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Reelin dorsal horn neurons co-express Lmx1b and are mispositioned in *disabled-1* mutant mice

Griselda M. Yvone,

Carmine L. Chavez-Martinez,

Amanda R. Nguyen,

Deborah J. Wang,

Patricia E. Phelps

Department of Integrative Biology and Physiology, UCLA, Terasaki Life Science Building, 610 Charles E. Young Drive East, Los Angeles, CA 90095-7239, USA

Abstract

Mice missing either Reelin or Disabled-1 (Dab1) exhibit dorsal horn neuronal positioning errors and display heat hypersensitivity and mechanical insensitivity. Reelin binds its receptors, Apolipoprotein E receptor 2 and Very-low-density lipoprotein receptor, leading to the recruitment and phosphorylation of Dab1 and activation of downstream pathways that regulate neuronal migration. Previously, we reported that 70% of Dab1 laminae I-II neurons co-expressed LIM homeobox transcription factor 1 beta (Lmx1b). Here we asked if Reelin-expressing dorsal horn neurons co-express Lmx1b, are mispositioned in *dab1* mutants, and contribute to nociceptive abnormalities. About 90% of Reelin-labeled neurons are Lmx1b-positive in laminae I-II, confirming that most Reelin and Dab1 neurons are glutamatergic. We determined that Reelin-Lmx1b and Dab1-Lmx1b dorsal horn neurons are separate populations, and together, comprise 37% of Lmx1b-positive cells within and above the Isolectin B4 (IB4) layer in wild-type mice. Compared to wild-type mice, *dab1* mutants have a reduced area of laminae I-II outer (above the IB4 layer), more Reelin-Lmx1b neurons within the IB4 layer, and fewer Reelin-Lmx1b neurons within the lateral reticulated area of lamina V and lateral spinal nucleus. Interestingly, both Reelin- and Dab1-labeled dorsal horn neurons sustain similar positioning errors in mutant mice. After noxious thermal and mechanical stimulation, Reelin, Lmx1b, and Reelin-Lmx1b neurons expressed Fos in laminae I-II and the lateral reticulated area in wild-type mice, and therefore

Please address correspondence to: Patricia E. Phelps, Ph.D., Dept. of Integrative Biology and Physiology, UCLA, Terasaki Life Science Building, 610 Charles Young Dr. E, Los Angeles, CA 90095-7239, USA, FAX: (310) 206-9184, Phone: (310) 825-7264, pphelps@physci.ucla.edu.

Author contributions

GMY: designed study, performed experiments, analyzed data, wrote and revised manuscript
CLCM: performed experiments, analyzed data, revised manuscript
ARN: performed experiments, revised manuscript
DJW: performed experiments, revised manuscript
PEP: designed study, analyzed data, wrote and revised manuscript

Competing interests

The authors declare no competing financial interests.

Data Accessibility

The datasets and images generated and analyzed during the current study are available from the first and corresponding authors on reasonable request.

participate in nociceptive circuits. Together, our data suggest that disruption of the Reelin-signaling pathway results in neuroanatomical abnormalities that contribute to the nociceptive changes that characterize these mutant mice.

Graphical Abstract

Reelin-Disabled-1 (Dab1) signaling regulates neuronal migration in the spinal dorsal horn. 90% of Reelin and 70% of Dab1-expressing laminae I-II neurons co-express Lmx1b and are glutamatergic. These Reelin and Dab1 neurons are separate subsets of Lmx1b neurons. In *dab1*$^{-/-}$, Reelin-Lmx1b neurons are mispositioned in similar nociceptive areas as Dab1-Lmx1b neurons in *Reln*$^{-/-}$ mice. They are increased in laminae I-II dorsal inner and reduced in lateral lamina V and lateral spinal nucleus.

Keywords

Dab1; lateral spinal nucleus; lateral lamina V; *reeler* ; spinal cord

Introduction

The canonical Reelin-signaling pathway involves the binding of secreted Reelin (Reln) to its two receptors, Apolipoprotein E receptor 2 (Apoer2) and Very-low-density-lipoprotein receptor (Vldlr), and the recruitment and phosphorylation of the adaptor protein Disabled-1 (Dab1). Phosphorylated Dab1 activates signaling pathways that regulate neuronal migration during embryonic development and then is degraded (Howell *et al.*, 1997; Arnaud *et al.*, 2003; Bock & Herz, 2003). In the absence of Reelin, Dab1 is not degraded but rather accumulates in neurons that migrate aberrantly and sustain positioning errors. Mice without Reelin (*Reln*$^{-/-}$), both Apoer2 and Vldlr (*Apoer2*$^{-/-}$/*Vldlr*$^{-/-}$), or Dab1 (*dab1*$^{-/-}$) display similar neuronal positioning errors in the central nervous system (Howell *et al.*, 1997; Trommsdorff *et al.*, 1999). Although expression levels of the components of the Reelin-Dab1-pathway decrease after birth, lower levels can regulate both postnatal dendritic morphology (Niu *et al.*, 2004; Matsuki *et al.*, 2008) and adult synaptic functions (Weeber *et al.*, 2002; Beffert *et al.*, 2005; Ventruti *et al.*, 2011).

The Reelin-signaling pathway has an established role in nociception, as the mutant mice (*Reln*$^{-/-}$, *dab1*$^{-/-}$) show functional changes including heat hypersensitivity and mechanical insensitivity (Villeda *et al.*, 2006; Akopians *et al.*, 2008; Wang *et al.*, 2012; Yvone *et al.*, 2017). Moreover, in the spinal cord, Reelin and Dab1 are concentrated in areas dedicated to pain transmission, i.e., laminae I-II, the lateral reticulated area of lamina V, and the lateral spinal nucleus (LSN; Villeda *et al.*, 2006). Recently we reported that 70% of Dab1-expressing neurons in the lumbar superficial dorsal horn are glutamatergic as they co-express the LIM-homeobox transcription factor 1-beta (Lmx1b; Cheng *et al.*, 2004; Yvone *et al.*, 2017). Evidence that Dab1 neurons are mispositioned in *Reln*$^{-/-}$ mice include: a compaction of laminae I-II outer (area dorsal to the Isolectin B4 layer; IB4), more Dab1 and Dab1-Lmx1b neurons within the IB4 region than *Reln*$^{+/+}$ mice, and fewer Dab1-Lmx1b neurons in the lateral reticulated area of lamina V and LSN than *Reln*$^{+/+}$ mice (Yvone *et al.*,

2017). These anatomical abnormalities likely contribute to the altered nociceptive functions in *Reln*$^{-/-}$ mice.

Study of the brain-sparing *Lmx1b* conditional knockout mice reported a large reduction in Reelin-positive cells in laminae I-II and insensitivity to mechanical stimulation in the mutants (Szabo *et al.*, 2015). As the *dab1*$^{-/-}$ mice exhibit mechanical insensitivity comparable to that of the conditional *Lmx1b* mutants, we first assessed Reelin expression in superficial dorsal horn neurons, and then asked whether the Reelin neurons showed positioning errors. As most neurons in laminae I-II are excitatory, including the Dab1 neurons, we tested if Reelin-labeled cells co-expressed Lmx1b. Based on our findings we then determined whether Reelin and Dab1 neurons were the same or separate populations of excitatory neurons. We also compared the locations of Reelin-expressing neurons in other nociceptive areas of the dorsal horn, the lateral reticulated area of lamina V and the LSN in *dab1*$^{+/+}$ and *dab1*$^{-/-}$ mice. Finally, we asked if the Reelin-expressing neurons participate in thermal and mechanical nociceptive circuits.

Materials and Methods

Animals

dab1* mice—dab1*^{lacZ} mice were a gift from Dr. Brian Howell (SUNY Upstate Medical University, Syracuse, NY) and established as a breeding colony at UCLA. The generation and characterization of the *dab1*^{lacZ} mice were described previously (Abadesco *et al.*, 2014), and they were genotyped according to Pramatarova *et al.* (2008). Dab1 protein expression is eliminated in these mutants that have abnormal pain responses similar to those of *Reln,* *dab1*KO, and *scrambler* mutants (Villeda *et al.*, 2006; Akopians *et al.*, 2008; Yvone *et al.*, 2017). The *dab1*^{lacZ} mice that we used to compare Reelin expression are identified in this study as *dab1*$^{+/+}$ and *dab1*$^{-/-}$ mice, and used for both thermal and mechanical (Fos) stimulation experiments (Yvone *et al.*, 2017).

*dab1*KO mice (BALB/cByJ *dab1*$^{-/-}$, gifts from Drs. J. Cooper, Fred Hutchinson Cancer Research Center, Seattle, WN, and B. Howell, SUNY Upstate Medical University, Syracuse, NY; Howell *et al.*, 1997) were bred at UCLA and genotyped using polymerase chain reaction as described by Brich *et al.* (2003). These mice were used for thermal (Fos) stimulation experiments (Wang *et al.*, 2019).

Apoer2*/*Vldlr* mice—Apoer2* (B6; 129S6-*Lrp8*^{tm1Her}, generous gift from Dr. T. Curran) and *Vldlr* (B6; 129S7-*Vldlr*^{tm1Her}, Jackson Laboratory) mice were generated from breeding colonies maintained at UCLA. *Apoer2*$^{-/-}$ and *Vldlr*$^{-/-}$ mutant mice were genotyped following protocols adapted from Frykman *et al.* (1995) and Trommsdorff *et al.* (1999). As *Apoer2*$^{-/-}$/*Vldlr*$^{-/-}$ mice usually do not survive to adulthood, we used the tissues from two, 30-day-old *Apoer2*$^{-/-}$/*Vldlr*$^{-/-}$ mice with their wild-type controls only to evaluate whether Reelin and Dab1 cells are the same or separate neuronal populations.

