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Protein Tyrosine Phosphatase Receptor Type O regulates development and function of the sensory nervous system

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Abstract

The roles of protein tyrosine phosphatases (PTPs) in differentiation and axon targeting by dorsal root ganglion (DRG) neurons are essentially unknown. The type III transmembrane PTP, PTPRO, is expressed in DRG neurons, and is implicated in the guidance of motor and retinal axons. We examined the role of PTPRO in DRG development and function using PTPRO$^{-/-}$ mice. The number of peptidergic nociceptive neurons in the DRG of PTPRO$^{-/-}$ mice was significantly decreased, while the total number of sensory neurons appeared unchanged. In addition, spinal pathfinding by both peptidergic and proprioceptive neurons was abnormal in PTPRO$^{-/-}$ mice. Lastly, PTPRO$^{-/-}$ mice performed abnormally on tests of thermal pain and sensorimotor coordination, suggesting that both nociception and proprioception were perturbed. Our data indicate that PTPRO is required for peptidergic differentiation and process outgrowth of sensory neurons, as well as mature sensory function, and provide the first evidence that RPTPs regulate DRG development.

Keywords

tyrosine phosphorylation; nociception; axon guidance; central afferent; pain

INTRODUCTION

The development of the sensory nervous system requires the differentiation of sensory neuron subtypes, coupled with the guidance of their axons to appropriate targets in the spinal cord. Both subtype differentiation and axon guidance are regulated by a family of receptor tyrosine kinases, the tropomyosin-related kinase (Trk) receptors, among others (Huang and Reichardt,

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2003; Masuda et al., 2008; Togashi et al., 2006). For example, differentiation and axon targeting of nociceptive neurons requires signaling through TrkA, and differentiation and targeting of proprioceptive neurons requires signaling through TrkC (Ben-Zvi et al., 2008; Genc et al., 2004; Marmigere and Ernfors, 2007). Although signaling through tyrosine phosphorylation requires the coordinated activity of tyrosine kinases together with protein tyrosine phosphatases (PTPs), essentially nothing is known about the roles of PTPs in sensory development.

Among vertebrate PTPs, the transmembrane receptor PTPs (RPTPs) are most clearly implicated in the regulation of axon guidance. RPTPs with cell adhesion molecule-like extracellular domains (type II and type III RPTPs) have been shown to be important for the guidance of a variety of axons in the central nervous system (Johnson and Van Vactor, 2003). Although two type II RPTPs, PTP-σ and LAR, are known to modulate Trk signaling (Faux et al., 2007; Yang et al., 2006), and to regulate the speed and efficiency of peripheral nerve regeneration (Van der Zee et al., 2003; Xie et al., 2001), their roles in sensory development have not been examined. The roles of type III RPTPs in sensory development and regeneration are unknown.

The type III RPTP known as Protein Tyrosine Phosphatase Receptor type O (PTPRO) is clearly implicated in the developmental guidance of axons. PTPRO is selectively expressed on developing neurons, their axons and their growth cones (Beltran et al., 2003; Bodden and Bixby, 1996; Ledig et al., 1999). In vitro, the PTPRO extracellular domain, consisting of 8 fibronectin type III repeats, inhibits adhesion and neurite outgrowth, causes growth cone collapse, and repels the growth cones of retinal axons (Stepanek et al., 2001). Further, loss of function experiments using RNAi and/or dominant-negative constructs show that PTPRO mediates guidance of motor axons and optic nerve axons in vivo (Shintani et al., 2006; Stepanek et al., 2005). PTPRO is strongly expressed on developing TrkC-positive and Trk-A positive sensory neurons (Beltran et al., 2003), but its functions in sensory differentiation and axon guidance have not been studied. To begin to address the role of PTPRO during development of mammalian sensory neurons, we examined these neurons in PTPRO$^{-/-}$ mice at the time of birth (P0). Sensory development was clearly altered by loss of PTPRO. PTPRO$^{-/-}$ mice had fewer nociceptive peptidergic neurons, while the total number of sensory neurons was unchanged. This difference persisted into the adult. Importantly, pathfinding in neonates was altered both in these nociceptive neurons and in proprioceptive neurons. Finally, adult PTPRO$^{-/-}$ mice performed abnormally on tests of thermal pain and of sensorimotor function. These results indicate that PTPRO is required for sensory neuronal differentiation and process outgrowth, as well as mature sensory function, including the perception of thermal pain.

RESULTS

Decreased number of nociceptive neurons in PTPRO-deficient mice

All populations of DRG sensory neurons, including peptidergic nociceptors, arise from neural crest stem cells in response to Wnt/β-Catenin signaling (Lee, 2004). Migratory neural crest cells from the dorsal neural tube then coalesce to form the developing DRG. Later in DRG development, the neuronal determination gene neurogenin1 (Ngn1) is required for the formation of TrkA-expressing cells (Bertrand, 2002), and Ngn2 is required for the generation of TrkC-expressing cells (Ma et al., 1999). TrkA, TrkB, and TrkC are receptors for the neurotrophins nerve growth factor (NGF), brain derived growth factor (BDNF) and neurotrophin-4/5 (NT 4/5), as well as neurotrophin-3 (NT-3), respectively (Bibel, 2000; Huang, 2001; Huang and Reichardt, 2003). Cells expressing Trk A include the populations that will develop into peptidergic and non-peptidergic nociceptive neurons (Woolf CJ, 2007). The majority of Trk B expressing cells develop into mechanoreceptive neurons, while most of the cells expressing Trk C become proprioceptive neurons.

Nociceptive neurons are small in diameter, and express TrkA at P0; in mature animals roughly 50% of these neurons express the neuropeptides calcitonin gene related peptide (CGRP) and/or substance P (Averill et al., 1995). In contrast, proprioceptive cells are large in diameter, comprise about 20% of the DRG neuronal population at birth, and express TrkC and parvalbumin (PV) (Ernfors et al., 1994). PTPRO is expressed on most TrkC-positive DRG neurons and some TrkA-positive DRG neurons at embryonic day 16 (Beltran et al., 2003), and PTPRO is still expressed in DRG neurons at P0 (unpublished). To determine the effects of PTPRO loss of function on these neuronal populations, we examined lumbar DRG from wildtype and PTPRO-deficient mice at P0, at which time neurogenesis and developmental cell death are complete in wildtype animals (Silos-Santiago et al., 1995). This period coincides with peak PTPRO expression in the brain (Beltran et al., 2003).

