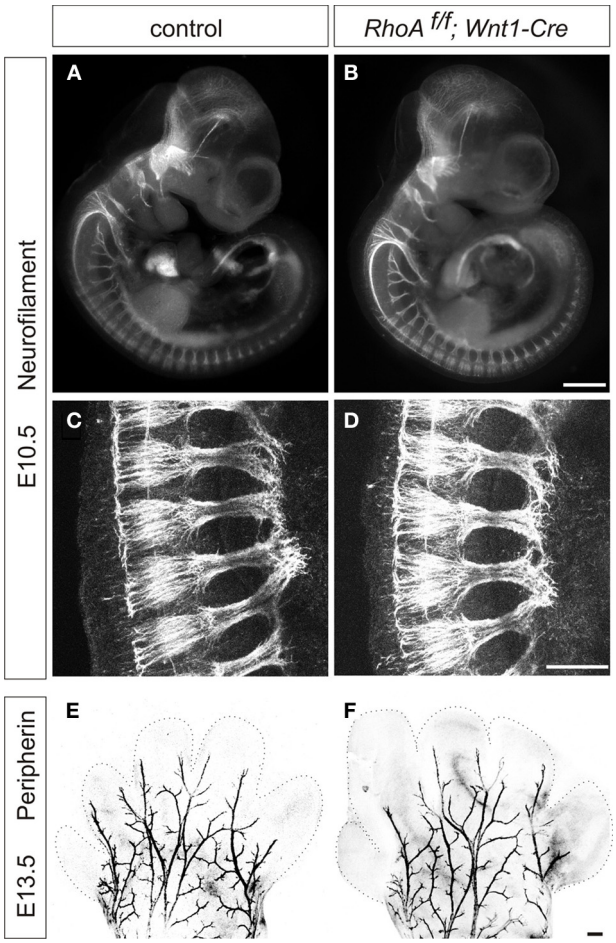

FIGURE 3 | Whole-mount neurofilament and peripherin staining. **(A–D)** Control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos were stained with anti-neurofilament (NF) antibody at E10.5. **(C,D)** show enlarged views of Plexus region. **(E,F)** Forelimbs of control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos were stained with anti-peripherin antibody at E13.5. There were no obvious defects in NF^{+} or peripherin^{+} axons in *RhoA*$^{f/f}$*; Wnt1-Cre* embryos compared to control embryos. Scale bars, 100 μm **(B,F)** and 20 μm **(D)**.