***Reln*$^{rl\text{-}Orl}$; *GAD67*^{GFP} mice—**Mice were generated as described in Abadesco *et al.* (2014) and genotyped as adapted from Hammond *et al.* (2006). The *Reeler Orleans* (*Reln*$^{rl\text{-}Orl}$) mutation causes defective Reelin secretion (de Bergeyck *et al.*, 1997). The Green

Fluorescent Protein (GFP) reporter was expressed under the control of the glutamic acid decarboxylase 67 (GAD67) gene in the *GAD67*$^{GFP/+}$ mice (Tamamaki *et al.*, 2003). These mice were intercrossed with *Reln*$^{rl\text{-}Orl}$ mice to ask if Reelin-labeled cells were GABAergic.

Reln* mice—Reln* (B6C3Fe-ala-*Reln*^{rl}) mice were obtained from Jackson Laboratory and a breeding colony was established. Mice were genotyped according to D'Arcangelo *et al.* (1996). We used *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice to analyze Dab1 neurons, and these mice were also used for mechanical (Fos) stimulation (Yvone *et al.*, 2017).

Fos stimulation

All experiments were approved by the Chancellor's Animal Research Committee at UCLA and conducted according to the National Institute of Health guidelines. Earlier studies of *Reln* or *dab1* mice found that their responses did not differ by sex (Villeda *et al.*, 2006; Akopians *et al.*, 2008). Fos stimulation parameters were similar to the ones described in previous reports (Yvone *et al.*, 2017; Wang *et al.*, 2019).

Thermal (heat) Fos stimulation—Age-matched sets of female mice were used (n=5–7 mice/genotype) and lightly anesthetized with sodium pentobarbital (50–60 mg/kg). 15 mins after anesthesia induction, the left hindpaw was dipped into 50ºC water for 3 sec/min for 10 min. One hour later, mice were re-anesthetized (80–100 mg/kg) and perfused.

Mechanical Fos stimulation—Age-matched male mice (n=5–7 mice/genotype) were used and lightly anesthetized as described for thermal Fos stimulation. The left hindpaw was stimulated with a padded alligator clip for 20 sec every 3 min for 15 min. After one hour, mice were re-anesthetized as above and perfused.

Tissue preparation and immunohistochemistry

Adult male and female mice were anesthetized (sodium pentobarbital, 100 mg/kg), perfused transcardially with 4% paraformaldehyde in 0.12 M phosphate buffer (PB), and post-fixed for 1–4 h at 4°C in the same fixative. Following an overnight wash with 0.12 M PB, spinal cords were dissected and cryoprotected in 30% sucrose in PB. Lumbar (L4–5) segments were blocked and frozen in Optimum Cutting Temperature embedding medium (Fisher Scientific #4585) and stored at −80°C. 25 μm thick, free-floating sections were used for most immunofluorescence experiments. Sections for Fos experiments were 20 or 40 μm thick.

Immunohistochemical procedures—Most Reelin immunofluorescence experiments used mouse anti-Reelin (G10; see Table 1 for primary antibody information) together with a Tyramide Signal Amplification (TSA) kit. Sections were incubated with 3% hydrogen peroxide and 10% methanol in 0.1M phosphate buffered saline (PBS) followed by a blocking solution of 1% nonfat milk, 0.2% gelatin, 10% normal donkey serum (NDS), and 0.5% Triton X-100 in PBS for 1 h. Sections were incubated overnight with mouse anti-Reelin at 4°C, followed by repeated washes in PBS and a 1 h incubation with biotinylated donkey anti-mouse IgG (1:800; Jackson Immunoresearch #715–065-150) in PBS. Multiple washes with TNT (0.1M Tris-HCl; 0.15M NaCl; 0.05% Tween) preceded a

1 h incubation with streptavidin-conjugated horseradish peroxidase (1:1,000; PerkinElmer #NEL750001EA) in TSA-specific blocking buffer (TNB; 0.1M Tris-HCl; 0.15M NaCl; 0.5% Blocking reagent; PerkinElmer #FP1020), and a 5-min incubation with TSA Plus Fluorescein (1:150; PerkinElmer #NEL741001KT) or Cyanine 5 (Cy5; 1:100; PerkinElmer #NEL745001KT).

To identify Dab1-labeled neurons, sections were incubated with 1% hydrogen peroxide and 0.1% sodium azide in PBST (0.1M PBS and 0.3% Triton X-100) followed by blocking with 5% NDS in PBST and Avidin-Biotin. Sections were then incubated for 48 hrs with rabbit anti-Dab1 (Table 1) and then washed in PBST and TNT, followed by a 1 h incubation with biotinylated donkey anti-rabbit IgG (1:1,000; Jackson Immunoresearch #711–065-152) in TNB. Multiple washes with TNT preceded a 1 h incubation with streptavidin-conjugated horseradish peroxidase in TNB, and a 5-min incubation with TSA Plus Cy5 (1:100; PerkinElmer #NEL745001KT). To determine if Reelin neurons expressed Lmx1b or Paired box 2 (Pax2), 5% NDS and 0.1% Triton X-100 in PBS was used as the blocking solution and followed by an overnight incubation with primary antibodies (Table 1). The standard TSA immunofluorescence protocol described above was then used.

To detect Fos expression in Reelin and Lmx1b-labeled neurons, a triple TSA immunofluorescence protocol was used. Sections were first incubated with TNB blocking solution followed by rabbit anti-Fos (Table 1) overnight. TSA plus Cy5 was used for Fos immunofluorescence, and then sections were incubated with goat anti-Reelin (Table 1) the second night. TSA plus Fluorescein was used for Reelin labeling, and finally sections were incubated with anti-Lmx1b (Table 1) overnight followed by TSA plus Cyanine 3 (Cy3; 1:150; PerkinElmer # NEL744B001KT).

To examine the locations of Reelin and Lmx1b-expressing neurons within and above the IB4 region, IB4 (Table 1) was visualized using a biotinylated IB4 conjugate as published (Yvone *et al.*, 2017). Routine immunofluorescence techniques were used in $Reln^{rl\text{-}Orl}$; $GAD67^{GFP}$ mice to localize GAD67 with chick anti-GFP as reported (Abadesco *et al.*, 2014). Most secondary antibodies were purchased from Jackson Immunoresearch including: biotinylated donkey anti-guinea pig (#706–065-148) and donkey anti-rabbit IgG (#711–065-152) for TSA experiments. Streptavidin-AlexaFluor 488 (Invitrogen, Molecular Probes #S32354) was used to visualize the IB4 conjugate, and donkey anti-chick Alexa Fluor 488 (#703–545-155) to detect GFP. Some sections were counterstained with Hoechst nucleic acid stain (1:500; Molecular Probes #H1398), mounted, dried, and coverslipped.

We used a Zeiss Laser Scanning Microscope (LSM800) with solid-state lasers 488nm, 561nm, and 640nm for triple-labeled images and with 488nm and 640nm lasers for double-labeled images. The 405nm laser was used to image Hoechst as a nuclear marker. Hemisections were captured with a 10x objective (numerical aperture 0.45), while high magnification images of the superficial dorsal horn, LSN and lateral lamina V were obtained with a 40x oil immersion lens (numerical aperture 1.4) with the pinhole aperture set to 1 Airy unit. The mediolateral width of laminae I-II, the LSN and lateral lamina V were scanned within the depth of the section where signals of all channels were detected to generate z-series of approximately 6–12 μm, with a z-separation of 3 μm.

ZEN (Zeiss Efficient Navigation) lite (v. 2.3) imaging software was used for all analyses. For the laminae I-II datasets, a single optical section with the highest signal for all channels was selected and analyzed (estimated thickness 1 μm). In most cases, the optical section selected was located in the center of the z-series. Laminae I-II were measured 120 μm from the dorsal border of grey and white matter (adapted from Yvone *et al.*, 2017). The fact that we only analyzed a single optical section may have underestimated the total number of neurons, especially those small in size. Due to the limited number of cells present in the LSN and lateral lamina V, 3–5 optical sections per z-series were analyzed. Lateral lamina V counts were sampled in a 17,000 μm^2 boxed region adjacent and medial to the LSN (illustrated in Yvone *et al.*, 2017), whereas the LSN counts were conducted in an outlined region as reported in Wang *et al.* (2012).

To determine co-localization, the Reelin and Lmx1b channels were viewed together and then separately by channel. Co-localization was determined by cytoplasmic Reelin encircling the Lmx1b-positive nucleus. Each Lmx1b-positive nucleus was then compared to the Hoechst-labeled profile. The remainder of the optical sections were used to confirm neuronal profiles. A Reelin-only cell was identified as a Hoechst-labeled nucleus surrounded by Reelin-labeled cytoplasm. For the Reelin-Lmx1b-IB4 dataset, both the IB4-positive area and the region above IB4 (laminae I-II outer) were outlined and cells that were immunoreactive for Reelin, Lmx1b, and Reelin-Lmx1b were counted. For all analyses, counts were restricted to cells with a diameter greater than 7 μm to ensure neuronal identity, a measurement determined by an analysis of anti-Neuronal Nuclei (NeuN) immunoreactivity (data not shown). Images were transferred to Photoshop for figure assembly.

Experimental design and statistical analyses

To determine the percentage of Reelin neurons that co-expressed Lmx1b in laminae I-II, we counted labeled neurons in 5 hemisections per mouse in *dab1*$^{+/+}$ and *dab1*$^{-/-}$ mice (n = 3 mice/genotype). The means of Reelin and Reelin-Lmx1b neurons were compared by genotype and cell type with 2×2 repeated measures ANOVA. Total numbers of Reelin neurons were compared with ANOVA. Post hoc *p*-values for all mean comparisons are reported. All statistical analyses in this study were performed with JMP 10 (SAS Inc.).