The total number of neurons in the L4 DRG was the same in wildtype and PTPRO$^{-/-}$ mice (Figure 1 G-I) suggesting that PTPRO is not necessary for neuronal determination in the DRG or for neuronal survival. The lack of effect on the birth of DRG neurons is consistent with previous studies suggesting that PTPRO is not expressed in neurons until they are post-mitotic (Beltran et al., 2003; Bodden and Bixby, 1996; Ledig et al., 1999). Immunohistochemical analysis with anti-CGRP and anti-PV was carried out to identify the nociceptive and proprioceptive sensory subpopulations, respectively. There were significantly fewer CGRP-immunoreactive neurons in the L4 DRG of PTPRO$^{-/-}$ mice ($p<0.003$; Figure 1A-C). In contrast, there was no difference in the number of PV-immunoreactive neurons (Figure 1D-F). There were no obvious differences between wildtype and PTPRO-deficient mice in the size or morphology of the DRG neurons or ganglia in the sections examined. Taken together, these data indicate that PTPRO is necessary for the appropriate development of the CGRP-expressing subpopulation of DRG neurons. Because neither the total population of neurons nor the TrkC/PV population was changed, the results suggest that some fraction of TrkA/peptidergic neurons failed to express CGRP despite their survival.

To determine whether this decrease in peptidergic neurons was transient, or persists into adulthood, we examined adult (P60) PTPRO$^{-/-}$ mice to determine the fate of the peptidergic neurons. We found that adult DRGs also exhibit a significant decrease in the number of CGRP + neurons compared to wild type controls ($p<0.03$; Fig. 2A-C). In fact, the decrease in CGRP + neurons appears substantially greater (ca. 50%) in the adult than at P0 (Fig. 1C, 2C). As is the case at P0, adult PTPRO$^{-/-}$ mice show no change in the total number of neurons compared to wild type controls (Fig. 2D-F). These data suggest that a loss of PTPRO results in an increase of other neuronal populations in the DRG at the expense of peptidergic nociceptive neurons.

Aberrant nociceptive axon projections in PTPRO$^{-/-}$ *spinal cord*

PTPRO regulates axon guidance in developing motor and retinal neurons (Shintani et al., 2006; Stepanek et al., 2005). To determine if PTPRO also regulates the guidance of sensory axons, we first examined the projections of nociceptive neurons in PTPRO$^{-/-}$ and wt mice. Axons from peptidergic nociceptors (which express Trk A) were examined in P0 mice by immunostaining for CGRP. In normal mice, CGRP-positive axons project to lamina I and the outer lamina II (IIo) of the dorsal horn of the spinal cord (Carlton et al., 2001; Carlton et al., 1987). A fraction of these axons decussates, projecting to the same laminae in the contralateral spinal cord (Ozaki and Snider, 1997). This typical pattern of projection was seen in our wt animals (Fig. 3A).

In contrast, peptidergic nociceptive projections in PTPRO$^{-/-}$ mice were abnormal in three major ways. First, the ventrolateral portion of the nociceptive projection was missing in the PTPRO-deficient mice (Fig. 3D; Supplemental Fig. 1). Second, the dorsal horn projections of CGRP immunoreactive axons in the PTPRO$^{-/-}$ mice appeared less organized than in wt mice, and projected beyond lamina IIo (Figure 3F; Supplemental Fig. 2). Quantification revealed that the

average width of this projection was significantly increased in the PTPRO$^{-/-}$ mice (wt, 26 ± 1.8 μm, n=3; PTPRO$^{-/-}$, 46 ± 3.0 μm; n=6, $p<0.006$). Finally, there was a decreased contralateral projection of CGRP-positive axons in the PTPRO$^{-/-}$ mice (Figure 3H; Supplemental Fig. 3). Each of these nociceptive guidance errors was consistently observed in the PTPRO$^{-/-}$ animals (n=7, wt; n=6, PTPRO$^{-/-}$). Thus, PTPRO is required for appropriate guidance of nociceptive axons. Preliminary evidence suggests that PTPRO$^{-/-}$ mice continue to demonstrate abnormal patterns of CGRP+ axonal projections in the adult (not shown).

Disorganized proprioceptive axonal projections in PTPRO$^{-/-}$ *spinal cord*

Although loss of PTPRO did not affect the number of proprioceptive neurons in lumbar sensory ganglia, PTPRO might nevertheless regulate the axonal projections of these neurons. To address this issue, we examined the central projections of PV immunoreactive afferents in PTPRO-deficient mice at P0. Normally, proprioceptive afferents enter the dorsal horn of the spinal cord and project to targets in the intermediate laminae and to the ventral horn motor column (Solbach and Celio, 1991; Zhang et al., 1990). In wt mice, these projections could be seen as tightly fasciculated bundles projecting to the intermediate zone, with further projections to the ventral horn motor neurons (Fig. 4A, C). In PTPRO-deficient mice, projections to the intermediate zone were less fasciculated, appeared disorganized, and were skewed toward the lateral spinal cord (Fig. 4B, D; Supplemental Fig. 4). This finding was consistent at lumbar spinal cord levels in each mutant animal examined.

Close examination of proprioceptive fibers in PTPRO-deficient mice revealed another abnormality. The relative density of projections to the intermediate and ventral horn was altered, such that intermediate zone projections were relatively denser compared to ventral projections (Fig. 4A, B). This finding suggests that fewer proprioceptive axons reach the ventral neuron pool in PTPRO-deficient mice. To corroborate this suggestion, we examined sensory axon projections using the fluorescent dye DiI. In wt animals, labeled axons projected to the dorsal horn, intermediate zone, and ventral horn, as expected (Fig. 4E). In PTPRO-deficient mice there was a relative paucity of projections to the ventral horn compared to intermediate zone projections (Fig. 4F), consistent with fewer axons reaching their motor neuron targets in the ventral horn. This observation was consistent in the animals examined. Thus, loss of PTPRO leads to abnormal fasciculation and inappropriate targeting of proprioceptive axons. Taken together, our results show that both nociceptive and proprioceptive neurons require PTPRO for axonal guidance during development.