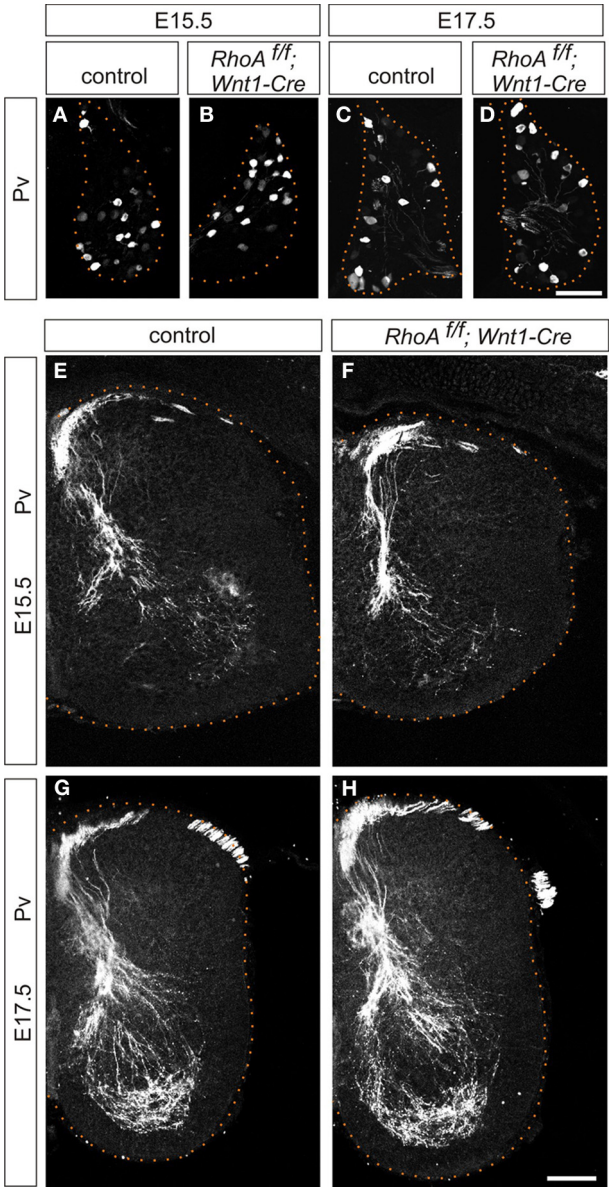

FIGURE 4 | Proprioceptive sensory neurons and proprioceptive axonal projections in the spinal cord of *RhoA*$^{f/f}$*; Wnt1-Cre* embryos. (A–D) Pv^{+} proprioceptive sensory neurons in DRGs from control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos at E15.5 and E17.5. **(E–H)** Pv^{+} proprioceptive axonal projections in the spinal cord of control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos at E15.5 and E17.5. Scale bars, 100 μm.

in *RhoA*$^{f/f}$*; Wnt1-Cre* embryos. There was no difference in the numbers of Pv^{+} proprioceptive sensory neurons in the DRG between control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos at E15.5 and E17.5 (**Figures 4A–D**). In control embryos at E15.5 and E17.5, the proprioceptive axons entered the spinal cord medially and projected to the ventral spinal cord (**Figures 4E,G**). There were no obvious defects in proprioceptive axonal projections at E15.5 and E17.5 in *RhoA*$^{f/f}$*; Wnt1-Cre* embryos compared to control embryos (**Figures 4E–H**). Thus, these data suggest that RhoA is not necessary for establishing proprioceptive axonal trajectories in the spinal cord.

RhoA IS NOT INVOLVED IN ESTABLISHING CUTANEOUS AXONAL PROJECTIONS

We next analyzed cutaneous sensory neurons in *RhoA*$^{f/f}$*; Wnt1-Cre* embryos. To do this we performed immunohistochemistry with an anti-TrkA antibody, which marks both cell bodies and axons of cutaneous sensory neurons during mouse embryogenesis. The numbers of TrkA^{+} cutaneous sensory neurons in the DRG were not changed between E13.5–E17.5 control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos (**Figures 5A,C,E,G,I,K**). We also analyzed cutaneous axonal projections in the spinal cord in *RhoA*$^{f/f}$*; Wnt1-Cre* embryos. Cutaneous axons did not penetrate the spinal cord at E13.5, but penetrated the spinal cord laterally and projected in the dorsal spinal cord at E15.5 and E17.5