To identify positioning errors and estimate the percentage of total Lmx1b neurons that were Reelin-positive in laminae I-II inner dorsal, we counted Reelin-, Lmx1b-, and double-labeled cells within the IB4 layer (lamina II inner dorsal) and above the IB4 layer (laminae I-II outer). 3 hemisections per mouse were analyzed in *dab1*$^{+/+}$ and *dab1*$^{-/-}$ mice (n = 4 mice/genotype). The means of Reelin-, Lmx1b-, Reelin-Lmx1b-labeled neurons were compared by genotype and area for each cell type with a 2×2 repeated measures ANOVA and post hoc *t*-tests. The means of total Reelin and total Lmx1b neurons were compared by genotype and area in a separate ANOVA. The significance of IB4 area measurements was analyzed with ANOVA. Percentages of Reelin-Lmx1b neurons out of total Reelin or total Lmx1b neurons were obtained by combining the numbers of cells above the IB4 layer (laminae I-II outer) and within the IB4 area (lamina II inner dorsal).

We also analyzed the total number of Lmx1b neurons from a previous dataset of Dab1 and Dab1-Lmx1b neurons within and above the IB4 region (Yvone *et al.*, 2017). Here we report

the percentage of Lmx1b neurons that co-expressed Dab1 in the superficial dorsal horn. Six hemisections per mouse were analyzed in *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice (n = 4 mice/genotype). ANOVA was used to compare the mean numbers of Lmx1b-positive neurons by genotype.

To estimate the numbers of Reelin, Reelin-Lmx1b and Lmx1b neurons in lateral lamina V and the LSN, we examined 3–10 hemisections per mouse in *dab1*$^{+/+}$ and *dab1*$^{-/-}$ mice (n = 4 mice/genotype). For each area, the means of Reelin, Lmx1b, and Reelin-Lmx1b neurons, together with total Reelin and Lmx1b neurons, were compared by genotype and cell type with a 2×3 repeated measures ANOVA and post-hoc *t*-tests.

Finally, we compared the number of total Fos neurons in unstimulated controls (n = 3 *dab1*$^{+/+}$ mice; 3–4 hemisections/mouse, and 4 *Reln*$^{+/+}$ mice; 3–5 hemisections/mouse) versus mice stimulated with noxious thermal (n = 4 *dab1*$^{+/+}$ mice; 4–5 hemisections per mouse) or mechanical stimulation (n = 4 *Reln*$^{+/+}$ mice; 4–6 hemisections per mouse). The sample size was too small to subdivide Fos-expressing neurons identified with Reelin, Lmx1b, and Reelin-Lmx1b. The means of total Fos neurons were compared by stimulation with ANOVA.

Results

90% of Reelin-expressing laminae I-II neurons co-express Lmx1b

Because the majority of laminae I-II interneurons are excitatory (Todd, 2010), we first asked whether Reelin cells in this area co-express the transcription factor Lmx1b, which is a glutamatergic neuron marker (Cheng *et al.*, 2004). The majority of Reelin-labeled neurons in laminae I-II co-localized with Lmx1b nuclei in *dab1*$^{+/+}$ (Fig. 1A–A3; arrowheads) and *dab1*$^{-/-}$ mice (Fig. 1B–B3; arrowheads). Single-labeled Reelin neurons also were detected (Fig. 1A1–2; arrows). Occasionally, large Reelin-Lmx1b neurons were found in laminae I-II of both genotypes (Fig. 1B1–3; large arrowheads), but the majority of double-labeled neurons were small. The Reelin-expressing neurons, with or without Lmx1b, were analyzed in laminae I-II and did not differ between genotypes (*dab1*$^{+/+}$ 34±0.6 neurons; *dab1*$^{-/-}$ 31±1.4 neurons; n=3 mice/genotype). These analyses revealed that 88–92% of Reelin-expressing laminae I-II neurons co-expressed Lmx1b (*dab1*$^{+/+}$ 88±5.1%; *dab1*$^{-/-}$ 92±1.5%), and therefore the majority of Reelin neurons in the adult superficial dorsal horn are glutamatergic.

Reelin- and Dab1-labeled laminae I-II neurons are separate subsets of the Lmx1b population

Because both Dab1 (Yvone *et al.*, 2017) and Reelin laminae I-II neurons co-express Lmx1b, we asked whether or not they represent the same subset of these excitatory neurons. To address this question, we examined *Apoer2;Vldlr* spinal cords, as both Reelin and Dab1 neurons can be visualized in both genotypes in these mice (Trommsdorff *et al.*, 1999). With triple immunolabeling, we showed that Reelin-Lmx1b and Dab1-Lmx1b neurons (Fig. 2A–A3 and 2B–B3) were distributed throughout laminae I-II. The outer superficial dorsal horn in the *Apoer2*$^{-/-}$*;Vldlr*$^{-/-}$ mouse showed evidence of neuronal compaction as a row of Reelin-Lmx1b neurons was occasionally observed in the double-receptor mutant

(Fig. 2B, white arrows) but not in the wild-type mouse (Fig. 2A, Akopians *et al.*, 2008). To demonstrate the specificity of the Reelin-Dab1-Lmx1b labeling, we examined *Reln*$^{-/-}$ dorsal horn and show that only Dab1 and Lmx1b proteins are expressed (Fig. 2C–C2), whereas in the *dab1*$^{-/-}$ dorsal horn, only Reelin and Lmx1b proteins are detected (Fig. 2D–D2). Importantly, results from the *Apoer2;Vldlr* mice show that Reelin- and Dab1-Lmx1b neurons represent separate subsets of Lmx1b-expressing neurons.

Reelin-Lmx1b neurons are mispositioned in *dab1* mutant laminae I-II inner dorsal

To assess whether the loss of the Reelin-signaling pathway in *dab1*$^{-/-}$ mice results in similar neuroanatomical rearrangements to those of *Reln*$^{-/-}$ mice (Yvone *et al.*, 2017), we examined Reelin neurons within the *dab1*$^{-/-}$ superficial dorsal horn. Specifically, we analyzed the distribution of Reelin and Lmx1b neurons within the IB4 layer, which receives nonpeptidergic nociceptive afferents that terminate in lamina II inner dorsal (Basbaum *et al.*, 2009). Measurements of the area overlaid by the IB4 terminals showed no differences between genotypes, but fewer Reelin-Lmx1b neurons were found in the *dab1*$^{+/+}$ than the *dab1*$^{-/-}$ IB4 terminal zones (Fig. 3A1–2, B1–2, C; *dab1*$^{+/+}$ 10±1.6 neurons; *dab1*$^{-/-}$ 16±1.5 neurons; p=0.022; n=4 mice/genotype). In contrast, the numbers of single-labeled Reelin (Fig. 3C; *dab1*$^{+/+}$ 2±0.6 neurons; *dab1*$^{-/-}$ 2±0.4 neurons) and Lmx1b neurons (Fig. 3C; *dab1*$^{+/+}$ 58±6.6 neurons; *dab1*$^{-/-}$ 59±3.5 neurons) in the IB4 area did not differ. Thus, our data show that despite both genotypes having similar areas of lamina II inner dorsal, *dab1*$^{-/-}$ mice had increased numbers of Reelin-Lmx1b neurons in this area compared to *dab1*$^{+/+}$ mice.

Next, we analyzed the area above the IB4 layer that receives peptidergic innervation, laminae I-II outer. We found that the size of this region in *dab1*$^{+/+}$ was larger compared to *dab1*$^{-/-}$ mice (Fig. 3A, B; *dab1*$^{+/+}$ 21,226±755 μm^2; *dab1*$^{-/-}$ 16,598±1,223 μm^2; p=0.006). Surprisingly, neither the number of Reelin, Lmx1b, or Reelin-Lmx1b neurons in laminae I-II outer differed between genotypes (Fig. 3C). Relatively few Reelin-expressing cells were present ventral to the IB4 layer (lamina II inner ventral; Fig. 3A1–2, B1–2), an area characterized by Protein Kinase C γ (PKCγ)-positive interneurons that responds to innocuous touch (Neumann *et al.*, 2008). Our data show that although the area of laminae I-II outer is smaller in *dab1*$^{-/-}$ than *dab1*$^{+/+}$ mice, the numbers of Reelin and Lmx1b neurons did not differ.

Nearly 40% of Lmx1b neurons within laminae I-II inner dorsal express Reelin or Dab1

To determine what percentage of the Lmx1b-positive neurons in the superficial dorsal horn were associated with Reelin signaling, we quantified the numbers of Reelin-, Lmx1b-, and Reelin-Lmx1b-expressing neurons in laminae I-II outer (above IB4 layer) and in lamina II inner dorsal (the IB4 layer). The numbers of Reelin-only and Lmx1b-only neurons in these combined areas (i.e., laminae I-II inner dorsal) did not differ. In contrast, there were fewer Reelin-Lmx1b neurons (*dab1*$^{+/+}$ 20±1.6; *dab1*$^{-/-}$ 31±1.6; p=0.004; n=4 mice/genotype; Table 2) and total Reelin neurons (*dab1*$^{+/+}$ 25±3.3; *dab1*$^{-/-}$ 35±1.9; p=0.04) in *dab1*$^{+/+}$ than in *dab1*$^{-/-}$ mice. The number of total Lmx1b neurons in this combined area, however, did not differ (Table 2). The Reelin-Lmx1b neurons in laminae I-II inner dorsal made up

83–88% of total Reelin-labeled neurons (*dab1*$^{+/+}$ 83±3.5%; *dab1*$^{-/-}$ 88±1.9%) and 17–24% of total Lmx1b neurons (*dab1*$^{+/+}$ 17±1.7%; *dab1*$^{-/-}$ 24±2.2%).

