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ABSENCE OF REELIN RESULTS IN ALTERED NOCICEPTION AND ABERRANT NEURONAL POSITIONING IN THE DORSAL SPINAL CORD

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Abstract—Mutations in *reeler*, the gene coding for the Reelin protein, result in pronounced motor deficits associated with positioning errors (i.e. ectopic locations) in the cerebral and cerebellar cortices. In this study we provide the first evidence that the *reeler* mutant also has profound sensory defects. We focused on the dorsal horn of the spinal cord, which receives inputs from small diameter primary afferents and processes information about noxious, painful stimulation. We used immunocytochemistry to map the distribution of Reelin and Disabled-1 (the protein product of the *reeler* gene, and the intracellular adaptor protein, Dab1, involved in its signaling pathway) in adjacent regions of the developing dorsal horn, from early to late embryonic development. As high levels of Dab1 accumulate in cells that sustain positioning errors in *reeler* mutants, our findings of increased Dab1 immunoreactivity in *reeler* laminae I–III, lamina V and the lateral spinal nucleus suggest that there are incorrectly located neurons in the *reeler* dorsal horn. Subsequently, we identified an aberrant neuronal compaction in *reeler* lamina I and a reduction of neurons in the lateral spinal nucleus throughout the spinal cord. Additionally, we detected neurokinin-1 receptors expressed by Dab1-labeled neurons in *reeler* laminae I–III and the lateral spinal nucleus. Consistent with these anatomical abnormalities having functional consequences, we found a significant reduction in mechanical sensitivity and a pronounced thermal hyperalgesia (increased pain sensitivity) in *reeler* compared with control mice. As the nociceptors in control and *reeler* dorsal root ganglia are similar, our results indicate that Reelin signaling is an essential contributor to the normal development of central circuits that underlie nociceptive processing and pain. © 2006 Published by Elsevier Ltd on behalf of IBRO.

Key words: Dab1, *reeler*, neuronal migration, dorsal horn, primary afferents, NK-1 receptor.

For over half a century Reelin and its associated signaling pathway have served as a model to study defects in neuronal positioning. Disruptions of the Reelin signaling pathway induce severe motor deficits—"suggestive of inebriation"—stemming from the disorganized lamination of the cerebellum and cerebral cortex (Falconer, 1951). During development of the *reeler* cerebral cortex the preplate fails to split and most cortical plate neurons assume incorrect, inverted laminar positions (Bielas et al., 2004; Caviness, 1976; Rice and Curran, 1999). In distinct contrast, several groups of spinal cord neurons migrate normally and to date, there are only two migratory errors identified in the *reeler* spinal cord (Phelps et al., 2002). Specifically, the cholinergic sympathetic and parasympathetic preganglionic neurons fail to stop appropriately, overshooting their normal position while the cholinergic interneurons are located correctly (Yip et al., 2000; Phelps et al., 2002). However, because the neuronal migration in both the cerebral cortex and spinal cord can be altered by blocking the function of Reelin using the CR-50 antibody, it appears that both positional errors result from the same migratory deficit (Nakajima et al., 1997; Yip et al., 2000).

The *reeler* gene is a naturally occurring autosomal recessive mutation that was identified and cloned by D'Arcangelo et al. (1995). Reelin, the protein product missing in the *reeler* mice, then was characterized as a large secreted extracellular matrix type protein (D'Arcangelo et al., 1995). Subsequently Reelin was found to be a high affinity ligand for both the very low density lipoprotein (Vldlr) and apolipoprotein E2 (ApoER2) receptors (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Trommsdorff et al., 1999). The adaptor protein Disabled-1 (Dab1), which binds the intracellular domain of Vldlr and ApoER2, undergoes tyrosine phosphorylation after Reelin binds to the receptors (Howell et al., 1997a,b; Trommsdorff et al., 1999). Phosphorylation of Dab1 in response to Reelin binding triggers a cytoplasmic signaling cascade that is essential for correct neuronal positioning and synaptogenesis in the developing nervous system (Rice and Curran, 2001). Cells that secrete Reelin are readily defined in wild-type mice whereas those that express Dab1 are identified in *reeler* mutants, which have 5-10-fold higher concentrations of Dab1 (Rice et al., 1998). One explanation for the high levels of Dab1 in mutants is that Dab1 must be phosphorylated before it can be ubiquitinated and degraded (Arnaud et al., 2003). Thus in wild-type mice Reelin downregulates Dab1, while in mutants Dab1 accumulates in specific neurons that sustain positioning errors, i.e. are incorrectly located and often misoriented.

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Abbreviations: ANOVA, analysis of variance; ApoER2, apolipoprotein E2 receptor; CGRP, calcitonin gene-related peptide; DAB, diaminobenzidine; Dab1, Disabled-1; DRG, dorsal root ganglion; E, embryonic day; LSN, lateral spinal nucleus; NeuN, Neuronal-specific protein; NK-1, neurokinin-1 receptor; P, postnatal day; TRPV1, transient receptor potential V1 (vanilloid receptor); Vldlr, Very low density lipoprotein receptor.

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Recently we localized Reelin-positive cells in the superficial dorsal horn in wild-type mice between embryonic day (E) 12.5 and postnatal day (P) 7 (Kubasak et al., 2004). Given that this region of the dorsal horn processes nociceptive information, we extended our analysis to the developmental distribution of both Reelin and Dab1 and the functional consequences of Reelin deletion. Importantly, because many spinal cord neurons migrate normally in *reeler* mutants, we hypothesized that any functional alterations produced by positioning errors in the *reeler* mutants will provide valuable information on the circuits through which particular aspects of pain processing are generated.

EXPERIMENTAL PROCEDURES

Animal and tissue preparation

The *reeler* mice (B6C3Fe *-a/a- Reln*^{rl}, Jackson Laboratory, Bar Harbor, ME, USA) were obtained from a breeding colony maintained at UCLA. Mice were studied at embryonic (E12.5–17.5), postnatal (P14-19) and adult (3–6 mo) ages. The adult *reeler* mutants used for sensory tests were healthy, but displayed ataxia, inability to rear on their hind limbs and were smaller than the wild-type controls. Mice were genotyped using polymerase chain reaction screening as adapted from D'Arcangelo et al. (1996). Pregnant mice were deeply anesthetized and embryos (E12.5–E14.5) were delivered by cesarean section and immersed in 4% paraformaldehyde. Older embryos (E15.5–E17.5) and postnatal animals were perfused through the heart with the same fixative. Tissue preparation followed the methods described in Kubasak et al. (2004). Adult L4-5 dorsal root ganglia (DRG) were dissected and embedded in a mixture of gelatin and sucrose as reported (Phelps et al., 1996).

Immunocytochemical procedures

We used a monoclonal anti-Reelin antibody (G10, 1:10,000; de Bergeyck et al., 1998; gift of Drs. Tom Curran and Susan Magdaleno, Memphis, TN, USA) as described previously to detect Reelin in embryos (Kubasak et al., 2004). To determine the distribution of Dab1 protein in the embryonic dorsal horn, we immunostained sections with a polyclonal Dab1 antiserum (2720, 1:15,000; gift of Dr. Joachim Herz, Dallas, TX, USA) and standard avidin–biotin methods (Phelps et al., 2002).