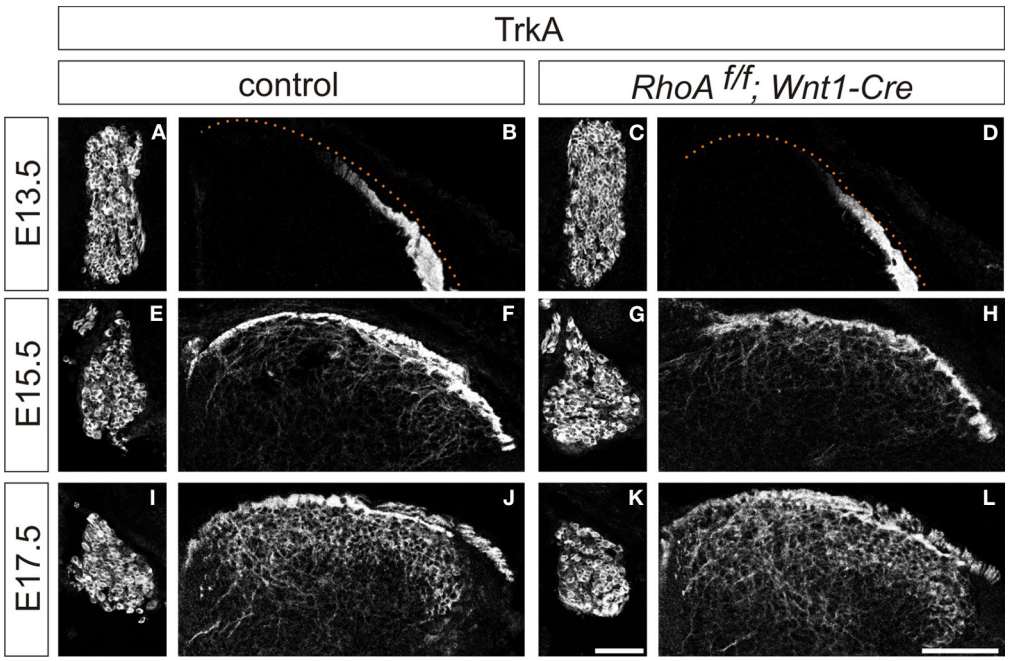

FIGURE 5 | Cutaneous sensory neurons and cutaneous axonal projections in the spinal cord of *RhoA*$^{f/f}$*; Wnt1-Cre* embryos. (**A,E,I** and **C,G,K**) TrkA^{+} cutaneous sensory neurons in DRGs from control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos at E15.5 and E17.5. (**B,F,J** and **D,H,L**) TrkA^{+} cutaneous axonal projections in the spinal cord of control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos at E15.5 and E17.5. Scale bars, 100 μm.

in control embryos (**Figures 5B,F,J**). *RhoA*-deficient cutaneous axons displayed axonal trajectories similar to control embryos at E13.5, E15.5, and E17.5 (**Figures 5D,H,L**). Thus, RhoA is not necessary for the establishment of proper cutaneous axonal projections in the spinal cord at these embryonic stages. During postnatal development, cutaneous sensory neurons are further subdivided into different groups, which project axons to different laminae within the dorsal spinal cord. These different types of neurons are marked by different molecular markers. For example, calcitonin-gene-related-peptide (CGRP)-positive thinly myelinated cutaneous axons terminate in lamina I and outer lamina II of the dorsal horn in the spinal cord (Lawson, 2002). Isolectin IB4-, a marker of some primary afferent C fibers, positive and non-myelinated cutaneous axons, terminate in lamina II (Molliver et al., 1997; Fang et al., 2006). Furthermore, vesicular glutamate transporter 1 (vGlut1)-positive myelinated cutaneous afferents terminate in laminae III–V (Todd et al., 2003). Since most *RhoA*$^{f/f}$*; Wnt1-Cre* mice died before birth with severe brain defects (Katayama et al., 2011; data not shown), we used another Cre driver mouse line, *Advillin-Cre* (da Silva et al., 2011), whose expression starts at E12.5 in the DRG (Hasegawa et al., 2007). *RhoA*$^{f/f}$*; Advillin-Cre* mice were born in normal numbers and survived into adulthood. We examined CGPR^{+}, IB4^{+}, and vGlut1^{+} axonal projections of cutaneous sensory neurons in the P8 spinal cord of *RhoA*$^{f/f}$*; Advillin-Cre* mice. There was clear laminar segregation of different classes of cutaneous sensory axons both in control and *RhoA*$^{f/f}$*; Advillin-Cre* mice (**Figure 6**). Therefore, RhoA is unlikely to be involved in regulating laminar specific cutaneous axonal projections in the spinal cord during early mouse postnatal stages.