Next, we quantified the number of Lmx1b neurons in laminae I-II inner dorsal to estimate the percentage of Lmx1b-positive neurons that co-expressed Dab1. Previously, we found that in *Reln*$^{-/-}$ mice, there were increased numbers of single-labeled Dab1 and Dab1-Lmx1b neurons only within lamina II inner dorsal (Yvone *et al.*, 2017). In the combined area of laminae I-II inner dorsal, however, only the single-labeled Dab1 neurons were increased in *Reln*$^{-/-}$ compared to *Reln*$^{+/+}$ mice (Table 3; *Reln*$^{+/+}$ 10±0.7; *Reln*$^{-/-}$ 12±0.6; p=0.03; n=4 mice/genotype). On the other hand, the numbers of Lmx1b-only, Dab1-Lmx1b, total Dab1 and total Lmx1b neurons did not vary significantly between genotypes. Dab1-Lmx1b neurons comprised 71–74% of total Dab1 neurons (*Reln*$^{+/+}$ 74±2.1%; *Reln*$^{-/-}$ 71±2.2%) and 20–22% of total Lmx1b neurons (*Reln*$^{+/+}$ 20±2.9%; *Reln*$^{-/-}$ 22±0.6%) in laminae I-II inner dorsal.

The Reelin-Lmx1b neurons in *dab1*$^{+/+}$ laminae I-II inner dorsal represent 17% of the total Lmx1b neurons, whereas Dab1-Lmx1b neurons in *Reln*$^{+/+}$ mice made up an average of 20% of the total Lmx1b neurons. Together, the Reelin-Lmx1b and Dab1-Lmx1b neurons composed about 37% of the total Lmx1b-positive neurons in laminae I-II inner dorsal in wild-type mice. In *dab1*$^{-/-}$ laminae I-II inner dorsal, Reelin-Lmx1b neurons comprised about 24% of the total Lmx1b neurons, whereas in *Reln*$^{-/-}$ laminae I-II inner dorsal, Dab1-Lmx1b represented an average of 22% of the total Lmx1b neurons. Our results show that a large number of Lmx1b neurons are associated with the Reelin-Dab1-signaling pathway in laminae I-II inner dorsal, particularly within the IB4 layer.

Most Reelin neurons in the laminae I-II are not inhibitory

Given that most Reelin neurons in the laminae I-II are excitatory, we asked if the remaining 8–12% of Reelin-labeled cells were inhibitory. We first assessed *Reln*$^{rl\text{-}Orl}$ mice interbred with a *GAD67*^{GFP} line that expresses GFP under the GAD67 promoter (Tamamaki *et al.*, 2003; Abadesco *et al.*, 2014). Reelin-expressing neurons in laminae I-II mostly did not express GAD67 (Fig. 4A–A2). We also co-labeled Reelin and Pax2, a transcription factor expressed by most inhibitory dorsal horn neurons (Lai *et al.*, 2016). Almost none of the Reelin-expressing laminae I-II dorsal horn neurons expressed Pax2 (Fig. 4B–B3), a finding that suggests that the remaining Reelin neurons are excitatory but do not co-express Lmx1b in adults.

Fewer Reelin-Lmx1b neurons are located in the lateral reticulated area and LSN of *dab1*$^{-/-}$ than *dab1*$^{+/+}$ mice

We next asked whether Reelin is expressed by neurons located in other nociceptive dorsal horn areas. The lateral reticulated area of lamina V (Fig. 5A) contains wide-dynamic range projection neurons that respond to noxious thermal and mechanical stimuli (Menétrey *et al.*, 1980, 1982), and for which there are few established markers. On average, we found more total Reelin neurons (i.e., with and without Lmx1b) in the area of lateral lamina V analyzed in *dab1*$^{+/+}$ compared to *dab1*$^{-/-}$ mice (Figs. 5A, G; *dab1*$^{+/+}$ 6.4±0.4 neurons; *dab1*$^{-/-}$ 4.6±0.5; p=0.009; n=4 mice/genotype). Our analyses showed that although the number of

Reelin-only neurons in lateral lamina V did not differ by genotype, there were more Lmx1b-only neurons in *dab1*$^{+/+}$ than in *dab1*$^{-/-}$ lateral reticulated area (Fig. 5G; *dab1*$^{+/+}$ 3.7±0.5; *dab1*$^{-/-}$ 1.8±0.3; p=0.002). Additionally, more Reelin-Lmx1b neurons were detected in *dab1*$^{+/+}$ than in *dab1*$^{-/-}$ lateral reticulated area (Fig. 5C–C2, D–D2; arrowheads; Fig. 5G; *dab1*$^{+/+}$ 2.0±0.2; *dab1*$^{-/-}$ 0.4±0.2; p=0.005). Overall, Lmx1b and Reelin-Lmx1b neurons are greatly reduced in *dab1*$^{-/-}$ lateral reticulated area and are likely to be incorrectly located in other dorsal horn areas.

The final nociceptive area analyzed was the lateral spinal nucleus (LSN), a small group of neurons within the dorsolateral funiculus that includes several large neurons that relay pain information rostrally to the brainstem and thalamus (Fig. 5B; Menétrey *et al.*, 1982; Burstein *et al.*, 1990). Here we found a difference between genotypes in the number of Reelin-only neurons (Fig. 5H; *dab1*$^{+/+}$ 1.4±0.5; *dab1*$^{-/-}$ 0.2±0.1; p=0.005). We also detected more Lmx1b-only neurons (Fig. 5H; *dab1*$^{+/+}$ 2.4±0.1 neurons; *dab1*$^{-/-}$ 1.1±0.3 neurons; p=0.003), and more Reelin-Lmx1b neurons (Fig. 5E–E2, F–F2; arrowheads; Fig. 5H; *dab1*$^{+/+}$ 1.7±0.2; *dab1*$^{-/-}$ 0.7±0.3; p=0.02) in *dab1*$^{+/+}$ compared to *dab1*$^{-/-}$ LSN. These results show consistent losses of Reelin-, Lmx1b-, and Reelin-Lmx1b neurons in the LSN in the absence of functional Dab1.

Reelin-, Lmx1b-, and Reelin-Lmx1b neurons participate in nociceptive circuits

Due to the presence of Reelin- and Reelin-Lmx1b neurons in dorsal horn areas involved in pain transmission, we asked if any of the Reelin-expressing neurons were activated by noxious thermal or mechanical stimulation. For these experiments, we used wild-type mice from both *Reln* and *dab1* lines to compare levels of Fos expression before and after noxious stimulation. Unstimulated wild-type controls showed only minimal Fos activation in the superficial dorsal horn (Fig. 6E–E3). After noxious heat stimulation, we found a few Reelin-Lmx1b neurons in *dab1*$^{+/+}$ mice that co-expressed Fos, the majority of which were in lamina I (Fig. 6A, B–B4, C–C4, white arrowheads), and occasionally within lamina II (Fig. 6B1–4, lower white arrowhead). Additionally, several Lmx1b neurons in laminae I-II were Fos-immunoreactive (Fig. 6B1–4, C1–4; red arrows; Yvone *et al.*, 2017). Additionally, after heat stimulation we detected a few large Reelin-Fos neurons (Fig. 6D–D2, D4, white arrows) and Lmx1b-Fos neurons (Fig. 6D, D2–4, red arrows) in the *dab1*$^{+/+}$ lateral reticulated area of lamina V, a region that contains many projection neurons. After heat stimulation, there were increased numbers of total Fos neurons in the superficial dorsal horn (unstimulated *dab1*$^{+/+}$ 4.3±0.7; stimulated *dab1*$^{+/+}$ 18±2; p=0.003) and lateral lamina V (unstimulated *dab1*$^{+/+}$ 1.4±0.5; stimulated *dab1*$^{+/+}$ 3.4±0.1; p=0.004) compared to the unstimulated controls.

After noxious mechanical stimulation we detected occasional triple-labeled Reelin-Lmx1b-Fos-expressing neurons primarily in *Reln*$^{+/+}$ lamina I (Fig. 7A, A1–4, white arrowheads). In addition, several Lmx1b-Fos neurons (Fig. 7A, A1–3, red arrows) and a few Reelin-Fos neurons (Fig. 7B, B1–4, white arrows) were observed in lamina II. In the *Reln*$^{+/+}$ lateral reticulated area of lamina V (Fig. 7C–C2), we found a cluster of large Reelin-Fos neurons in serial optical sections (Fig. 7C–C1, white arrows). Another example from *dab1*$^{+/+}$ lateral lamina V showed serial optical sections of a large Reelin-Lmx1b-Fos neuron (Fig. 7D–D3, E–E3, white arrowhead) alongside a large Lmx1b-Fos neuron (Fig. 7D–D., E–E3,

red arrow). Compared to unstimulated controls, we detected greater numbers of total Fos neurons after mechanical stimulation in the superficial dorsal horn (unstimulated *Reln*$^{+/+}$ 4.9±1.3; stimulated *Reln*$^{+/+}$ 13.4±2.5; p=0.025) and in lateral lamina V (unstimulated *Reln*$^{+/+}$ 0.8±0.3; stimulated *Reln*$^{+/+}$ 2.9±0.3; p=0.002). These results suggest that Reelin and Lmx1b are useful markers for lateral lamina V neurons and that Reelin-expressing neurons participate in both thermal and mechanical pain circuits.