To compare chemically unique cell types within laminae I–II of the dorsal horn, we analyzed three to six pairs of adult age- and sex-matched *reeler* and control mice after their nociceptive testing was completed. Nonadjacent cervical, thoracic, and lumbar coronal sections from each animal were processed. The immunostaining patterns for the neurokinin-1 (NK-1; 1:10,000, Novus; Littleton, CO) and vanilloid receptors (transient receptor potential V1, TRPV1; 1:12,000–25,000, gift of Dr. David Julius, San Francisco, CA, USA) were localized following established methods (Caterina et al., 2000; Malmberg et al., 1997; Tominaga et al., 1998). To assess the distribution of nociceptors in the DRG and small diameter primary afferents in the spinal cord we used an antibody to calcitonin gene related peptide (CGRP; 1:30,000, Chemicon; Temecula, CA, USA) and the lectin IB4 (1:400; Vector Laboratories, Burlingame, CA, USA), which marks primary afferent neurons of the so-called non-peptide population of nociceptors (Snider and McMahon, 1998). To identify subpopulations of interneurons of lamina II we processed tissue for calbindin (1:150,000, Swant; Bellinzona, Switzerland), calretinin (1:50,000, Swant) and parvalbumin (1:100,000, Swant). The protocol for parvalbumin immunostaining was identical to that described for choline acetyltransferase (Phelps et al., 2002), except for additional avidin–biotin blocking steps (Vector Laboratories) for 15 min each followed by a buffer wash before the primary antiserum. The protocols for CGRP, calbindin and calretinin were similar to parvalbumin except that a Vector Elite Rabbit Kit was used. For calbindin immunofluorescent labeling, we used an Alexa Fluor 594 goat anti-rabbit secondary antibody (1:200; Molecular Probes, Eugene, OR, USA). IB4 was visualized as reported previously (Runyan et al., 2005).

We conducted double-labeling experiments to determine if NK-1 receptors are expressed by Dab1-positive neurons in *reeler* mutants. Sections from three pairs of wild-type and mutant mice were cut 30 μm thick, slide mounted, incubated with NK-1 antiserum (1:5000, Novus, Littleton, CO, USA), processed as described above and developed with diaminobenzidine (DAB) intensified with Ni–glucose oxidase, yielding a black product. After glycerin coverslipping, NK-1-labeled cells were photographed, washed, and subsequently processed with anti-Dab1 antiserum (1:8000). Following the second immunocytochemical procedure, DAB was developed with 0.02 M imidazole yielding a contrasting amber–brown product. For double-labeling controls, NK-1 experiments were followed by Dab1 procedures in which normal serum was substituted for the primary antibody. No evidence of brown reaction product was detected (data not shown). For fluorescent double-labeling experiments, sections were incubated with a polyclonal guinea-pig anti-NK-1 (1:1000; Chemicon) and Dab1 following the above protocol with the exception that we used an Alexa Fluor 488 goat anti-guinea-pig and Alexa Fluor 594 goat anti-rabbit secondary antibodies for NK-1 and Dab1 localization respectively.

To map the overall cellular distribution of neurons in the dorsal horn we used a monoclonal antibody to the neuronal-specific protein NeuN (1:900, Chemicon) with standard avidin–biotin peroxidase immunocytochemical methods (Houser and Esclapez, 2003). To localize NeuN in double-labeling experiments, we used an Alexa Fluor 488 goat anti-mouse antibody (1:200; Molecular Probes). Neurons of the lateral spinal nucleus (LSN) were analyzed in 40 μm coronal sections from six pairs of *reeler* and control mice at cervical, thoracic, and lumbar levels. Each LSN was photographed in eight to 15 sections/animal/level, randomized, counted blindly by three investigators, and the results were averaged. The number of cells per segmental level was averaged by animal and the overall mean was obtained for mutants and control mice. Statistical significance was evaluated with a student's *t*-test.

Behavioral analysis

We assessed mechanical nociception in 11 pairs of 3–6 month old wild-type and *reeler* mice (six male and five female pairs) with von Frey hairs using the up–down paradigm of Chaplan et al. (1994). Mice were placed on an elevated wire mesh and acclimated to the testing environment for 1 h. Calibrated monofilaments (3.61–4.56 g) were applied in series to the plantar aspect of the left hindpaw. In the absence of a paw withdrawal response to the initially selected hair, a stronger stimulus was used; if the paw was withdrawn, then the next weaker stimulus was chosen. The resulting pattern of positive and negative responses was tabulated and the 50% response threshold was determined from the data. Statistical significance of the mean threshold values was evaluated with the Mann-Whitney test.

Mutant and wild-type mice were tested for thermal nociception using the Hargreaves' paw withdrawal test (model 336G stimulator, IITC, Inc., Woodland Hills, CA, USA). Mice were placed in clear plastic tubes on a glass floor and habituated to the test environment for 1 h. A radiant heat source was focused onto the plantar surface of each hindpaw and the latency to withdraw from the heat stimulus was recorded (Hargreaves et al., 1988). An optimal response time for male and female animals was determined at 14% intensity after analyzing responses at a range of intensities (10% to 25%) prior to the actual testing. The percent