PTPRO$^{-/-}$ mice have decreased responses to thermal pain stimuli

Loss of PTPRO led to deficiencies in both differentiation and axonal targeting of lumbar nociceptive neurons. To determine whether nociceptive function is compromised in PTPRO-deficient mice, we used a hot plate test to measure hindpaw responses to thermal pain stimuli in adult wt and PTPRO$^{-/-}$ mice. Reaction times until the first pain response (lick, step, or jump) were measured at 55°C (O'Callaghan and Holtzman, 1975). PTPRO-deficient mice had average reaction times 3 times as long as the wt animals ($p<0.001$; Fig. 5A), suggesting severely compromised sensitivity to thermal pain. Thus abnormal development of nociceptive neurons and their projections is correlated with abnormal thermal pain sensitivity in PTPRO-deficient mice.

PTPRO$^{-/-}$ mice have decreased sensorimotor function

Because PTPRO regulates axon guidance by both hindlimb proprioceptive neurons (this report) and motor neurons (Stepanek et al., 2005), we examined hindlimb sensorimotor function in PTPRO-deficient mice. For this purpose we used a battery of proprioceptive/motor exams that included the rotorod exam, tests of hindlimb placement, and tests of locomotor coordination requiring appropriate balance and paw placement (Dunham and Miya, 1957; Kunkel-Bagden

et al., 1993; Romero et al., 2001; Taylor et al., 2001). Several abnormalities were observed in the PTPRO$^{-/-}$ mice. In the rotarod test, mean time until fall was significantly decreased in PTPRO$^{-/-}$ mice compared to wt mice, suggesting decreased coordination and/or strength ($p<0.001$; Fig. 5B). In the beam walk, PTPRO$^{-/-}$ mice made significantly fewer correct hindlimb steps than wt animals, though these differences were subtle ($p<0.05$; Fig. 5C). Although these tests cannot distinguish proprioceptive from motor deficits, they suggest that PTPRO$^{-/-}$ mice have deficits in coordination. Therefore, further tests of proprioceptive placement were performed.

Deficits of proprioceptive placement in PTPRO$^{-/-}$ *mice*

For the hindlimb placement exam, the ability of the mouse to place its hindlimbs properly onto a level surface was scored in adult PTPRO$^{-/-}$ and wt mice. PTPRO$^{-/-}$ mice showed a substantial decrease in proper hindlimb placement compared to wt animals ($p<0.005$; Fig. 5D). These findings are likely due, at least partially, to proprioceptive deficits in PTPRO-deficient mice. PTPRO$^{-/-}$ mice also took slightly more time than wt mice to place their hindlimbs ($p<0.05$; Fig. 5E). No differences were seen between wt and PTPRO$^{-/-}$ mice in a gridwalk test of motor coordination (Fig. 5F). The differences in hindlimb placement speed, together with the differences on the beam walk test, suggest that PTPRO deficiency also leads to subtle but measureable differences in motor function.

DISCUSSION

We have found that loss of the type III RPTP, PTPRO, leads to deficits in the development of sensory neurons. In particular, peptidergic nociceptive neurons differentiated in smaller numbers and projected abnormally within the dorsal horn, while proprioceptive neurons were present in normal numbers but made guidance errors within the spinal cord and appeared not to reach ventral motor neuron targets to the normal extent. These anatomical abnormalities were associated with behavioral abnormalities, most notably in a substantial loss of thermal pain responses. Our findings represent the first evidence for the role of a specific PTP in the development of DRG neurons, and in nociceptive development in particular.

PTPRO is expressed in a fraction of nociceptive (TrkA-positive) neurons by E16 (Beltran et al., 2003). Because loss of PTPRO led to a decrease in numbers and inappropriate targeting of CGRP+ (presumed TrkA+) neurons, it is possible that the CGRP+ neurons are the population that normally expresses PTPRO. The decrease in CGRP+ neurons with no significant change in total numbers implies a loss of normal CGRP expression in some nociceptive neurons, similar to what happens in nociceptive neurons that continue to express the transcription factor Runx1 postnatally (Chen et al., 2006a; Kramer et al., 2006). It may be that PTPRO participates in one or more signals that inhibit Runx1 expression in developing nociceptive neurons. We presume that the increased density of CGRP+ fibers in the inner part of lamina II results from an alteration of guidance signals normally regulated by PTPRO in CGRP+ neurons. Interestingly, the deficit in thermal pain responses seen in PTPRO-deficient mice is as least as great as that seen in mice lacking Runx1 (Chen et al., 2006b).

Proprioceptive projections appeared disorganized in PTPRO-deficient spinal cords, and fewer seemed to reach the motor neurons in the ventral horn. There was also a tendency for these projections to be skewed laterally compared to wt axons. This phenotype is similar to, but less severe than, that seen in mice lacking the transcription factors Runx3 or ER81 (Arber et al., 2000; Inoue et al., 2002). Perhaps PTPRO expression is regulated by Runx3/ER81, or perhaps PTPRO helps to regulate the activity of signaling proteins which are in turn regulated by expression of these transcription factors. A battery of behavioral tests indicated subtle but significant deficits in sensorimotor function in PTPRO-deficient mice. These results are consistent with the alterations seen in proprioceptive axon targeting in these mice. For example,

skewing of projections toward the lateral edge of the cord could result in a degradation of synapse specificity (Ladle et al., 2007). Sensorimotor abnormalities are also consistent with our earlier observations that PTPRO regulates the guidance of hindlimb motor axons in the chick (Stepanek et al., 2005). At present, we cannot differentiate effects on these behaviors due to sensory abnormalities from those due to motor abnormalities. It will clearly be interesting to examine the development of the motor system in PTPRO$^{-/-}$ mice.