Discussion

In this study, we identified the Reelin-expressing neurons in nociceptive areas of the adult dorsal horn of *dab1*$^{+/+}$ mice and established that 88% of Reelin neurons in laminae I-II, 35% of those in the lateral reticulated area of lamina V, and 59% in the LSN are double-labeled with Lmx1b. Together with previous studies (Cheng *et al.*, 2004; Dai *et al.*, 2008; Häring *et al.*, 2018), these findings show that the Reelin-Lmx1b neurons are glutamatergic. Importantly, the Reelin-Lmx1b and Dab1-Lmx1b neurons make up separate subsets of Lmx1b neurons and when combined, comprise almost 40% of the adult population of Lmx1b-labeled neurons in laminae I-II inner dorsal. The positioning errors in the *dab1*$^{-/-}$ dorsal horn are illustrated in figure 8B and compared to the wild-type mouse in figure 8A: 1) The area of laminae I-II outer was reduced in *dab1*$^{-/-}$ mice, but the numbers of Reelin-Lmx1b neurons did not differ, 2) The area of lamina II inner dorsal, the IB4 layer, was similar between genotypes, but *dab1*$^{-/-}$ mice had more Reelin-Lmx1b neurons than wild-type mice, 3) Laminae I-II outer is compacted and the IB4 layer is shifted dorsally in *dab1*$^{-/-}$ mice, and 4) Fewer Reelin-Lmx1b neurons were found in the lateral reticulated area of lamina V and LSN in *dab1*$^{-/-}$ than wild-type mice. Additionally, we found that Reelin-, Lmx1b, and Reelin-Lmx1b neurons in *dab1*$^{+/+}$ and *Reln*$^{+/+}$ laminae I-II inner dorsal and the lateral reticulated area expressed Fos after noxious thermal and mechanical stimulation, and therefore participate in nociceptive circuits. Together with our previous study (Yvone *et al.*, 2017), we implicate Reelin, Dab1, and Lmx1b neurons in the dorsal horn pain circuits and highlight the importance of Reelin-signaling in dorsal horn organization and function.

Reln and *dab1* mutant dorsal horns show common neuroanatomical abnormalities

Our results show a distribution of mispositioned Reelin-Lmx1b neurons in the *dab1*$^{-/-}$ dorsal horn (Fig. 8B) that is similar to the Dab1-Lmx1b neurons in the same nociceptive areas of *Reln*$^{-/-}$ mice (Fig. 8C; Yvone *et al.*, 2017). Compared to other areas of the nervous system, it was surprising to find that both Reelin-Lmx1b and Dab1-Lmx1b neurons were interspersed in the same dorsal horn locations, expressed the same neurotransmitter, and were both mispositioned. In the adult cerebral cortex, for example, Reelin is expressed by GABAergic interneurons, whereas Dab1 is expressed by excitatory cortical projection neurons (Howell *et al.*, 1997; Alcántara *et al.*, 1998; Rice *et al.*, 1998; Abadesco *et al.*, 2014). Our results in the dorsal horn show that several subtypes of glutamatergic neurons sustain similar positioning errors in both *dab1* and *Reln* mutant mice.

The cells missing from the mutant lateral reticulated area and LSN are likely located in the superficial dorsal horn as increased numbers of Reelin-Lmx1b and Dab1-Lmx1b neurons are detected in the IB4 area (Yvone *et al.*, 2017; current study). We also reported increased

numbers of Neurokinin-1-receptor (NK1R) neurons, including NK1R-Dab1, NK1R-Lmx1b and NK1R-Dab1-Lmx1b-expressing neurons, in the superficial dorsal horn of *Reln*$^{-/-}$ versus *Reln*$^{+/+}$ mice (Wang *et al.*, 2019). It is important to note that generally the loss of Reelin signaling predominantly influences the positioning of Dab1 neurons, but other types of neurons also may be affected. Interestingly, Lmx1b, which is expressed in the early-born dorsal interneuron dI5 and late-born excitatory dIL$_B$ dorsal spinal cord neurons in embryos (Gross *et al.*, 2002; Müller *et al.*, 2002), was implicated in neuronal migration and survival of superficial dorsal horn neurons (Ding *et al.*, 2004). The similarities in the positioning errors between Reelin, Dab1, and Lmx1b dorsal horn neurons, therefore, are likely due to interactions between the Lmx1b and Reelin signaling pathways.

The role of the Reelin-signaling pathway in nociception

Dorsal horn neurons can be classified by morphology, neuropeptide or neurotransmitter expression, and transcriptional profiling (Gutierrez-Mecinas *et al.*, 2016; Todd, 2017; Häring *et al.*, 2018). Neurochemical markers such as Neurotensin, Neurokinin B, and Gastrin-releasing peptide can identify many of the excitatory superficial dorsal horn neurons as they are largely non-overlapping. However, since markers like Gastrin-releasing peptide and Somatostatin are expressed by multiple populations (Gutierrez-Mecinas *et al.*, 2016, Häring *et al.*, 2018), transcriptomic data can be used to further categorize these neurons. Häring *et al.* (2018) showed that Reelin-expressing neurons belong to the glutamatergic clusters Glut8 and Glut9. The Glut8 neurons also were identified by the expression of *Neuromedin U Receptor 2* (*Nmur2*), a G-protein-coupled receptor, whereas the Glut9 cluster also expressed *Neuropeptide FF* (*Npff*; Häring *et al.* 2018). Neurons that express *Nmur2* (Yu *et al.*, 2003; Zeng *et al.*, 2006) and *Npff* are both localized within the superficial dorsal horn (Kivipelto and Panula, 1991; Panula *et al.*, 1999; Gutierrez-Mecinas *et al.*, 2019) and implicated in nociceptive responses (Yu *et al.*, 2003; Zeng *et al.*, 2006; Torres *et al.,* 2007; Gutierrez-Mecinas *et al.,* 2019). Together, our results and those from Häring *et al.* (2018) confirm that Reelin-labeled superficial dorsal horn neurons are glutamatergic and further implicate Reelin in nociceptive functions.

Reln$^{-/-}$ and *dab1*$^{-/-}$ mice exhibited distinct nociceptive abnormalities: thermal hypersensitivity and a profound loss of mechanical sensitivity (Villeda *et al.*, 2006; Akopians *et al.*, 2008; Yvone *et al.*, 2017). Recently, we showed that the ablation of mispositioned NK1R-expressing neurons in laminae I-II with a substance P analog conjugated to saporin abolished the heat hypersensitivity in *dab1*$^{-/-}$ mice without altering their mechanical insensitivity. Additionally, we reported that many NK1R-expressing neurons co-express Lmx1b, and after noxious heat stimulation, several NK1R-positive neurons co-express Fos (Wang *et al.*, 2019). These results imply that incorrectly positioned NK1R-expressing neurons are the underlying cause of their heat hypersensitivity (Wang *et al.*, 2019).

We postulate that the mechanism of mechanical insensitivity displayed by *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mice includes an overall reduction of the efficacy in interneuron circuits. The substantial neuronal compaction of laminae I-II inner dorsal in mutants, i.e., more Reelin-Lmx1b (current study) and Dab1-Lmx1b neurons (Yvone *et al.*, 2017) within the IB4

layer, could contribute to the loss of mechanical sensitivity. In addition, large lateral lamina V neurons that express Dab1-Lmx1b or Reelin-Lmx1b also are mispositioned in *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mice, respectively. Because some of these neurons co-expressed Fos, i.e. were activated following mechanical stimulation, they could contribute to the profound mechanical insensitivity seen in these mutants.

The loss of Reelin-Dab1 signaling also results in defects in dendritic development and synaptic modulation, which may disrupt dorsal horn circuits. In better studied areas such as the hippocampus and cerebral cortex, the loss of the Reelin-Dab1 signaling led to dendritic stunting and reduced spine density in the Dab1-expressing neurons (Niu *et al.*, 2004; Niu *et al.*, 2008; Olson *et al.*, 2006; Matsuki *et al.*, 2008). The Reelin-signaling pathway is also known to be important for regulating the development of postsynaptic structures and modulating synaptic plasticity (Beffert *et al.*, 2005; Herz and Chen, 2006; Niu *et al.*, 2008; Ventruti *et al.*, 2011). There is substantial evidence that disrupting specific dorsal horn circuits elicits nociceptive abnormalities such as mechanical insensitivity (Wang *et al.*, 2013; Duan *et al.*, 2014, Peirs *et al.*, 2015, Szabo *et al.*, 2015). Our data show that the loss of Reelin signaling leads to ectopic Reelin and Dab1 excitatory neurons in laminae I-II inner dorsal and a reduction of Reelin- and Dab1-Lmx1b neurons in lateral lamina V. These alterations, in turn, may disrupt normal dorsal horn organization and the relay of nociceptive information to supraspinal targets. As Reelin and Dab1 continue to be expressed in the adult dorsal horn, our results contribute to the characterization of the neurons that express these proteins and implicate them in processing of noxious stimuli.

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Abbreviations

Apoer2	Apolipoprotein E receptor 2
Dab1	Disabled-1
GAD67	Glutamic Acid Decarboxylase 67
GFP	Green Fluorescent Protein
IB4	Isolectin B4
Lmx1b	LIM-homeobox transcription factor 1 beta
LSN	lateral spinal nucleus
NDS	normal donkey serum
NK1R	Neurokinin-1 Receptor

Nmur2	Neuromedin U Receptor 2
Npff	Neuropeptide FF
Pax2	Paired box 2
PKCγ	Protein Kinase C γ
PB	phosphate buffer
TSA	Tyramide Signal Amplification
Reln	Reelin
Vldlr	Very-low-density lipoprotein receptor