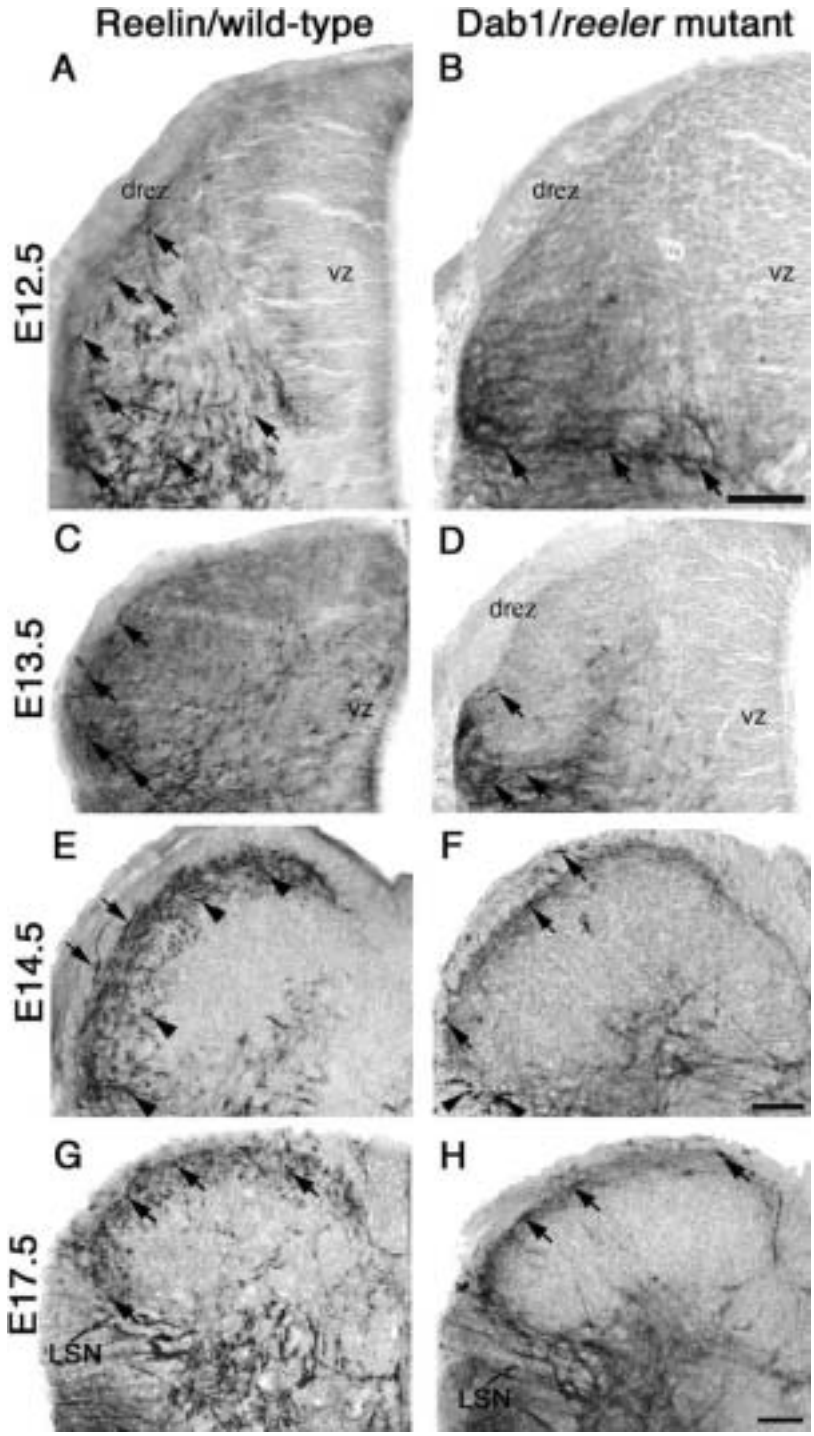

Fig. 1. Reelin (A, C, E, G) and Dab1 (B, D, F, H) are expressed in adjacent regions of the developing wild-type and mutant dorsal horns. Images are oriented with dorsal toward the top and medial to the right in this and subsequent transverse dorsal hemisections. (A, B) E12.5: Reelin-positive cells (arrows, A) are initially detected along the base of the dorsal horn between the ventricular zone (vz) and the edge of the dorsal root entry zone (drez). A band of Dab1-positive cells and processes (arrows, B) is stretched along the base of the mutant dorsal horn. (C, D) E13.5: Both Reelin-positive cells (arrows, C) in wild-type and Dab1-positive cells (arrows, D) in *reeler* mice are concentrated along the ventral and lateral borders of the dorsal horn. (E, F) E14.5: Reelin-labeled cells are localized in laminae I (arrows, E), II (arrowheads, E), and in lateral lamina V. Dab1-positive cells (arrows, F) are concentrated in lamina I, with a few cells detected in the future lateral spinal nucleus (LSN) (arrowheads, F), and in lateral lamina V. (G, H) E17.5: Reelin-positive cells (arrows) are maintained within laminae I–II. Several large Reelin-labeled cells are integrated into the future LSN (G). Dab1-positive cells persist in laminae I (arrows, H), V and in the LSN. Scale bar=50 μm A and B, C–F, G and H.

intensity of the heat source was calculated by testing control mice and setting their response time to the 10–15 s range. The heat source has a cut off time of 20 s to prevent injury. Data from male and female mice were examined separately and combined as there were few differences. Statistical significance of the mean values was evaluated with an ANOVA. After testing mice were perfused for morphological analyses.

RESULTS

Pattern of Reelin expression in the superficial dorsal horn

We used immunocytochemistry in coronal sections from wild-type mice to map and analyze the expression pattern of Reelin and detected Reelin-immunoreactive cells in E12.5 dorsal horn (arrows, Fig. 1A). Labeled neurons were scattered near the base of the dorsal horn, with a few cells located near the dorsal root entry zone. On E13.5, Reelin-labeled cells (arrows, Fig. 1C) were concentrated along the ventrolateral border of the dorsal horn, and by E14.5 they occupied the mediolateral extent of laminae I–II (Fig. 1E). Large Reelin-positive cells with mediolaterally arborizing dendrites were seen in lamina I (arrows, Fig. 1E) and resemble Waldeyer cells (Kubasak et al., 2004; Scheibel and Scheibel, 1968). Small round Reelin-labeled cells occupied lamina II (arrowheads, Fig. 1E) and distinct immunoreactive cells also were present in the lateral region of lamina V. At E17.5 we observed a high concentration of diffuse extracellular Reelin and several large Reelin-positive cells just lateral to the dorsal horn, in the LSN (Fig. 1G), which consists of a group of neurons in the dorsolateral funiculus that relay nociceptive information to higher brainstem and thalamic structures (Burstein et al., 1990b; Molander and Grant, 1995; Olave and Maxwell, 2004).

Dab1 expression is detected in *reeler* dorsal horn

To determine whether the abnormal expression of Dab1 in the cerebral and cerebellar cortices of *reeler* mice is recapitulated in the dorsal horn, we examined embryonic Dab1 expression in *reeler* mutants. At E12.5, we found high levels of Dab1 in bands of cells and processes (arrows, Fig. 1B) that extended from the ventricular zone to the lateral edge of the dorsal horn. On E13.5, Dab1-immunoreactive cells (arrows, Fig. 1D) clustered along the lateral boundary of the dorsal horn with a few cells detected beneath the dorsal root entry zone. By E14.5 many Dab1-labeled cells were distributed across the superficial border of lamina I (arrows, Fig. 1F; Supplemental Fig. 1B). At this age we also observed Dab1-positive cells in the region of the LSN (arrowheads, Fig. 1F), and within the deep dorsal horn, including the lateral part of lamina V (Supplemental Fig. 1B). In contrast, we did not detect Dab1 immunoreactivity in wild-type E14.5 dorsal horn (Supplemental Fig. 1A). At E17.5, Dab1 immunoreactivity persisted in lamina I, lamina V, and the LSN (Fig. 1H). In addition, we localized Dab1-positive cells (arrows, Fig. 2A) in the dorsal midline of the spinal cord, just ventral to the dorsal column white matter. At early postnatal and adult ages, we detected Dab1-immunoreactive cells in wild-type and *reeler* LSN

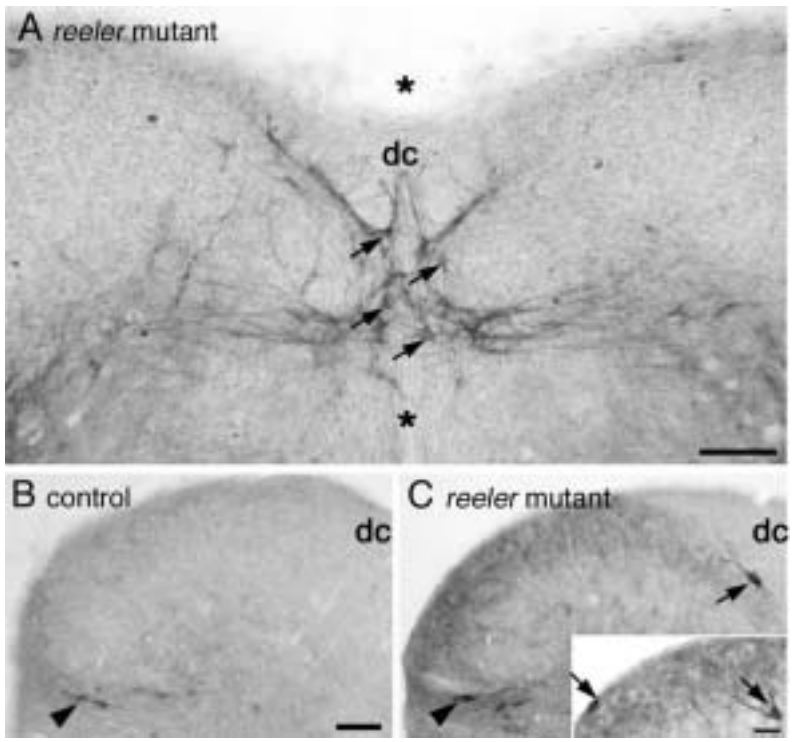

Fig. 2. Dab1-labeled cells are found in *reeler* (A, C) and wild-type (B) dorsal horns. (A) On E17.5, Dab1-positive cells (arrows) are detected within the dorsal midline (between asterisks), just beneath the future dorsal columns (dc). (B, C) A few Dab1-positive cells (arrowheads) are found in the LSN of both wild-type (B) and *reeler* (C) P14 thoracic spinal cords. Additional Dab-1 containing neurons (arrows) are detected in the *reeler* superficial dorsal horn (inset, C). Scale bar=50 μm A, B and C; Scale bar=25 μm inset in C.