RhoA IS NOT REQUIRED FOR Sema3A-MEDIATED AXONAL REPULSION

Since it has been reported that RhoA is required for Sema3A-mediated growth cone collapse of DRG sensory neurons using a knockdown approach (Wu et al., 2005; Hengst et al., 2006), we examined Sema3A-mediated axonal repulsion of DRG neurons from *RhoA*$^{f/f}$*; Wnt1-Cre* embryos. To do this we performed co-cultures of E12.5 DRG explants from control or *RhoA*$^{f/f}$*; Wnt1-Cre* embryos with 293T cells expressing GFP and/or Sema3A. E12.5 DRG axons from control embryos were repelled by Sema3A (**Figure 7B**). Similarly, *RhoA*-deficient DRG axons were also repelled by Sema3A (**Figure 7D**). This is in comparison to the unperturbed axonal growth of either set of DRGs in the presence of only GFP-transfected 293T cells (**Figures 7A,C**). These data suggest that RhoA itself is not essential for Sema3A-mediated DRG axonal repulsion.

RhoC IS UP-REGULATED IN THE DRG IN THE ABSENCE OF RhoA

Since RhoA has other related family members (Wennerberg and Der, 2004; Wheeler and Ridley, 2004), they may have a redundant function with RhoA in the DRG. Therefore, we examined the expression of RhoC by Western Blot analysis, since RhoC seems to be functionally the closest family member to RhoA (Wennerberg and Der, 2004; Wheeler and Ridley, 2004). We found that RhoC was significantly up-regulated in the DRG from *RhoA*$^{f/f}$*; Wnt1-Cre* embryos compared to control embryos (**Figure 8**). These results suggest that RhoC may compensate for RhoA in the DRG.

DISCUSSION

In this study we show that the conditional deletion of *RhoA* in the DRG using either *Wnt1-Cre* or *Advillin-Cre* drivers does not have