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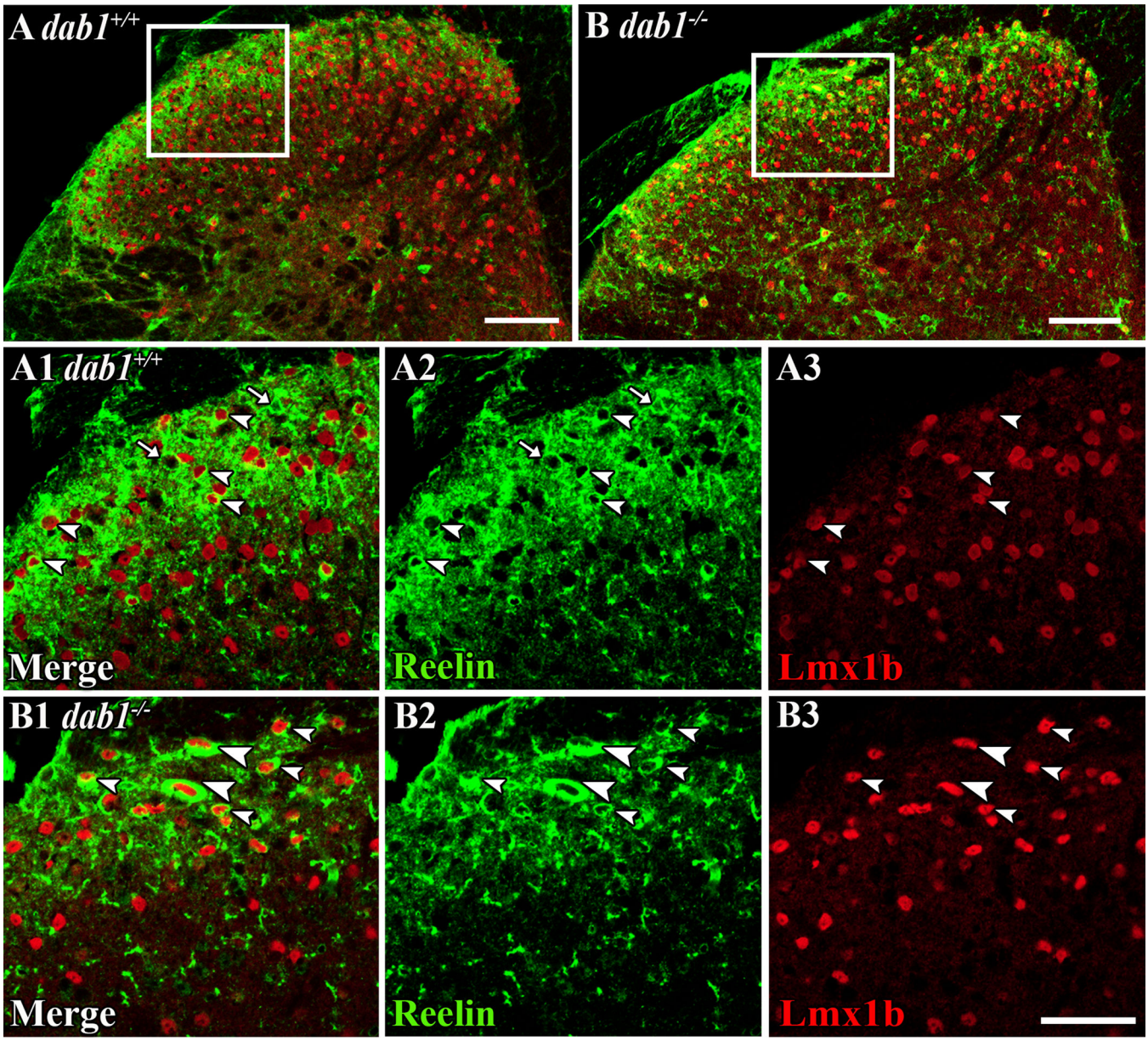

Figure 1. Most Reelin-expressing laminae I-II neurons co-express Lmx1b

Confocal images of *dab1*$^{+/+}$ (***A; A1–3***) and *dab1*$^{-/-}$ (***B; B1–3)*** lumbar dorsal horns show many Reelin neurons (green cytoplasm) that co-express Lmx1b (red nuclei). Boxes in ***A*** and ***B*** are enlarged in ***A1–3*** and ***B1–3***, with individual channels in ***A2–3*** and ***B2–3***. ***A-B,*** Most Reelin-Lmx1b cells in laminae I-II are small interneurons (***A1–3***; small arrowheads), but a few large neurons are seen in *dab1* mutants (***B1–3***; large arrowheads). Single-labeled Reelin neurons are marked with arrows. Images are oriented with medial to the right and dorsal toward the top in this and subsequent figures. Scale bars: ***A, B,*** 100 μm; ***A1–3; B1–3,*** 50 μm.

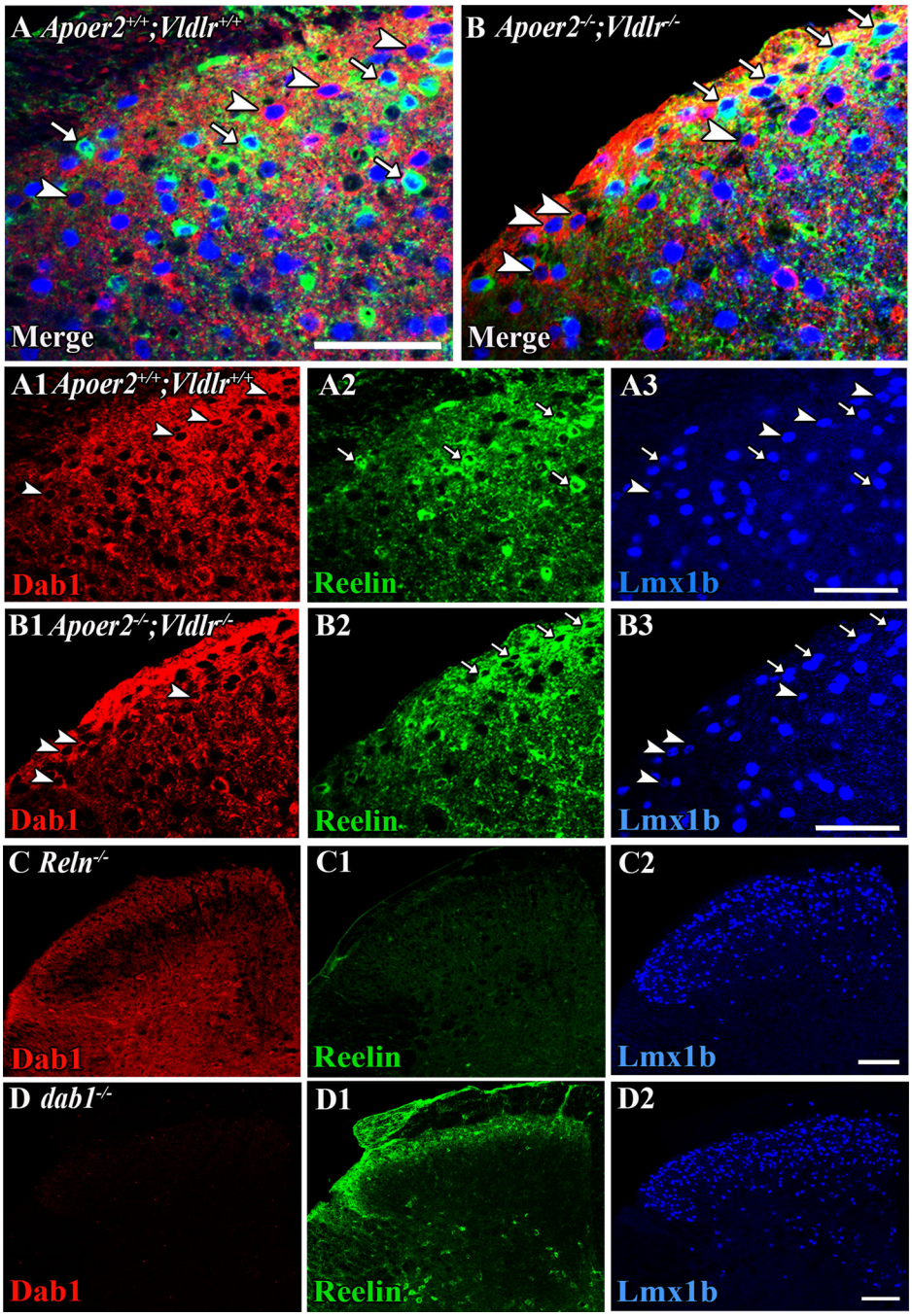

Figure 2. Reelin- and Dab1-Lmx1b neurons in laminae I-II are different subsets of Lmx1b neurons

Reelin-Dab1-Lmx1b expression in *Apoer2*$^{+/+}$*;Vldlr*$^{+/+}$ (***A-A3***) and *Apoer2*$^{-/-}$*;Vldlr*$^{-/-}$ (***B-B3)*** mice, and the negative controls including *Reln*$^{-/-}$ (***C-C2***) and *dab1*$^{-/-}$ ***(D-D2)*** mice. Enlargements of laminae I-II show the distribution of Reelin-Lmx1b (green cytoplasm with blue nuclei, arrows) and Dab1-Lmx1b (red cytoplasm with blue nuclei, arrowheads) within laminae I-II of *Apoer2*$^{+/+}$*;Vldlr*$^{+/+}$ (***A-A3***) and *Apoer2*$^{-/-}$*;Vldlr*$^{-/-}$ (***B-B3)*** mice. Enlarged areas are from the middle of the superficial dorsal horn. ***A-B,*** In both wild-type and double-

receptor knockout mice, the Reelin-Lmx1b and Dab1-Lmx1b neurons are interspersed within laminae I-II and represent separate subsets of glutamatergic neurons. The Reelin-Lmx1b neurons appear compacted in the *Apoer2*$^{-/-}$*;Vldlr*$^{-/-}$ laminae I-II (***B-B3,*** row of 4 arrows). ***C,*** In *Reln*$^{-/-}$ mice, Dab1 (red) and Lmx1b (blue) are expressed, whereas Reelin (green) is virtually absent. ***D***, In *dab1*$^{-/-}$ mice, only Reelin (green) and Lmx1b (blue) are expressed, and Dab1 immunoreactivity (red) is absent. Scale bars: ***A-A3, B-B3,*** 50 μm; ***C-C2, D-D2,*** 100 μm.