(Fig. 2B, C), and in the superficial dorsal horn of *reeler* mice (Fig. 2C).

Positioning errors exist in *reeler* dorsal horn

Given the developmental expression of Reelin and Dab1 in the superficial dorsal horn of wild-type and *reeler* mice respectively, we then investigated the adult dorsal horn to characterize neuronal positioning errors in *reeler* mutants. To examine the overall neuronal distribution in the dorsal horn, we studied NeuN-labeled preparations in cervical, thoracic, and lumbar segments of control and *reeler* mice. In control animals, we found that neurons were evenly distributed along the mediolateral extent of lamina I, at all levels of the spinal cord (Figs. 3A, 4A). By contrast, mutant dorsal horns exhibited a distinct compacted pattern of neurons in lamina I (arrows, Figs. 3D, 4B, C). To determine if these rather striking positional errors in the mutants were restricted to lamina I, we subsequently studied the distribution of characteristic markers of lamina II interneurons, including calbindin (Fig. 3B, E), calretinin (data not shown) and parvalbumin (data not shown). We found no apparent differences in the density or position of neurons identified with these calcium-binding proteins. To further characterize the aberrant neuronal distribution in lamina I, control and *reeler* dorsal horns were double-labeled for NeuN and calbindin (Fig. 3C, F). In these preparations, it was clear that in addition to the densely compacted lamina I (arrows, Fig. 3D), the mutants had a neuron-sparse region between lamina I and II outer (asterisks, Figs. 3F, 4B, C), a feature not normally found in wild-type dorsal horns (Figs. 3C, 4A).

Although the afferent distribution in the cerebral and cerebellar cortices is thought to be generally normal in *reeler* mutants, having observed aberrant neuronal positioning in lamina I, we next investigated the distribution of primary afferents that target these dorsal horn neurons. Specifically, we examined the distribution of peptide-containing (substance P/CGRP) and nonpeptide-containing (lectin IB4) thin primary afferents (C-fibers) that terminate in laminae I–II outer and II, respectively (Ribeiro-da-Silva, 1995; Basbaum, 1999). Taking advantage of the laminar specificity of these markers we examined the distribution of CGRP-immunoreactive primary afferents in control (Fig. 4D) and *reeler* mice (Fig. 4E, F). The CGRP-positive terminals in control animals were restricted to a dense band throughout the mediolateral extent of laminae I and II outer (Fig. 4D). Although we found CGRP afferents comparably positioned in the cervical and lumbar superficial dorsal horn of *reeler* mutant mice (Fig. 4E, F), some afferents in the medial region of cervical lamina I exhibited an ectopic bilaminar distribution (arrows, Fig. 4E).

The distribution of the IB4 lectin appeared somewhat abnormal in cervical segments of both control and mutant mice. For example, in most animals we observed IB4 reaction product in the cervical dorsal column white matter (Fig. 4G, H). However, IB4-labeled axons terminated in their normal inner lamina II target in both mutant and control mice (Fig. 4G, H). As with the abnormal CGRP pattern of termination, we also observed more aberrant IB4-labeled processes at cervical levels (Fig. 4G, H) than in lumbar spinal cord (Fig. 4I).

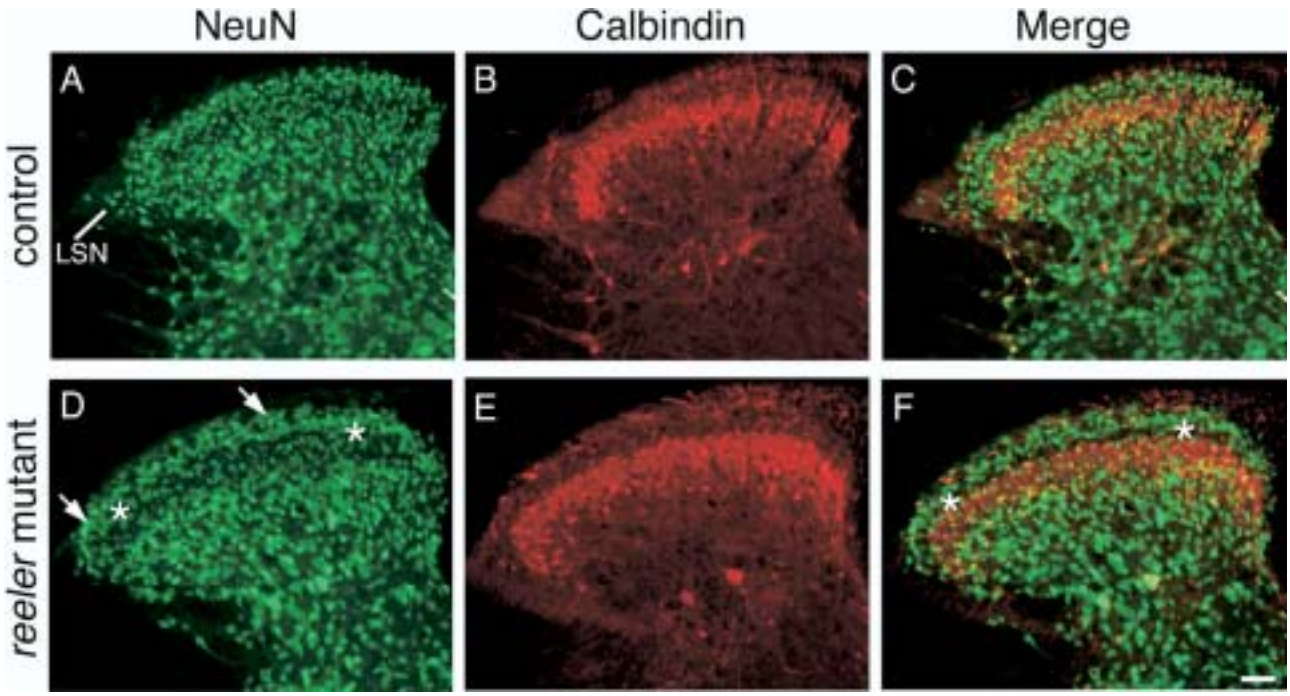

Fig. 3. Neuronal distribution in the lumbar dorsal horn in control (A–C) and *reeler* mice (D–F). (A, D) NeuN-positive cells in control animals (A) exhibit a homogenous cellular distribution in the superficial dorsal horn, while in *reeler* mutants, lamina I cells (arrows, D) appear compacted, with fewer cells between lamina I and lamina II outer (asterisks). (B, E) A few calbindin-positive cells reside in lamina I but the majority are found in lamina II in both control (B) and *reeler* (E) mice. (C, F) In double-labeled NeuN and calbindin sections, mutants exhibit an unusual neuron-sparse region (asterisks, F) between lamina I and the calbindin-positive lamina II cells not seen in controls (C). Scale bar=50 μm A–F.