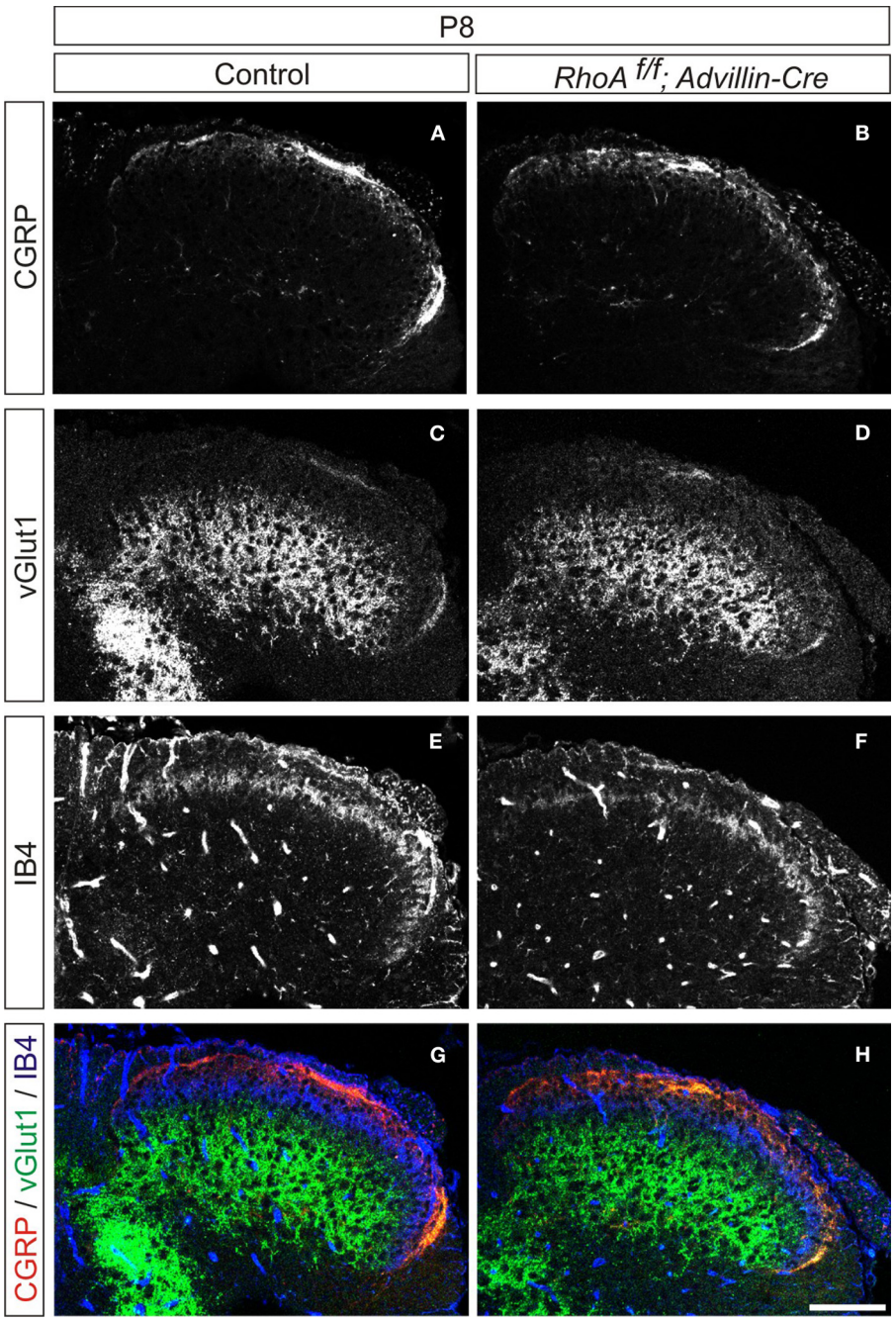

FIGURE 6 | Organization of cutaneous afferent projections in *RhoA*$^{f/f}$*; Advillin-Cre* mice. (A,B) CGRP expression, **(C,D)** vGlut1 expression, **(E,F)** IB4-binding, and **(G,H)** merged views of CGRP (red), vGlut1 (green), and IB4-binding (blue) in P8 control and *RhoA*$^{f/f}$*; Advillin-Cre* mice. Scale bar, 100 μm.

any obvious effect on either peripheral or central projections of DRG sensory neurons. In addition, loss of RhoA in the DRG does not change responses of DRG axons to Sema3A. Furthermore, RhoC protein is increased in the DRGs of *RhoA*$^{f/f}$*; Wnt1-Cre* embryos compared to control embryos. This suggests that RhoA itself is not required for axon guidance of DRG sensory neurons and that the related protein RhoC may compensate for loss of RhoA function.

LOSS OF RhoA DOES NOT CAUSE ANY OBVIOUS DEFECTS IN PROPRIOCEPTIVE OR CUTANEOUS AXONAL PROJECTIONS

Many previous *in vitro* and *in vivo* studies have suggested important roles for RhoA in axon guidance (Giniger, 2002; Guan and Rao, 2003; Gallo and Letourneau, 2004; Heasman and Ridley, 2008; Hall and Lalli, 2010). However, it remained unknown whether RhoA is in fact required for axon guidance events *in vivo* in the mammalian nervous system, since a loss-of-function