The roles of RPTPs in sensory neuronal differentiation and axon targeting have not previously been examined. However, the type IIa (LAR-family) PTPs, LAR and PTP-σ, are known to regulate the function of Trk receptors and to affect regeneration of adult peripheral sensory axons (Faux et al., 2007; McLean et al., 2002; Van der Zee et al., 2003; Xie et al., 2001; Yang et al., 2006). It will be worthwhile to examine the extent to which these RPTPs may act with PTPRO to control the differentiation and axon guidance of developing DRG neurons. Type IIa and type III RPTPs are known to cooperate and compete in the regulation of axon guidance in other situations (Jeon et al., 2008; Stepanek et al., 2005).

We do not know what tyrosine-phosphorylated substrates mediate the effect of PTPRO on DRG neuronal differentiation and axon guidance. Since Trk receptors control both of these processes, they are obvious candidates. Previous data suggest that TrkC, for example, is a PTPRO substrate (Hower et al., 2009). Tyrosine phosphorylation also regulates signaling through a variety of other axon guidance-related receptors, including receptors for netrins, semaphorins, and L1-family cell adhesion molecules (Franco and Tamagnone, 2008; Lemmon et al., 1989; Round and Stein, 2007; Walsh and Doherty, 1997). Each of these families of receptors is known to affect the guidance of sensory axons within the dorsal spinal cord (Ben-Zvi et al., 2008; Masuda et al., 2008; Perrin et al., 2001; Togashi et al., 2006).

Although PTPRO-deficient mice have not previously been examined for neurodevelopmental defects, PTPRO is known to affect neurite outgrowth in vitro (Stepanek et al., 2001). Moreover, PTPRO is expressed in retinal ganglion cells and motor neurons, and regulates the growth and guidance of the axons from these neurons in the chick (Ledig et al., 1999; Beltran et al., 2003; Shintani et al., 2006; Stepanek et al., 2005). PTPRO is expressed in a variety of postmitotic neurons during developmental times coinciding with axon growth (Beltran et al., 2003; Yahagi et al., 1996), and may therefore regulate axon targeting in other developing neurons.

EXPERIMENTAL METHODS

Animals

The generation of the original PTPRO$^{-/-}$ mouse strain has been described (Wharram et al., 2000); these animals were a generous donation from Dr. Roger Wiggins (University of Michigan). To eliminate difficulties with interpretation arising from mixed genetic backgrounds, we backcrossed PTPRO$^{-/-}$ mice onto a 100% 129P3/J background using the MaxBax speed congenic service (Charles River Laboratories, Wilmington, MA). P0 mice were examined to perform the DRG cell counts and central afferent labeling. Adult 5 month old mice were used to perform the behavioral tests of nociception and proprioception. PCR-based genotyping was performed with the following primers: for the wild-type allele, 5′-AAA CCT TAA ACT CCT GAT CCT CCT GCC TCC-3′ and 5′-CAC TGA ATC AAA ATG TCC CAC CCA TGT TTC-3′; for the targeted RO mutant allele 5′ GCC TTC TAT CGC CTT CTT GAC GAG TTC TTC-3′ and 5′-CAC TGA ATC AAA ATG TCC CAC CCA TGT TTC-3′. Note that the reverse primer for both PCR reactions was common to wildtype and targeted alleles. Genotype was determined by PCR product sizes of 307 and 570bp for wildtype and the targeted RO mutant alleles, respectively. All animal procedures were approved by the institutional animal care and use committee of the University of Miami.

Immunostaining for cell counts and central afferent axon projections

Postnatal day 0 (P0) mice were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS), pH 7.4 at 4°C. Next, the lumbar spinal column was dissected and preserved in Bouin's postfix solution for 24 hours and washed for 2 days in 70% ethanol. The tissue was dehydrated, paraffin embedded, and 20μm transverse serial sections were taken. The ribs were used as landmarks to identify the lumbar spinal column, and sections were carefully mapped to locate the lumbar level 4 dorsal root ganglia (L4 DRGs). Sections were deparaffinized, rehydrated, treated for 6 min at RT with 1%H_2O_2 in PBS, and incubated in 10mM sodium citrate pH 6.0 at 95°C for epitope retrieval (Shi et al., 1993) before blocking for 1 hr at RT in protein blocking solution (DAKO Cytomation; Glostrup, Denmark). Every 4th section was stained with the following: hematoxylin and eosin (HE), rabbit anti-parvalbumin (Swant, Bellinzona, Switzerland; 1:500) or rabbit anti-CGRP (Bachem T-4238; 1:250) diluted in PBS containing 5% normal goat serum and 0.1% Triton X-100 (TBS). The sections were incubated at 4 °C for 24 hr for the anti-CGRP antibody and 72 hr for the anti-parvalbumin antibody, washed with TBS, then incubated with biotinylated anti-rabbit secondary antibody (1:300 in TBS) for 1 h at RT. After washing with TBS, the sections were incubated with the peroxidase conjugated avidin—biotin complex (Vectastain Elite ABC kit; Vector Laboratories) for 1 h at RT and the reactions were visualized with 3, 3′-diaminobenzidine-nickel (DAB-nickel; Vector Laboratories). Wildtype and PTPRO$^{-/-}$ littermates were processed simultaneously to reduce variability in staining. Five animals (10 DRG) for each experimental group were examined for parvalbumin and CGRP staining. For HE staining, 4 animals (8 DRG) for each experimental group were examined. P60 (adult) mice were perfused as described for P0 mice. Next, the lumbar spinal cord and their DRG were dissected and carefully removed with the aid of a free-standing surgical microscope (Carl Zeiss). Then lumbar spinal cord and DRG were fixed in 4% PFA for 1 hr then washed in PBS and placed in 20% sucrose/PBS overnight at 4°C. After 24hr, the tissue was embedded in OCT (Tissue-Tek®), and 20μm frozen sections were prepared onto slides. Slides were allowed to dry in room air for 1-2 hr then washed with PBS, blocked with 5% normal goat serum in PBS, and incubated overnight in anti-CGRP (Bachem T-4238; 1:250) diluted in PBS containing 5% normal goat serum and 0.1% Triton X-100 (TBS) at 4°C. After 24hr, the slides were washed with PBS, labeled with secondary antibody Alexa 594 (1:200; Molecular Probes) at RT for 2 hours, and then dried and coverslipped. HE staining was performed in every other section as done with the P0 mice. For quantification of the width of CGRP projections, measurements were made in 3 standard positions within the projection on one section for each animal (using the Photoshop Measure tool) and averaged.