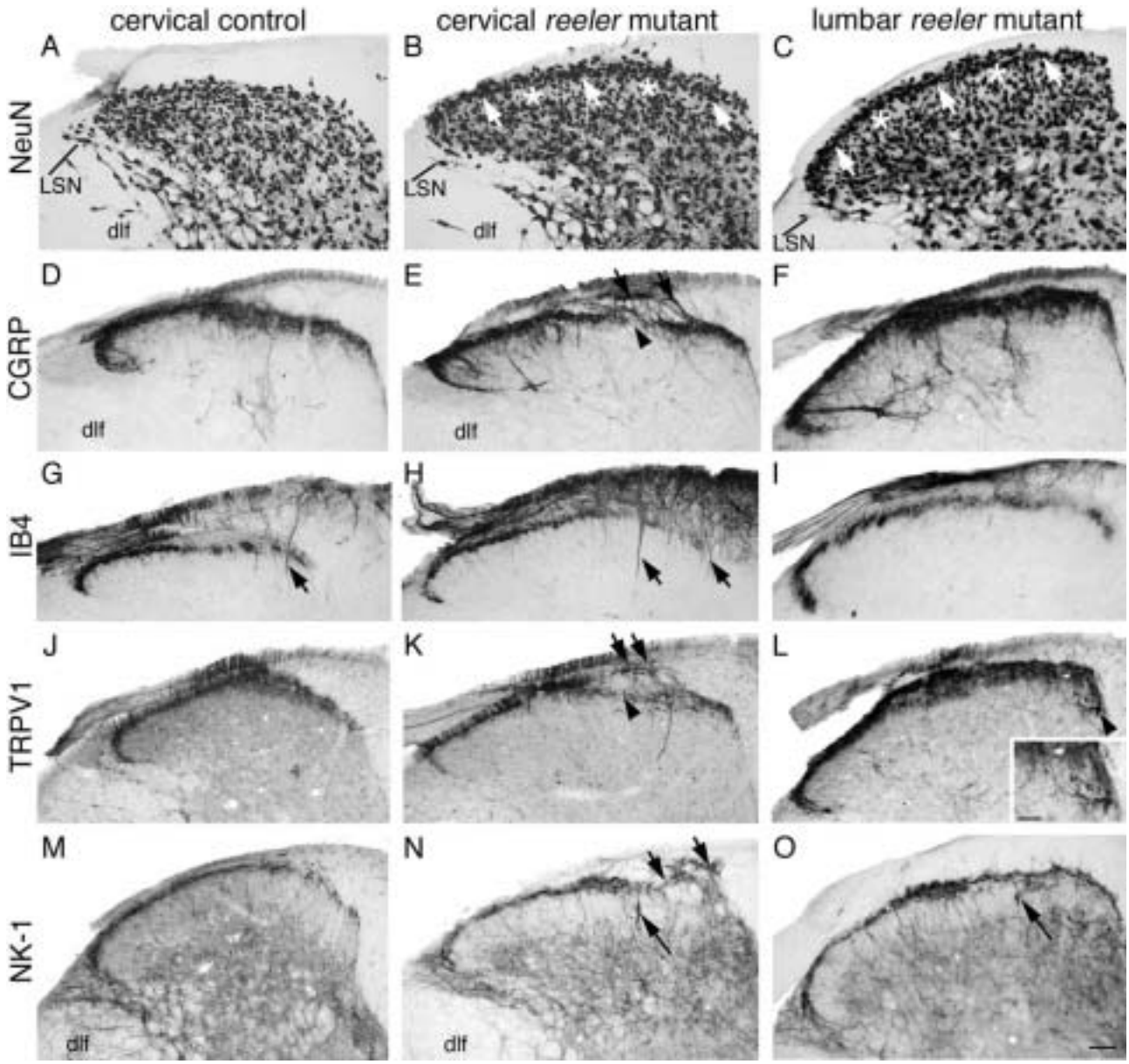

Fig. 4. Disruptions exist in the cervical (center column) and lumbar (right column) superficial dorsal horn of *reeler* mutants compared with controls (left column). (A–C) NeuN immunocytochemistry of the superficial dorsal horn and the LSN. A cellular compaction of lamina I (arrows, B, C), a cell-sparse region beneath the compaction (asterisks, B, C), and a neuronal reduction within the LSN (B, C) are found in *reeler* mutants compared with controls (A). The dorsolateral funiculus (dlf) is labeled for orientation. (D–F) The CGRP-positive afferents in control animals appear as a solid band along lamina I and II outer (D), while the primary afferents in mutants at cervical levels are missing areas in medial lamina I (arrowhead, E) or are ectopically positioned (arrows, E). A solid band of CGRP-labeled afferents is present at lumbar levels of *reeler* mice (F). (G–I) IB4-labeled afferents enter through the dorsal roots and terminate in lamina II in both control (G) and *reeler* mice (H, I). Additional unidentified IB4-positive processes reside in the dorsal columns (G–I) and at cervical levels bypass lamina II and extend into the deep dorsal horn (arrows, G, H). (J–L) The heat sensitive vanilloid receptor (TRPV1) is distributed mainly in lamina I. Occasionally *reeler* lamina I neurons are ectopically displaced in cervical (K) but not lumbar spinal levels (L). TRPV1-positive axonal clusters (arrowhead, L, inset) are detected in medial lamina II at *reeler* lower lumbar levels. (M–O) NK-1 receptor expression in control mice is concentrated in a band within lamina I (M) while *reeler* mutants exhibit an ectopic bilaminar distribution at cervical levels (arrows, N). Additionally, mutants display NK1-labeled cells in both cervical (long arrow, N) and lumbar (long arrow, O) lamina II. Scale bar=50 μm A–O; Scale bar=25 μm inset in L.

To evaluate markers of subsets of primary afferent nociceptors we also immunostained for the heat sensitive vanilloid receptor, TRPV1, which is activated by capsaicin, the pungent ingredient in hot peppers (Tominaga et al., 1998) (Fig. 4J–L). The expression pattern of TRPV1 afferents in *reeler* mutants clearly illustrates the abnormal positional shift of the afferent distribution in the medial part of lamina I of the cervical dorsal horn (arrows, Fig. 4K) not seen at other segmental levels (Fig. 4L). Lower lumbar sections from both mutant and control mice display an

unusual pattern of TRPV1 afferents within medial lamina II (Fig. 4L, inset). Taken together, these studies illustrate that although the overall primary afferent projection pattern in the superficial dorsal horn at all levels of the spinal cord is comparable between wild-type and *reeler* mutants, we consistently observed a bilaminar split at cervical levels in the medial region of lamina I.

Furthermore, because the NK-1 receptor is the target of the substance P-releasing nociceptors, we also examined the distribution of NK-1 receptor immunoreactive neurons in the dorsal horn. Neurons in wild-type animals that express the NK-1 receptor are concentrated in lamina I (Figs. 4 M, 5A); a few large neurons with dorsally-directed dendrites are found in laminae III–IV (Brown et al., 1995; Littlewood et al., 1995). We found NK-1 receptor immunoreactive neurons comparably positioned in *reeler* mutants at all spinal cord levels and also detected a number of distinct NK-1 receptor-positive cell bodies in lamina II or at the border between laminae II–III, which have dendrites that project into lamina I (Figs. 4N, O, 5B, inset; Supplemental Fig. 2B, E). Additionally, consistent with the abnormal distribution of CGRP axons in mutant mice, we identified differences in the positioning of the medial lamina I cells bearing NK-1 receptors in cervical segments of *reeler* mutants (arrows, Fig. 4N).