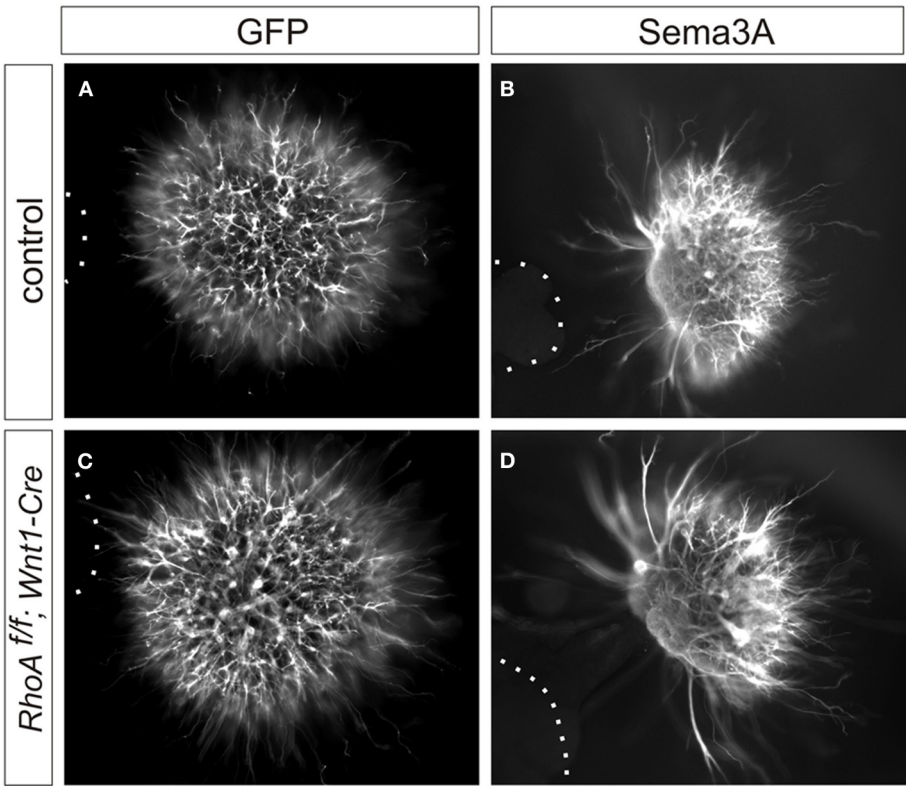

FIGURE 7 | Sema3A-induced DRG axonal repulsion *in vitro*. (A–D) Co-culture of DRG explants from control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos with GFP **(A,C)**, or Sema3A and GFP **(B,D)** transfected cell aggregates. The axons of DRG neurons were examined by anti-TuJ1 antibody. Both DRG axons from control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos were repelled by Sema3A. Dotted lines outline aggregates of 293T cells.

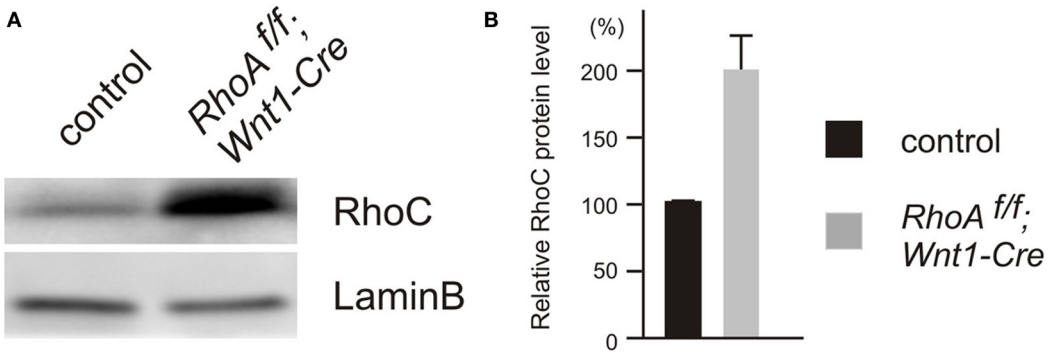

FIGURE 8 | Expression of RhoC in DRGs from E12.5 control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos. (A) RhoC protein was evaluated by Western blotting using DRGs from E12.5 control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos, and RhoC protein was up-regulated in DRGs from *RhoA*$^{f/f}$*; Wnt1-Cre* embryos compared to control DRGs. **(B)** The quantification of levels of RhoC protein expression in DRGs from E12.5 control and *RhoA*$^{f/f}$*; Wnt1-Cre* embryos. We determined expression of LaminB protein as an internal control ($n = 4$).

approach had not been performed to test the physiological roles of RhoA in axon guidance. In this study, we found that *RhoA* mRNA is strongly expressed in the DRG during mouse development, and we deleted *RhoA* in DRG neurons using either *Wnt1-Cre* or *Advillin-Cre* drivers. Surprisingly, we found that RhoA is not essential for mouse DRG neurons to project and reach their peripheral and central targets. First, we found that deletion of *RhoA* using *Wnt1-Cre* mice did not cause any aberrant peripheral axonal projections of sensory neurons that were detectable using anti-neurofilament and anti-peripherin antibodies. Second, there was no obvious disruption in the axon guidance of central projections of proprioceptive or cutaneous sensory axons. Therefore, it appears that RhoA itself is not crucial for the proper axonal pathfinding of DRG sensory neurons.