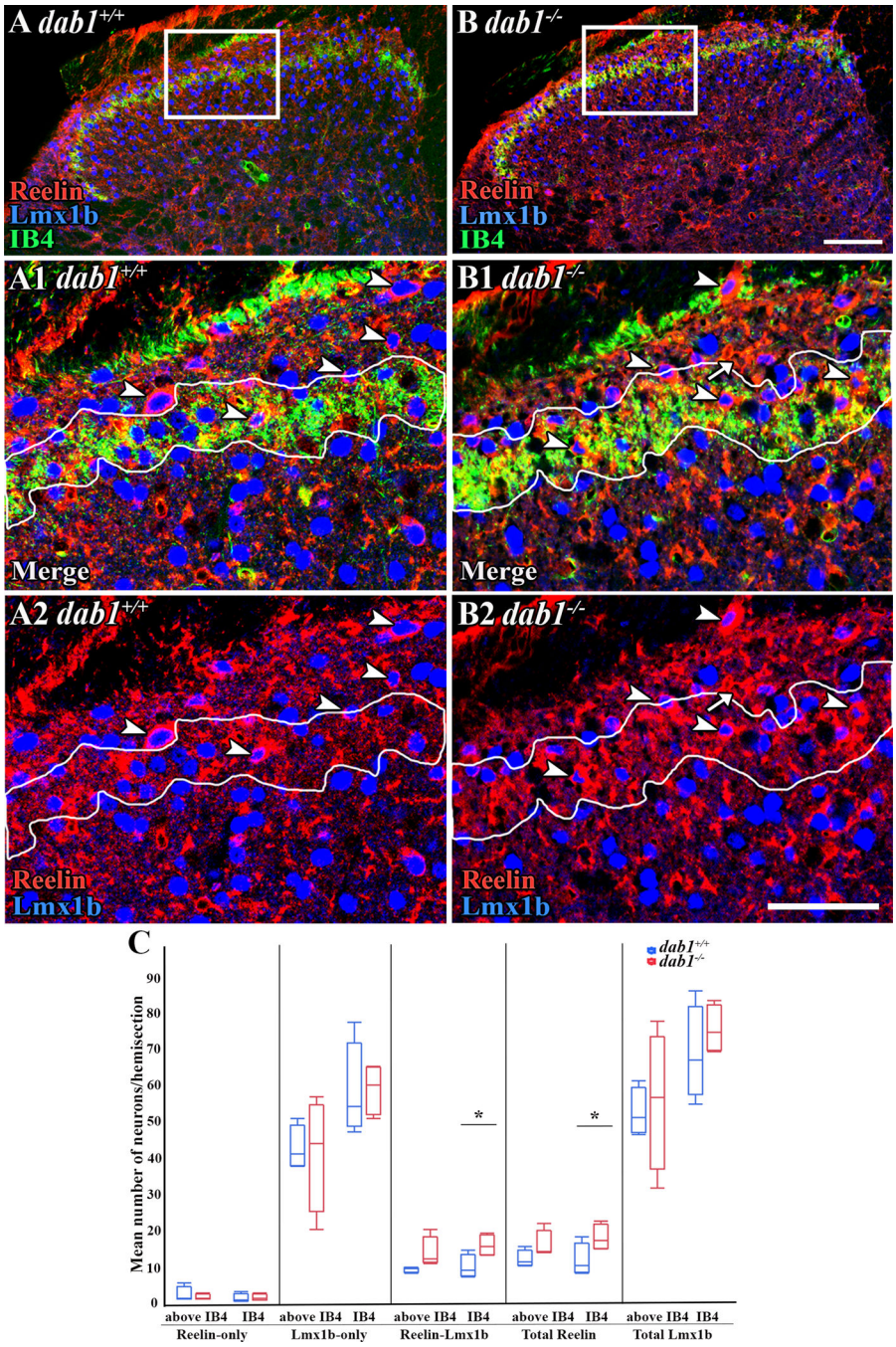

Figure 3. Reelin-Lmx1b neurons are mispositioned within the IB4 terminal zone

Reelin (red) and Lmx1b (blue) neurons are distributed within and above the IB4 (green) band, representing lamina II inner dorsal and laminae I-II outer, respectively. Boxes in ***A*** and ***B*** are enlarged in ***A1–2*** and ***B1–2***. Reelin-Lmx1b cells are marked with arrowheads; Single-labeled Reelin cells are marked with arrows. ***A-B,*** The IB4 terminal zone is outlined in ***A1–2*** and ***B1–2*** and does not differ in area between *dab1*$^{+/+}$ and *dab1*$^{-/-}$ mice. The area of laminae I-II outer is reduced in *dab1*$^{-/-}$ compared to *dab1*$^{+/+}$ mice. ***C,*** Within the IB4 terminal zone, the numbers of single-labeled Reelin and Lmx1b neurons do not differ

between genotypes, but the number of double-labeled Reelin-Lmx1b neurons is higher in *dab1*$^{-/-}$ than *dab1*$^{+/+}$ mice. Scale bars: ***A, B,*** 100 μm; ***A1–2, B1–2,*** 50 μm.

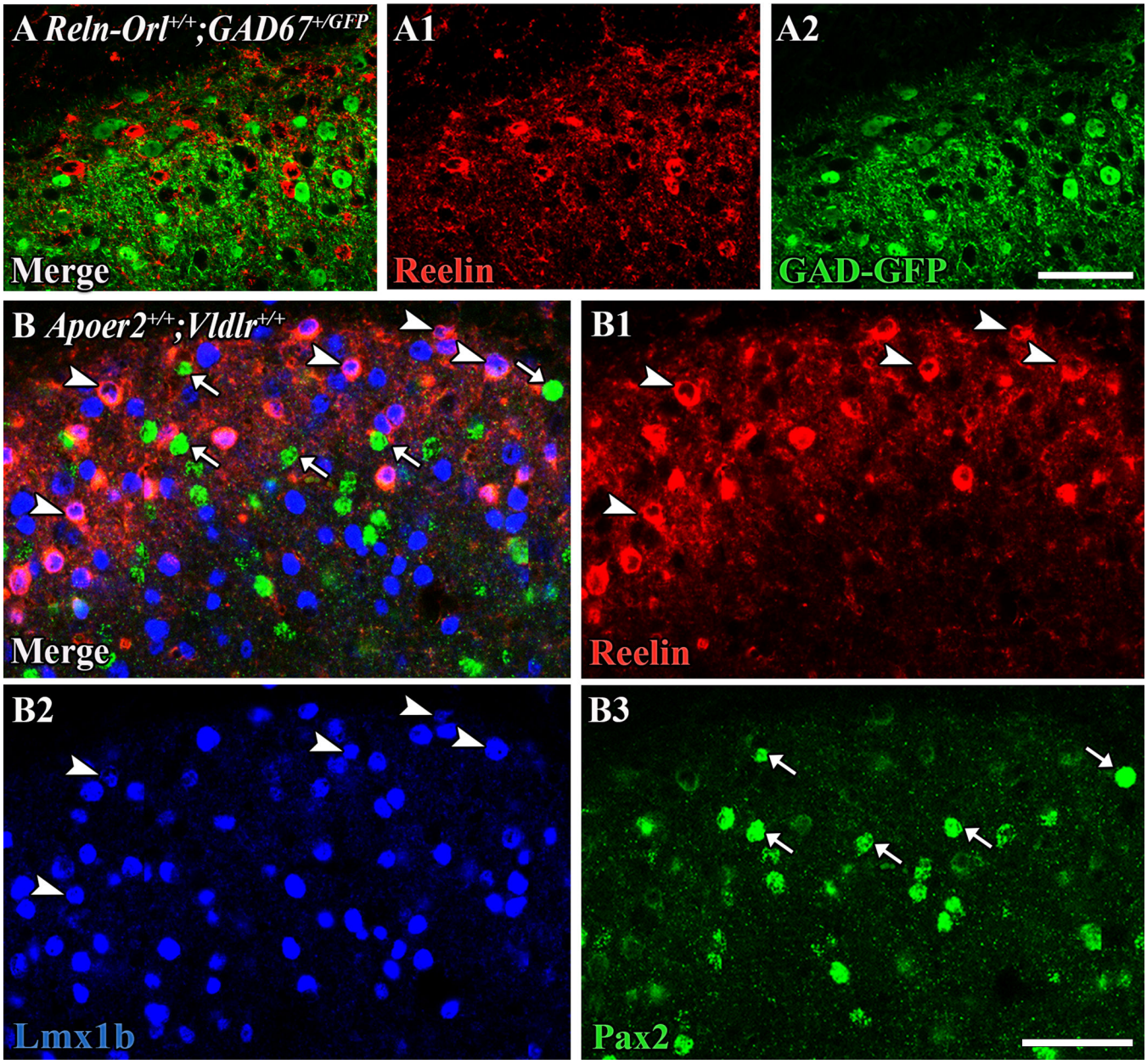

Figure 4. Most Reelin-labeled laminae I-II cells are not inhibitory

A-A2, Dorsal horns from *Reln-Orl*$^{+/+}$*; GAD67*$^{GFP/+}$ mice had little to no co-localization of Reelin (red) and GAD67^{GFP} (green). ***B-B3***, Experiments with Reelin (red), Lmx1b (blue), and Pax2 (green) show many co-labeled Reelin-Lmx1b neurons (arrowheads), but no evidence of Reelin cells expressing Pax2 (arrows). Scale bars: ***A, B***; ***A1–2, B1–3,*** 50 μm.