Finally, as the neurons that migrate incorrectly in *reeler* mutants contain high levels of Dab1, we asked if the Dab1 immunoreactive neurons found in laminae I–III also might express NK-1 receptors. Initial experiments detected NK-1 receptor-positive cell bodies located in laminae I (Fig. 5G; Supplemental Fig. 2A), II (Fig. 5C, E) and III (Fig. 5C) with labeled dendrites that project into lamina I. Subsequent

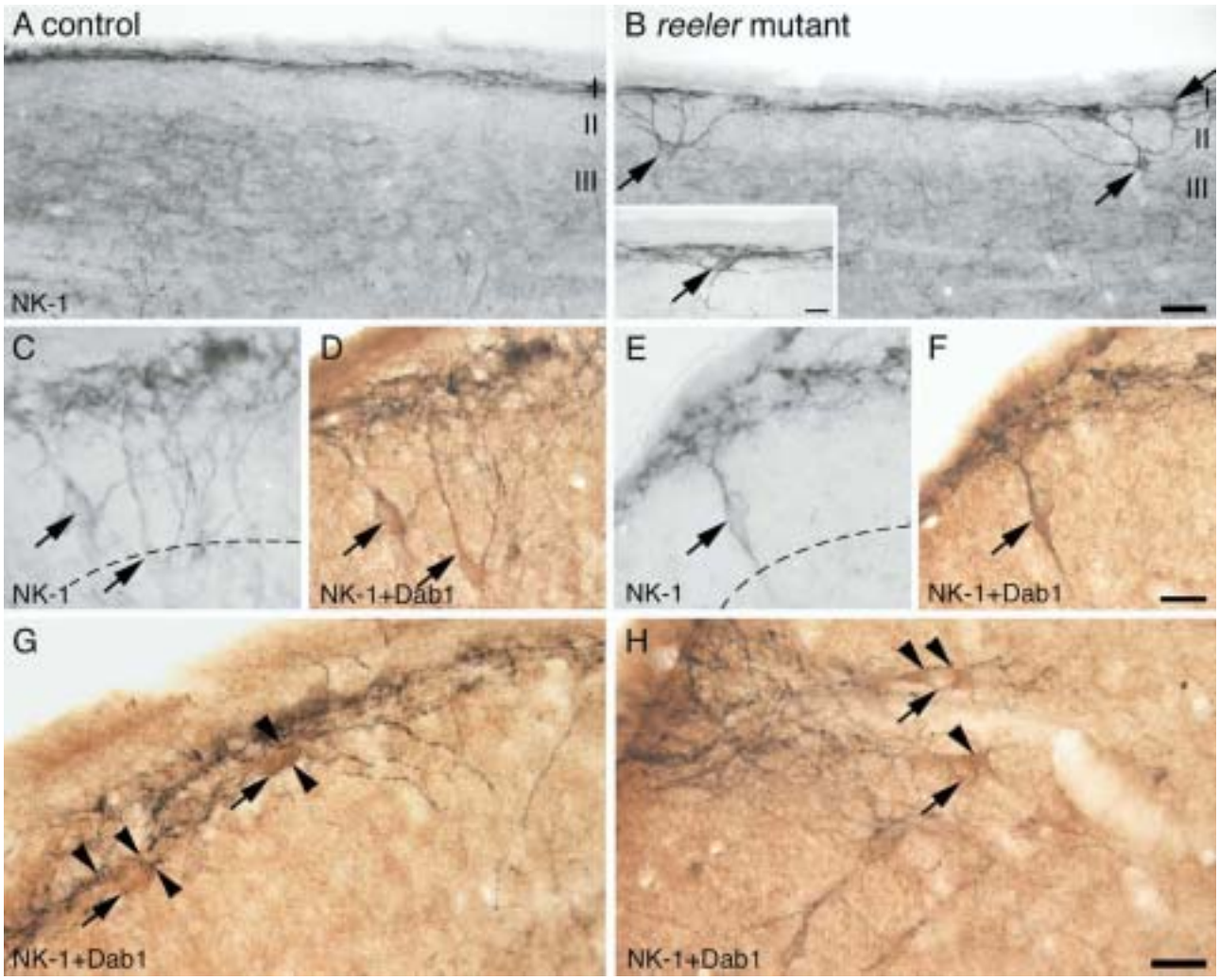

Fig. 5. NK-1 receptors delineate laminae I–III cells in *reeler* thoracic (A, B) or lumbar (C–H) dorsal horns from parasagittal (A, B) or transverse (C–H) sections. (A, B) In P19 *reeler* mice, NK-1 receptors outline large cell bodies located within lamina II or at the lamina II–III border and their dendrites that extend to lamina I (arrows, B). NK-1 receptor-positive cells also reside within lamina I (B, inset) of mutant mice. (C–F) Laminae II–III cells express NK-1 receptors (black chromogen, arrows, C, E) and contain high levels of Dab1 (amber–brown chromogen, arrows, D, F) in P14 *reeler* dorsal horn. In these experiments, NK-1 immunoreactivity was conducted first and photographed (C, E) before Dab1 immunoreactivity was localized (D, F). Dashed line marks laminae II–III border. (G, H) Many Dab1-positive lamina I (arrows, G) and LSN neurons (arrows, H) in *reeler* mice bear NK-1 receptors (arrowheads, G,H). Scale bar=50 μm A and B; Scale bar=25 μm C–F, G and H.

Table 1. Average number of neurons found in the LSN

Animal	Level	Cell count
Control	Cervical	9.0±1.1
	Thoracic	8.6±1.8
	Lumbar	8.9±1.2
reeler	Cervical[a]	3.5±0.8
	Thoracic[b]	4.0±1.2
	Lumbar[c]	5.0±1.4

Data (mean±SD) expressed per LSN from six pairs of control and mutant mice.
[a] Cervical, $t=9.55$, $df=5$, $P<0.0005$.
[b] Thoracic, $t=5.08$, $df=5$, $P<0.005$.
[c] Lumbar, $t=5.26$, $df=5$, $P<0.005$.

Dab1 localization confirmed that many of these NK-1 bearing neurons are Dab1 immunoreactive in *reeler* mice (Fig. 5D, F). Such double-labeled neurons are not detected in the dorsal horn of wild-type mice (data not shown). Mutant mice also have Dab1-positive somata in the dorsal funiculus that do not express NK-1 receptors and NK-1 receptor-labeled cells in the deep dorsal horn that do not contain Dab1 (Supplemental Fig. 2). In addition, some of the Dab1 containing neurons in the LSN bear NK-1 receptors in *reeler* mice (Fig. 5H; Supplemental Fig. 2F).

Positioning errors exist in the LSN of *reeler* mutants

Given the strong Dab1 expression in the LSN we postulated that this nociceptive region also might sustain positional errors in *reeler* mutants. To assess this, we immunolabeled sections from cervical, thoracic, and lumbar spinal cord levels for NeuN in six pairs of control and *reeler* mice (Fig. 4A, B). Each LSN averaged nine neurons per section, at all three segmental levels of the control spinal cord (Table 1). In contrast, in the *reeler* spinal cord the number of cells present in the LSN was significantly reduced. The *reeler* mutant averaged 3.5 cells in the cervical region, a 60% decrease in the overall cellular density of LSN neurons. In the thoracic region the mutants averaged four cells per hemisection, which is equivalent to a greater than 50% reduction compared with control mice. Lastly, in the lumbar region the *reeler* LSN averaged five cells, representing a 40% reduction (Table 1).

Nociceptors appear normal in the *reeler* DRG

The small diameter neurons of the DRG relay nociceptive information from the periphery to the superficial dorsal horn of the spinal cord. Thus in addition to evaluating the primary afferent distribution in the dorsal horn of *reeler* mice, we examined the CGRP, IB4, and TRPV1-labeled

