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

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INTRODUCTION

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; Wnt1-Cre mice. Furthermore, a co-culture assay using DRG explants from Rhoa/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
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 RhoAf/w; Wnt1-Cre or RhoAf/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 for 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.

**IMMUNOFLUORESCENCE**

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), 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 manufacturer’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 and was 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 RhoAf/w; 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 RhoAf/w; Wnt1-Cre embryos (Figure 2). As shown in Figure 2, RhoA was greatly reduced in RhoAf/w; Wnt1-Cre embryos compared to control embryos. The expression of Cdc42 as well as Rac1 in the DRG was not changed in RhoAf/w; Wnt1-Cre embryos compared to control embryos (Figure 2).

We first examined the peripheral projections of DRG sensory neurons of E10.5 RhoAf/w; 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 RhoAf/w; Wnt1-Cre embryos.
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\textsuperscript{ff}; Wnt1-Cre embryos (Figures 3E,F). Although we cannot exclude the subtle defects in peripheral axons in RhoA\textsuperscript{ff}; 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\textsuperscript{ff}; 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),
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FIGURE 3 | Whole-mount neurofilament and peripherin staining. (A–D) Control and RhoAf/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 RhoAf/f; Wnt1-Cre embryos were stained with anti-peripherin antibody at E13.5. There were no obvious defects in NF+ or peripherin+ axons in RhoAf/f; Wnt1-Cre embryos compared to control embryos. Scale bars, 100 µm (B,F) and 20 µm (D).

in RhoAf/f; Wnt1-Cre embryos. There was no difference in the numbers of Pv+ proprioceptive sensory neurons in the DRG between control and RhoAf/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 RhoAf/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 RhoAf/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 RhoAf/f; Wnt1-Cre embryos (Figures 5A,C,E,G,I,K). We also analyzed cutaneous axonal projections in the spinal cord in RhoAf/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.
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 3D,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; 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; 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; Advillin-Cre mice. There was clear laminar segregation of different classes of cutaneous sensory axons both in control and RhoA+/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; Wnt1-Cre embryos. To do this we performed cocultures of E12.5 DRG explants from control or RhoA+/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; 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
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\(^{+/f}\); 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

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**FIGURE 6 | Organization of cutaneous afferent projections in RhoA\(^{+/f}\); 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\(^{+/f}\); Advillin-Cre mice. Scale bar, 100 \(\mu\)m.
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; Katsukawa et al., 1997; Taniguchi et al., 1997; Gu et al., 2003; Huettl et al., 2011). Our results showing no obvious defects in fasciculation of peripheral motor and sensory axons (Behar et al., 1996; Katsukawa et al., 1997; Taniguchi et al., 1997; Gu et al., 2003; Huettl et al., 2011). Our results showing no obvious defects in fasciculation of peripheral motor and sensory axons (Behar et al., 1996; Katsukawa et al., 1997; Taniguchi et al., 1997; Gu et al., 2003; Huettl et al., 2011).

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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