RhoA-DEFICIENT SENSORY AXONS STILL RESPOND TO Sema3A

The small Rho GTPases have been shown to control axon guidance in part through semaphorin-plexin signaling (Kruger et al., 2005; Tran et al., 2007). For example, *RhoA* mRNA is localized to axons and growth cones of DRG sensory neurons, and this localization is mediated by an axonal targeting element located in the 3′ untranslated region of *RhoA* (Wu et al., 2005). Sema3A induces intra-axonal translation of *RhoA* mRNA, and this local RhoA translation has been suggested to be necessary for Sema3A-mediated growth cone collapse using a knockdown approach (Wu et al., 2005; Hengst et al., 2006). However, our loss-of-function study together with DRG explants cultured with Sema3A expressing 293T cells reveals that RhoA itself is not necessary for Sema3A-mediated axonal repulsion. The difference in the approach between loss-of-function and acute knockdown by siRNA or particular assay parameters to examine growth cone collapse or axonal repulsion may explain this discrepancy. Loss of Sema3A or its receptor neuropilin1 (Npn1) causes defects in fasciculation of peripheral motor and sensory axons (Behar et al., 1996; Kitsukawa et al., 1997; Taniguchi et al., 1997; Gu et al., 2003; Huettl et al., 2011). Our results showing no obvious defects in fasciculation of *RhoA*-deficient sensory axons (**Figure 3**) further support the idea that Sema3A-Npn1 signaling is present in *RhoA$^{f/f}$; Wnt1-Cre* mice.

RhoC EXPRESSION IS UP-REGULATED IN THE DRGs FROM *RhoA$^{f/f}$; Wnt1-Cre* EMBRYOS

There are many different types of small Rho GTPases, and the Rho GTPases with the most similar structure to that of RhoA are RhoB and RhoC (Wennerberg and Der, 2004; Wheeler and Ridley, 2004). RhoA, RhoB, and RhoC are identical in approximately 85% of their protein sequence (Wennerberg and Der, 2004; Wheeler and Ridley, 2004), however, the localization of RhoB is different from that of RhoA and RhoC. RhoA and RhoC are both localized in the cytoplasms or at the plasma membrane, whereas RhoB is generally found in the endosomal membranes since RhoB has a unique C-terminal lipid modifications and controls endosomal trafficking of membrane receptors (Adamson et al., 1992; Wheeler and Ridley, 2004; Heasman and Ridley, 2008). In addition, RhoB seems to have a growth inhibitory effect, whereas RhoA and RhoC have the opposite effect (Du and Prendergast, 1999; Chen et al., 2000; Wennerberg and Der, 2004). Thus, RhoB is likely to be functionally distinct from RhoA and RhoC. We also found that RhoC is significantly up-regulated in the DRG from *RhoA$^{f/f}$; Wnt1-Cre* embryos. Therefore, RhoC might be able to compensate for RhoA in the DRG. The analysis of *RhoA* and *RhoC* double mutants will give us valuable information about the possibility of the functional redundancy between RhoA and RhoC in the nervous system in the future.

In conclusion, our findings presented here using a loss-of-function approach demonstrate that RhoA itself is not essential for axonal projections of DRG sensory neurons and that RhoC may compensate for RhoA in the DRG. Further studies will reveal whether RhoA itself is required for the axon guidance of other types of neurons in the nervous system and whether RhoA and RhoC have redundant functions in the DRG and other regions of the mammalian nervous system.

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