Cell Counts

Examiners were blinded to genotype. For P0 mice, the HE stain identified the nucleus of each neuron in the DRG for the total neuron count; the parvalbumin and CGRP immunoreactive neurons comprised the proprioceptive and nociceptive counts, respectively. All counts were performed using StereoInvestigator software (MicroBrightfield, Williston, VT) with an optical fractionator to calculate the total number in each ganglion. The animal number and total DRGs examined for each cell count were as follows. CGRP: wt, N=4 animals, 8 DRG; PTPRO$^{-/-}$ N=5, 10 DRG; Parvalbumin: wt, N=5, 10 DRG; PTPRO$^{-/-}$ N=5,10 DRG; HE counts: wt, N=4, 8 DRG; PTPRO$^{-/-}$ N=4, 8 DRG. For P60 mice, CGRP+ neurons were counted in 4-6 sections per animal, and the average of these counts is reported. CGRP positively stained cells were defined as those with greater than 2X the background fluorescent intensity, and for HE staining, neurons were defined as large dark cells with a clearly stained nucleus. CGRP: wt N=2 animals, 2 DRG; PTPRO$^{-/-}$, N=2, 4 DRG; HE counts: N=3, 5 DRG.

DiI central afferent labeling

P0 mice from each experimental group were perfused with 4% PFA in 0.1 M PBS, pH 7.4 at 4°C. A 2mm×2mm square of nitrocellulose saturated with 1,1′, di-octadecyl-3, 3, 3′, 3′, tetramethylindocarbocyanine perchlorate (DiI) was inserted adjacent to the lumbar spinal column and allowed to incubate for 2 wks at 37°C in 4% PFA. The lumbar spinal column was dissected, embedded in 4% agar/8% sucrose, and serially sectioned on a vibratome at 200μm. Transverse sections were mounted with aqueous medium, coverslipped, and examined under a Nikon fluorescence microscope.

Nociceptive and proprioceptive function testing

Hot plate analgesia. Pain reflexes in response to a thermal stimulus were measured using a Hot Plate Analgesia Meter (IITC Life Science). The surface of the hot plate was heated to a constant temperature of 55°C. Adult mice (3 months old) were placed on the hot plate, and the latency to respond with either a hind paw lick, hind paw flick, or jump (whichever came first) was measured by deactivating the timer when the response was observed. Examiners were blinded to genotype. **Rotarod**. Mice were placed on a rotating rod (IITC Life Science) with an accelerating velocity (0 to 30rpm), facing the direction opposite the direction of rotation to encourage locomotion. Mice were allowed to stay on the rod for up to 180 s or until they fell off. Prior to the timed trials, mice were trained twice daily on the rotarod at a ramp speed from 0 to 30rpm over a total of 180s for 14 days. It took about 2 weeks of training for mice to reach a performance plateau. After the training period, mice were subjected to 1 timed trial a day for 10 consecutive days. **Beam walk**. The number of hindlimb slips/stepping errors and the total steps were counted while animals walked across a 24 in. wooden rod (1 in. diameter). Each animal underwent 4 to 5 trials; the total number of errors and total steps were recorded and scored as a percentage correct steps [100 × (total steps- steps with errors)/(total steps)]. **Grid walk**. The number of hindlimb slips thru the wire mesh and total steps were counted while animals walked across a 24 in. long wire mesh (0.25 in. mesh). Every animal underwent 4 to 5 trials per testing day. The test was recorded and scored as a percentage of correct steps as for the beam walk. **Hindlimb paw placement. A. accuracy**-mice were held by the tail with forelimbs on a raised platform and hindpaws above and parallel to the edge of the platform before gently letting go of the tail. The test was scored as 0 (unable to place foot on platform), 1 (able to place foot on platform but with delay/difficulty/asymmetry), or 2 (able to place foot on platform without delay/difficulty/asymmetry). Animals underwent 4 trials and the test was scored by adding the measured score/maximum score×100=accuracy score. **B. speed**-the number of video frames (30 frames/s) was counted to obtain the time from the moment the examiner let go of the tail until the paw was successfully placed onto the platform. Mice unable to place a hindpaw onto the platform were not timed. All of the proprioceptive tests were videotaped and scored in slow motion by examiners blinded to phenotype. For the hot plate analgesia and rotarod exams, age-matched adult males (3 months old) wild type (n=6) and PTPRO$^{-/-}$ (n=10) mice were examined. For the beam, mesh, hindlimb placement, and hindlimb stepping reflex tests, age-matched adult males (5 months old) wild type (n=10) and PTPRO$^{-/-}$ (n=10) were examined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PTP, protein tyrosine phosphatase; RPTP, receptor PTP; CGRP, calcitonin gene-related peptide; PV, parvalbumin; Trk, tropomyosin-related kinase; PTPRO, Protein Tyrosine Phosphatase Receptor type O.