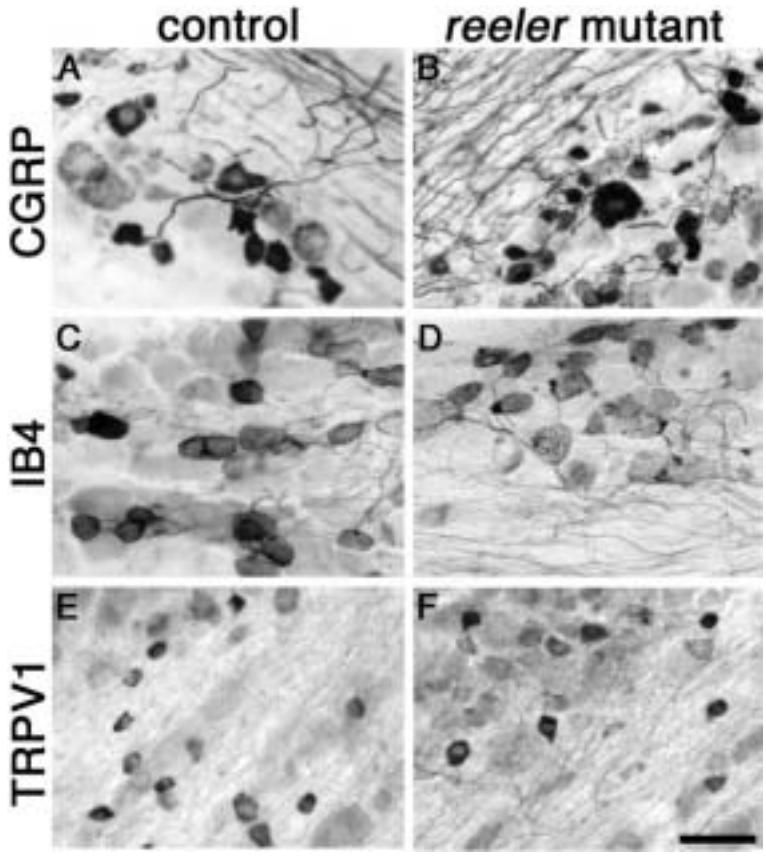

Fig. 6. Nociceptive DRG neurons identified with CGRP (A, B), IB4 (C, D), and TRPV1 (E, F) immunoreactivity in wild-type control (A, C, E) and *reeler* (B, D, F) mice. Both small- and medium-sized CGRP-positive DRG neurons (A, B) and small IB4-labeled DRG neurons (C, D) are found in both genotypes. TRPV1-immunoreactive nociceptors in control and *reeler* DRG are similar and small in size. Scale bar=50 μm A–F.

cell bodies in lumbar (L4-5) DRG. We observed no striking differences in the distribution or average size of the three populations of nociceptors (Fig. 6A–F), a finding that contrasts with the abnormalities identified in the *reeler* dorsal horn.

reeler mutants exhibit reduced sensitivity to noxious mechanical stimuli

The positional errors observed in *reeler* mice occurred in the superficial dorsal horn and in the LSN, both areas known to contain projection neurons that convey nociceptive information to rostral targets (Burstein et al., 1990a,b; Giesler et al., 1979; Molander and Grant, 1995; Olave and Maxwell, 2004). Therefore, in the next series of studies, we tested the hypothesis that the anatomical alterations occurring in these areas due to lack of Reelin expression are associated with changes in nociception in the mutant mice.

In these studies, we compared mechanical nociceptive thresholds in 11 pairs of age- and sex-matched wild-type and mutant mice. Using a series of von Frey monofilaments to test mechanical nociception of the plantar surface of the left hindpaw of each mouse, we found a striking increase of the mechanical threshold, in the mutant mice. The 50% threshold was 0.3 g (2.9 mN) in control animals, and almost 10 times higher, 2.2 g (21.6 mN) in *reeler* mutants, a difference that was highly significant (Mann-Whitney test; $z=4.025$, $df=10$, $P<0.0001$). Additionally, Fig. 7 shows that when groups were divided by sex, we also found significant differences between the control and mutant mice. These findings illustrate that there is a greatly reduced sensitivity to mechanical stimulation in *reeler* mutants.

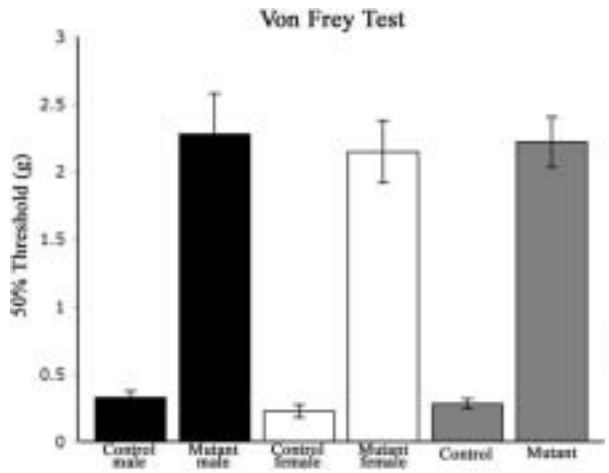

Fig. 7. The *reeler* mutants exhibit decreased mechanical sensitivity and these observations remain consistent when animals are divided by sex. The 50% threshold is 0.3 g (2.9 mN) for control males, 2.3 g (22.6 mN) for mutant males, 0.2 g (2.0 mN) for control females, and 2.1 g (20.6 mN) for mutant females. Both the differences observed in males ($z=2.929$, $df=5$, $P<0.005$) and females ($z=2.643$, $df=4$, $P<0.01$) are statistically significant. When these results are combined, the 50% threshold is 0.3 g (2.9 mN) in control and 2.2 g (21.6 mN) in mutant animals, a statistically significant difference ($z=4.025$, $df=10$, $P<0.0001$).

reeler mutants exhibit thermal hyperalgesia

Having found a significant difference in mechanical nociception between wild-type and mutant mice we next evaluated *reeler* sensitivity to thermal stimulation using the Hargreaves' paw withdrawal test, which permits the analysis to be performed in freely moving mice. In contrast to the profound reduction of mechanical sensitivity, we found that there was greatly enhanced thermal nociceptive processing in *reeler* mutants. Specifically, we found a significantly shorter withdrawal latency to the noxious thermal stimulation in 11 pairs of age- and sex-matched *reeler* mutants compared with control mice. On average, controls had a latency to withdraw of 11.33 s while mutant animals had an average latency to withdraw of 6.38 s (Table 2). This difference is highly significant. When the groups were divided by sex, we observed an average difference of 5.94 s between control and mutant males, while an average difference of 3.92 s was observed between control and mutant females (Table 2). These findings demonstrate significant thermal hyperalgesia in *reeler* mutants compared with control mice.

DISCUSSION

In the present study we used immunocytochemistry to characterize Reelin and Dab1 expression in the developing dorsal horn so as to identify positional errors in regions that process nociceptive information. During embryonic development Reelin and Dab1 are expressed in nearby neurons within the superficial dorsal horn, lateral lamina V, and in the LSN, areas associated with the processing of nociceptive information. In addition to identifying notable positional errors and moderate alterations in the distribution of small diameter primary afferents (presumed nociceptors) in the superficial dorsal horn, we observed significant reductions in the number of cells present in the LSN at all spinal cord levels. Importantly, in behavioral tests we showed that *reeler* mice display a profound increase in the mechanical nociceptive threshold and a significant thermal hyperalgesia. These behavioral abnormalities are the first to be defined in this well known mouse model, which is generally considered to manifest motor and coordination deficits.

Reelin and Dab1 are implicated in dorsal horn development

Reelin expression is found within laminated CNS structures, including the cerebral cortex, cerebellum, retina and hippocampus, and is thought to influence neuronal positioning and/or synaptogenesis in these regions (Caviness and Rakic, 1978; Lambert de Rouvroit and Goffinet, 1998; Rice and Curran, 2001). Given the previously defined contribution of Reelin in laminated structures, Kubasak et al. (2004) postulated that Reelin may be involved in the development of the dorsal horn, which is also a cytoarchitecturally laminated region.

Our previous investigation suggested that the large Reelin-positive cells in lamina I might be Waldeyer's cells, which raised the possibility that these Reelin-positive cells

Table 2. Thermal hyperalgesia is found in *reeler* mutants

Genotype	Sex	*n*	r, Plantar response	l, Plantar response	Mean response
Control	Combined	11	11.13±2.41	11.54±2.20	11.33±2.41
	Male	6	12.84±1.74	12.45±1.81	12.65±1.71
	Female	5	9.42±1.64	10.63±1.67	10.02±2.00
reeler	Combined	11	6.72±1.41	6.03±2.15	6.38±1.78[a]
	Male	6	7.07±2.00	6.35±1.24	6.71±1.62[b]
	Female	5	6.43±1.67	5.76±2.26	6.10±1.93[c]

Hargreaves' paw withdrawal test carried out on both right and left hindpaws. Mean response time±SD reported in seconds and statistical significance analyzed with ANOVA.