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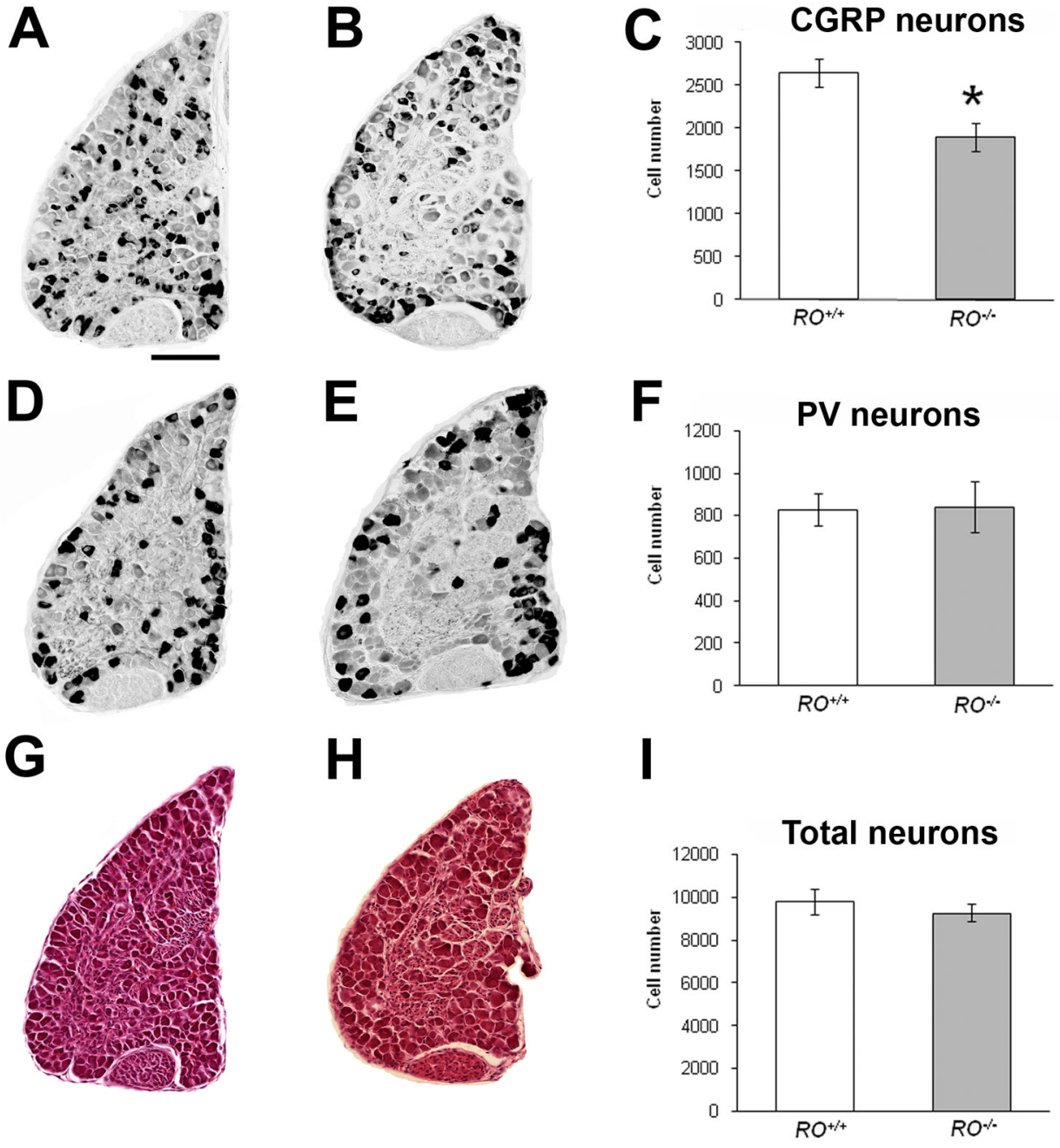

Figure 1. Decreased number of nociceptive neurons in PTPRO$^{-/-}$ mice

(**A-C**) P0 L4 DRG sections stained for CGRP to label nociceptive neurons. (**A**), wild type (n=3 animals; 6 DRG); (**B**), PTPRO$^{-/-}$ (n=5 animals; 10 DRG). PTPRO$^{-/-}$ DRG had fewer nociceptive neurons (**C**). (**D-F**) P0 DRG sections stained for parvalbumin to label proprioceptive neurons. (**D**), wild type (n=5 animals; 10 DRG); (**E**), PTPRO$^{-/-}$ (n=5 animals; 10 DRG). (**G-I**) All neurons were identified in HE stained sections. (**G**), wild type (n=4 animals; 8 DRG); (**H**), PTPRO$^{-/-}$ (n=4 animals; 8 DRG). Neither proprioceptive neuron counts nor total neuron counts were altered in the PTPRO$^{-/-}$ mice (**F,I**). Two-tailed Student's t tests were used for this and all statistical tests, to test for differences between wt and PTPRO$^{-/-}$. *, $p < 0.003$. Scale bar, 100μm.