[a] $F = 35.77$, $df=10$, $P<0.0001$.

[b] $F = 22.44$, $df=4$, $P<0.015$.

[c] $F = 17.44$, $df=5$, $P<0.019$.

may influence neuronal positioning in the dorsal horn laminae. Altman and Bayer (1984) proposed that the marginal cells of Waldeyer may be responsible for establishing the migratory pattern of the dorsal horn. Specifically, they suggested that the earliest generated Waldeyer cells would remain on the outside of the dorsal horn while the cells born later would occupy the deeper laminae. The adjacent expression domains of Reelin and Dab1 in the superficial dorsal horn and LSN in the present investigation further support this possibility. As in the cortex and cerebellum, the expression of Reelin in the superficial dorsal horn may influence the subsequent migration of Dab1-positive neurons. By identifying the adjacent expression of Reelin and Dab1, our observations implicate the Reelin-signaling pathway in the proper positioning of neurons within laminae I–III and the LSN.

Reelin is involved in neuronal positioning within nociceptive areas of the spinal cord

Our study focused first on lamina I and the LSN, areas known to contain projection neurons that convey nociceptive information to rostral CNS targets (Burstein et al., 1990a,b; Gauriau and Bernard, 2004; Giesler et al., 1979; Olave and Maxwell, 2004). The distinct neuronal compaction of lamina I, in conjunction with the significant neuronal loss observed in the LSN of *reeler* mutants, highlights the importance of Reelin in establishing proper neuronal positioning in areas involved in nociceptive signaling throughout the spinal cord.

The loss of LSN neurons in *reeler* mice is reminiscent of results from previous studies which showed that a majority of the sympathetic and parasympathetic preganglionic neurons in the *reeler* spinal cord migrate past their normal intermediolateral targets to reside in more medial locations (Kubasak et al., 2004; Phelps et al., 2002; Yip et al., 2000, 2003). We suggest that some of the missing LSN neurons also fail to stop migrating and instead either migrate back to the spinal cord midline or alternatively, enter into the dorsal horn. The presence of Dab1-positive cells in both the dorsal midline of the spinal cord and in laminae I–III at older embryonic and postnatal ages is consistent with the hypothesis that both migratory errors occur. Additional studies are necessary to further characterize the normal migration pattern of the LSN neurons in mice and subsequently to compare it to the *reeler* mutants.

We detected Dab1 in the postnatal LSN of both wild-type and *reeler* mice, a time when neuronal migration is complete. Similar to our findings, Rice et al. (2001) reported that postnatal AII amacrine cells are Dab1-positive in both control and mutant mice. Additionally, their study showed a decrease in rod-derived retinal responses in *reeler* mutants as well as abnormal synaptic distributions on both rod bipolar and AII amacrine cells, results suggesting that the Reelin signaling pathway is active in the retina during synaptogenesis (Rice and Curran, 2001; Rice et al., 2001). Thus, one possible explanation of Dab1 localization in postnatal LSN is that the Reelin signaling pathway contributes to functions related to neuronal circuit formation in the dorsal horn.

Reelin is involved in nociception

The presence of highly abnormal mechanical and thermal nociception in *reeler* mutants implicates Reelin in the development of nociceptive processing. The specification of nociceptive neurons occurs early in development and precedes the formation of synaptic contacts with their targets (Fitzgerald, 2005; Kubasak et al., 2004). The establishment of laminae I, II and the LSN are essential components in the development and subsequent processing of noxious and thermal sensitivity in mammals (Burstein et al., 1990b; Fitzgerald, 2005; Olave and Maxwell, 2004; Puskár et al., 2001). Neurons in lamina I receive input directly from Aδ and C primary afferent fibers, including both nociceptors and thermoreceptors, and are the main source of output from the superficial dorsal horn (Snider and McMahon, 1998). To date the absence of Reelin has been implicated in altered synaptogenesis in both the hippocampus and retina (Borrell et al., 1999; Del Río et al., 1997; Rice et al., 2001; Weeber et al., 2002). Therefore, the possibility should be considered that either lamina I or the LSN projection neurons fail to establish proper connections with the corresponding primary afferents, dorsal horn interneurons, and supraspinal CNS targets. The identification of mispositioned Dab1-positive neurons expressing NK-1 receptors in *reeler* mutants together with the proposed role of Reelin in synaptogenesis, suggests that

nociceptive processing may be altered in this cellular group. Additionally, the presence of NK-1 receptors on Dab1 containing neurons provides a link between the anatomical abnormalities and functional consequences detected in *reeler* mice.

The remarkable behavioral results that we found in the *reeler* mutants, namely thermal hyperalgesia and significantly reduced mechanical sensitivity, could result from disruptions in physiologically distinct primary afferent fibers, or in their connections with second order neurons of the superficial dorsal horn. As no remarkable differences were detected between nociceptors in the mutant and control DRG, our results to date implicate defects in CNS circuitry. Previous studies have defined morphologically and physiologically distinct lamina I neurons (Han et al., 1998; Puskár et al., 2001). Conceivably it is the connections with these different classes of nociresponsive projection neurons that underlie the unusual phenotypes manifest in *reeler* mutants. Based on these results, we suggest that Reelin acts in a cell specific manner to disrupt the positioning of individual populations of nociceptors, resulting in abnormal connectivity with subpopulations of neurons in lamina I and the Dab1-positive cells expressing NK-1 receptors in laminae I–III. Contributions to the altered mechanical and/or thermal nociceptive processing in *reeler* mutants also may be attributed to the reduction or misplacement of projection neurons within the LSN. Together, the nociceptive defects and aberrant neuronal positioning within laminae I–III and the LSN may help elucidate the importance of cellular positioning to the establishment of normal nociceptive processing in the spinal cord.

For the most part, the specificity of synaptic connections in *reeler* mice is thought to be preserved throughout the CNS. In fact, despite the positioning defects, the afferents that innervate the sympathetic preganglionic neurons appear to maintain their proper connectivity (Yip et al., 2003). On the other hand, because there is no simple behavioral test to evaluate the integrity of these connections, how functionally normal the system is remains to be determined. The presence of compacted lamina I cells and ectopically positioned Dab1-positive neurons in laminae I–III easily could contribute to a reduction in the dendritic development as previously observed in hippocampal pyramidal and dentate granule cells (Niu et al., 2004) and in somatic motor neurons (Phelps et al., 2002). The loss of cells in the LSN also may contribute to distortions in synaptic connectivity either from the projection neurons to their brainstem targets, or from spinal cord interneurons that project to LSN neurons. Although the connections may be generally correct, the dramatic behavioral changes in mechanical and thermal nociception suggest that substantial disruptions in neuronal connectivity are present.

What makes this particular Reelin model unique from other areas previously studied is its restricted location, defined afferent distribution, and the presence of a profound behavioral manifestation, that appears to result from a localized disruption of circuits that underlie the processing of nociceptive messages. It will be of particular interest to utilize this model in the setting of persistent injury and to decipher if the typical development of mechanical hypersensitivity will occur in *reeler* mutants. Future studies of the *reeler* superficial dorsal horn also will enable new investigations not only concerning mechanisms of neuronal migration in general, but more specifically also on the contribution of Reelin to the development of neuronal connections that underlie complex pain behaviors.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.neuroscience.2006.01.042.

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