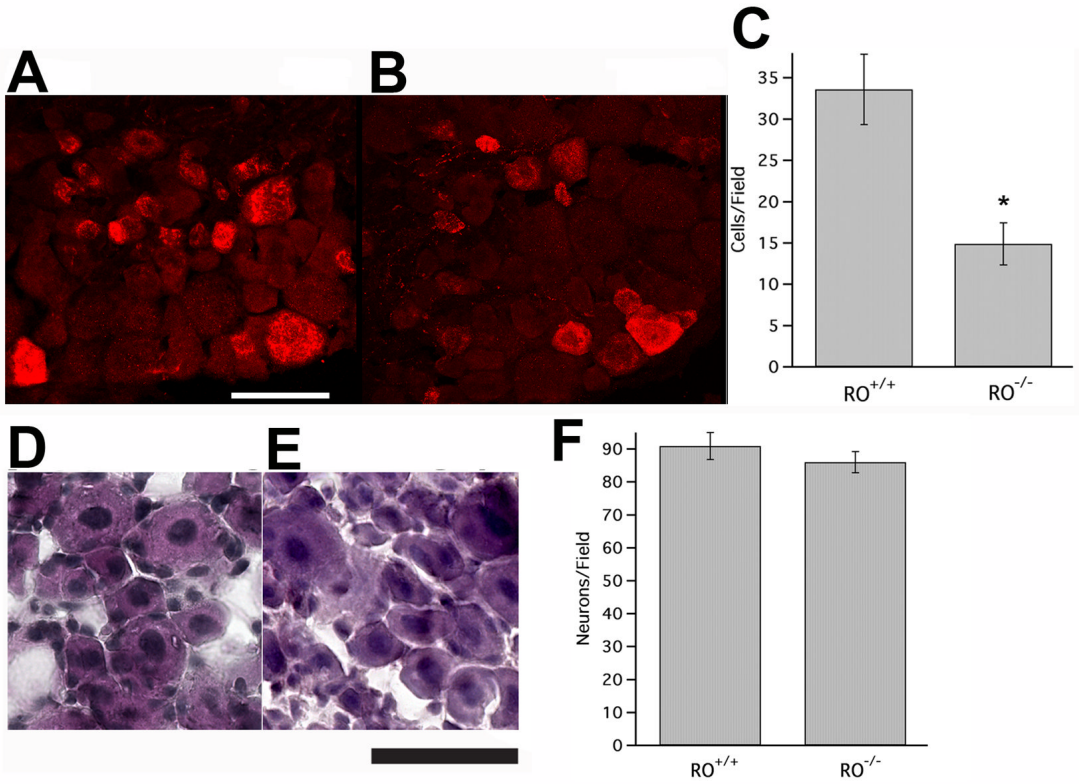

Figure 2. Decreased number of nociceptive neurons in adult (P60) PTPRO$^{-/-}$ mice

(**A,B**) Confocal fluorescent micrographs of wt (**A**) and PTPRO$^{-/-}$ (**B**) P60 L4 DRG sections stained for CGRP to label nociceptive neurons. PTPRO$^{-/-}$ DRG demonstrate a >50% decrease in the number of nociceptive neurons per section (**C**). N=2 animals, 2 DRG, wt; N=2 animals, 4 DRG, PTPRO$^{-/-}$.*, $p < 0.02$. (**D, E**) Bright field micrographs of wt (**D**) and PTPRO$^{-/-}$ (**E**) P60 L4 DRG sections stained for HE. There is no difference in the number of neurons per section between PTPRO$^{-/-}$ and wt (**F**, $p>0.3$). N=2 animals, 2 DRG, wt; N=3 animals, 5 DRG, PTPRO$^{-/-}$. Scale bar, 50μm.

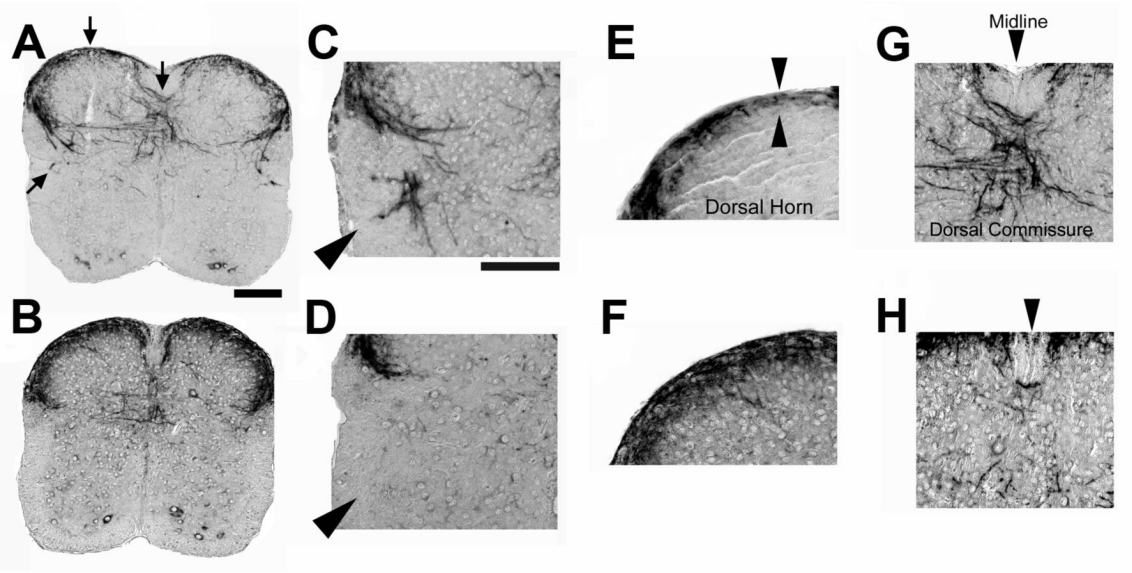

Figure 3. Aberrant nociceptive projections in PTPRO$^{-/-}$ mice

Transverse sections of lumbar spinal cord stained with CGRP antibody to label nociceptive afferents. (**A, C, E, G**, wt; **B, D, F, H**, PTPRO$^{-/-}$). The three small arrows in **A** point to areas in which projections are abnormal in PTPRO$^{-/-}$ mice. PTPRO$^{-/-}$ mice are missing the ventral-medial projection, show abnormally spread projections past lamina IIo, and have a decreased number of decussating axons in the dorsal commissure (**B**). Magnified views of the three areas of abnormal CGRP axon projection are shown in **C**-**H**. (**C, D**) Arrowhead points to ventral-medial projection representing innervation from the hindlimb/paw. (**E, F**) Large arrowheads (**E**) point to the outer borders of laminae I (top) and the outer portion of laminae II (bottom). CGRP+ axons appear less organized and project strongly beyond the border of lamina II in the PTPRO$^{-/-}$ animals. (**G, H**) Arrowhead points to midline. Fewer axons cross the midline in the PTPRO$^{-/-}$ mice. The defects shown are typical of those observed in all PTPRO$^{-/-}$ animals examined (n=7, wt; n=6, PTPRO$^{-/-}$). Scale bar, 100μm.

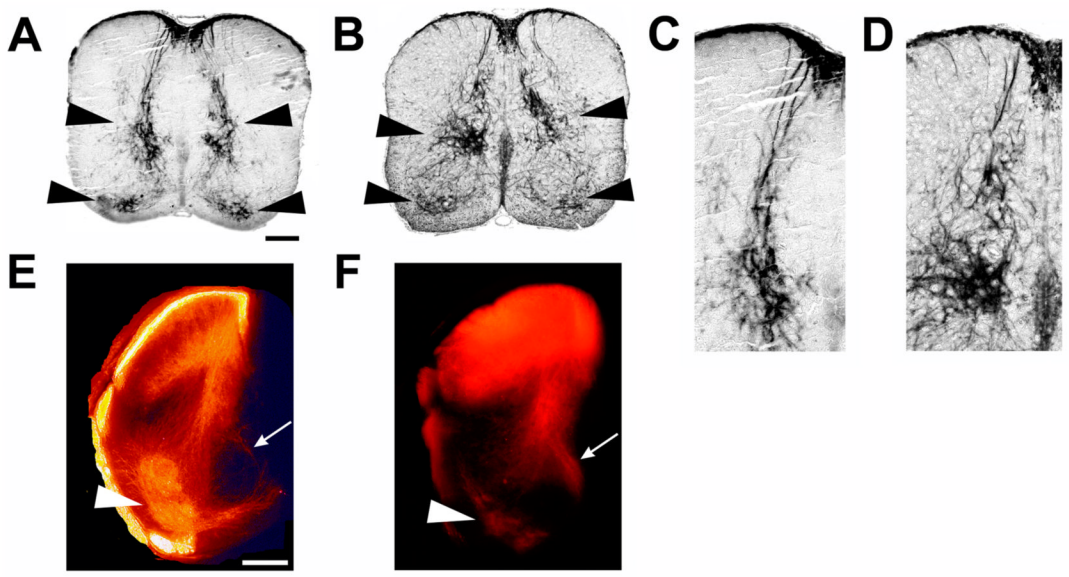

Figure 4. Aberrant proprioceptive projections in PTPRO$^{-/-}$ mice

(**A-D**) P0 spinal cord sections stained with parvalbumin antibody to label proprioceptive projections in wt (**A, C**) and PTPRO$^{-/-}$ (**B, D**) mice. Arrowheads point to areas of aberrant axon projections. Disorganized and defasciculated axonal projections in PTPRO$^{-/-}$ mice are obvious in the magnified views (**C, D**). Additionally, the ratio of intermediate zone axonal projections to those in the ventral horn is higher in the PTPRO$^{-/-}$ mice. Both of these patterns were observed in all animals examined (n=7, wt; n=6, PTPRO$^{-/-}$). (**E, F**) DiI labeled axon projections in P0 lumbar spinal cord. Fewer axons in PTPRO$^{-/-}$ mice reach their ventral horn motor neuron targets (arrowheads) compared to targets in the intermediate zone (arrows). Scale bars, 100μm.

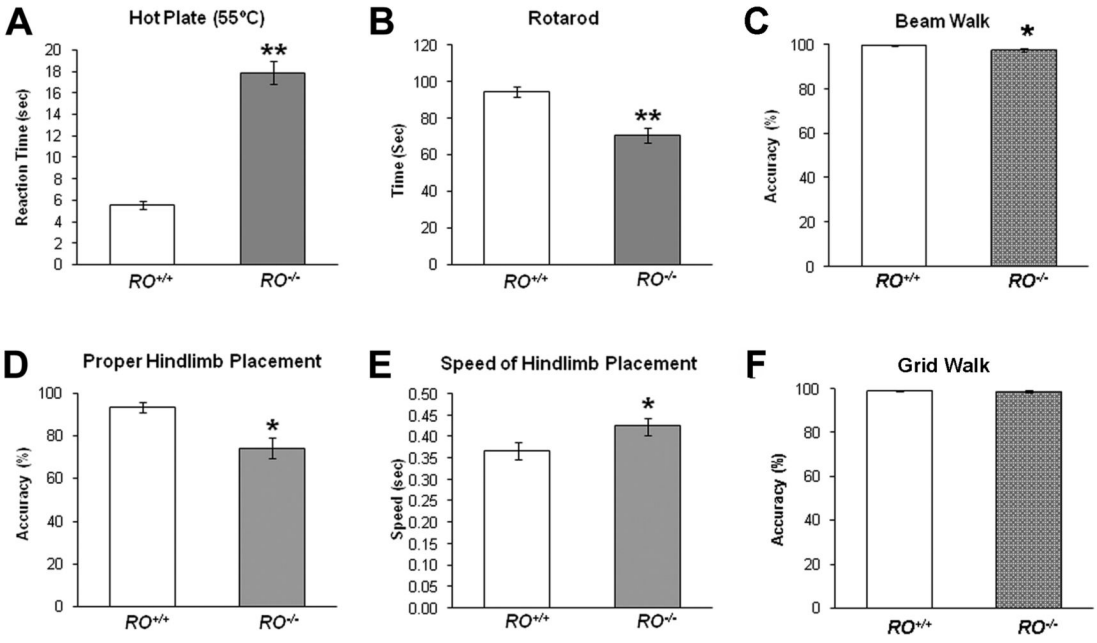

Figure 5. Nociceptive and sensorimotor deficits in PTPRO$^{-/-}$ mice

(**A**) To test nociceptive function, reaction times until pain response were measured with a 55° C hot plate analgesia in adult wt (n=6) and PTPRO$^{-/-}$ (n=10) mice. PTPRO$^{-/-}$ mice had reaction times 3X as long as wt mice. (**B-F**) represent sensorimotor exams. (**B**) Mean time until fall from the rotarod was measured in adult wt (n=6) and PTPRO$^{-/-}$ (n=10) mice over 10 trials (one trial daily); no differences between timed trials was observed within each experimental group (data not shown). The PTPRO$^{-/-}$ mice had significantly decreased times on the rotarod. (**C**) Hindlimb stepping errors were measured in a beam walk test in adult wt (n=10) and PTPRO$^{-/-}$ (n=10) mice. PTPRO$^{-/-}$ mice had slightly more errors. (**D**) Ability to place hindlimbs accurately was scored in adult wt (n =10) and PTPRO$^{-/-}$ (n =10) mice. PTPRO$^{-/-}$ mice had significant decreases in accuracy. (**E**) PTPRO$^{-/-}$ mice required more time for hindlimb placement. (**F**) No differences were seen between wt and PTPRO$^{-/-}$ mice in the grid walk test. *, $p < 0.05$; **, $p < 0.001$. Data presented as mean ± SEM.