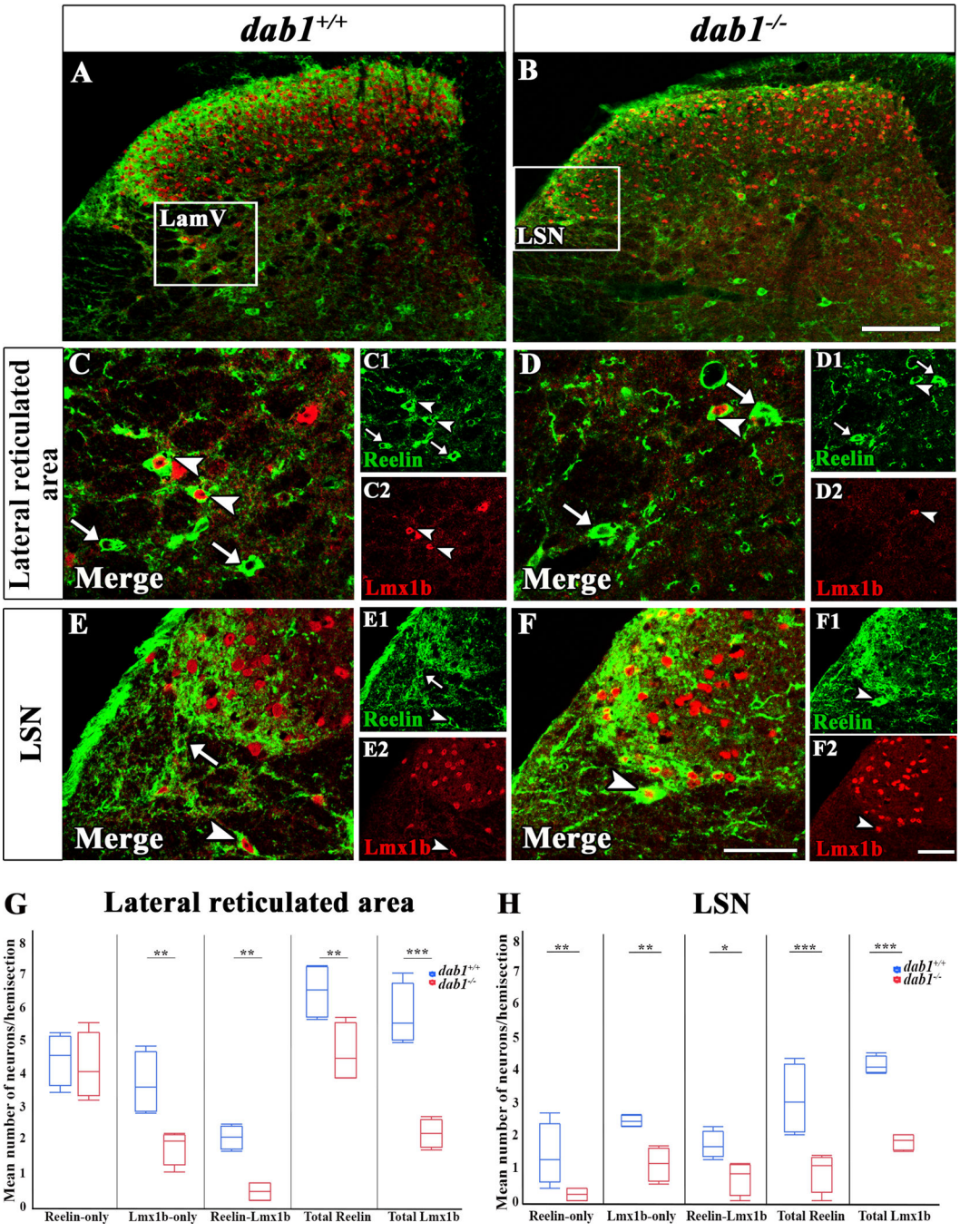

Figure 5. Lateral reticulated area of lamina V and LSN: Reelin-Lmx1b neurons are mispositioned in *dab1*$^{-/-}$ mice

Reelin (green) and Lmx1b (red) are co-expressed in neurons in the *dab1*$^{+/+}$ (***A, C, E***) and *dab1*$^{-/-}$ (***B, D, F***) lateral reticulated area (***C-D***) and LSN (***E-F***). Double-labeled cells are marked with arrowheads, and single-labeled Reelin neurons are marked with arrows. ***A-B,*** Box in ***A*** outlines the lateral reticulated area sampled and is enlarged in ***C***. Box in ***B*** marks the LSN and is enlarged in ***F***. ***C-D***, In the *dab1*$^{+/+}$ lateral reticulated area (***C***), there are more Reelin-Lmx1b neurons than in *dab1*$^{-/-}$ mice (***D***). Large Reelin-positive neurons are found in

the lateral reticulated area. ***E-F***, Single-labeled Reelin neurons are rarely detected and only a few Reelin-Lmx1b neurons are found in *dab1*$^{+/+}$ (***E***) and *dab1*$^{-/-}$ LSN (***F***). ***G,*** *dab1*$^{+/+}$ mice contain more Lmx1b, Reelin-Lmx1b, total Reelin, and total Lmx1b neurons in the lateral reticulated area than *dab1*$^{-/-}$ mice. ***H,*** The *dab1*$^{+/+}$ LSN has more single- and double-labeled neurons than the *dab1*$^{-/-}$ LSN. Scale bars: ***A, B,*** 100 μm; ***C-F***; ***C1–2, D1–2, E1–2, F1–2***, 50 μm.

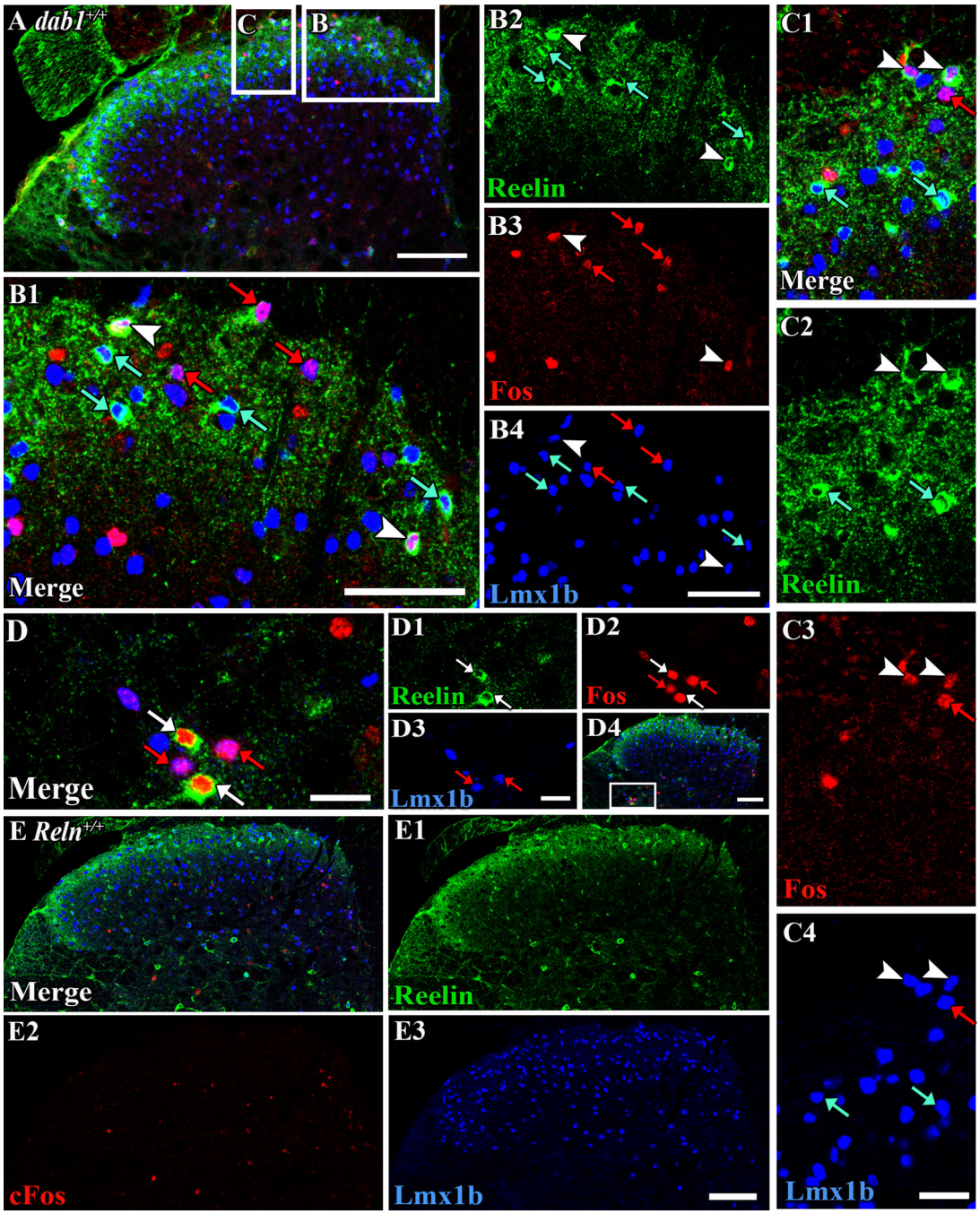

Figure 6. Noxious thermal stimulation induced Fos expression in Reelin, Lmx1b, and Reelin-Lmx1b neurons

Immunofluorescence labeling after thermal stimulation illustrates Reelin (green) and Lmx1b (blue) neurons that express Fos (red) in *dab1*$^{+/+}$ laminae I-II (***A***; ***B-B4; C-C4***), in *dab1*$^{+/+}$ lateral reticulated area of lamina V (***D-D4***), and in *Reln*$^{+/+}$ unstimulated controls (***E-E3***). ***A-C,*** Boxed areas in ***A*** are enlarged in ***B1–4*** and ***C1–4***. Several triple-labeled neurons (white arrowheads) are found together with Reelin-Lmx1b neurons (light blue arrows). Lmx1b-Fos neurons (red arrows) are also present. ***D,*** Boxed area in ***D4*** shows the *dab1*$^{+/+}$ lateral

reticulated area enlarged in ***D-D3***. Two Reelin-Fos neurons (white arrows) are detected together with Lmx1b-Fos (red arrows) neurons. ***E-E3,*** In the superficial dorsal horn of an unstimulated *Reln*$^{+/+}$ mouse, Reelin and Lmx1b are strongly expressed, but only a few neurons express Fos. Images captured at the same exposure as the sections from stimulated mice. Scale bars: ***A; D4; E-E3,*** 100 μm; ***B1; B2–4,*** 50 μm; ***C1–4; D; D1–3,*** 25 μm.

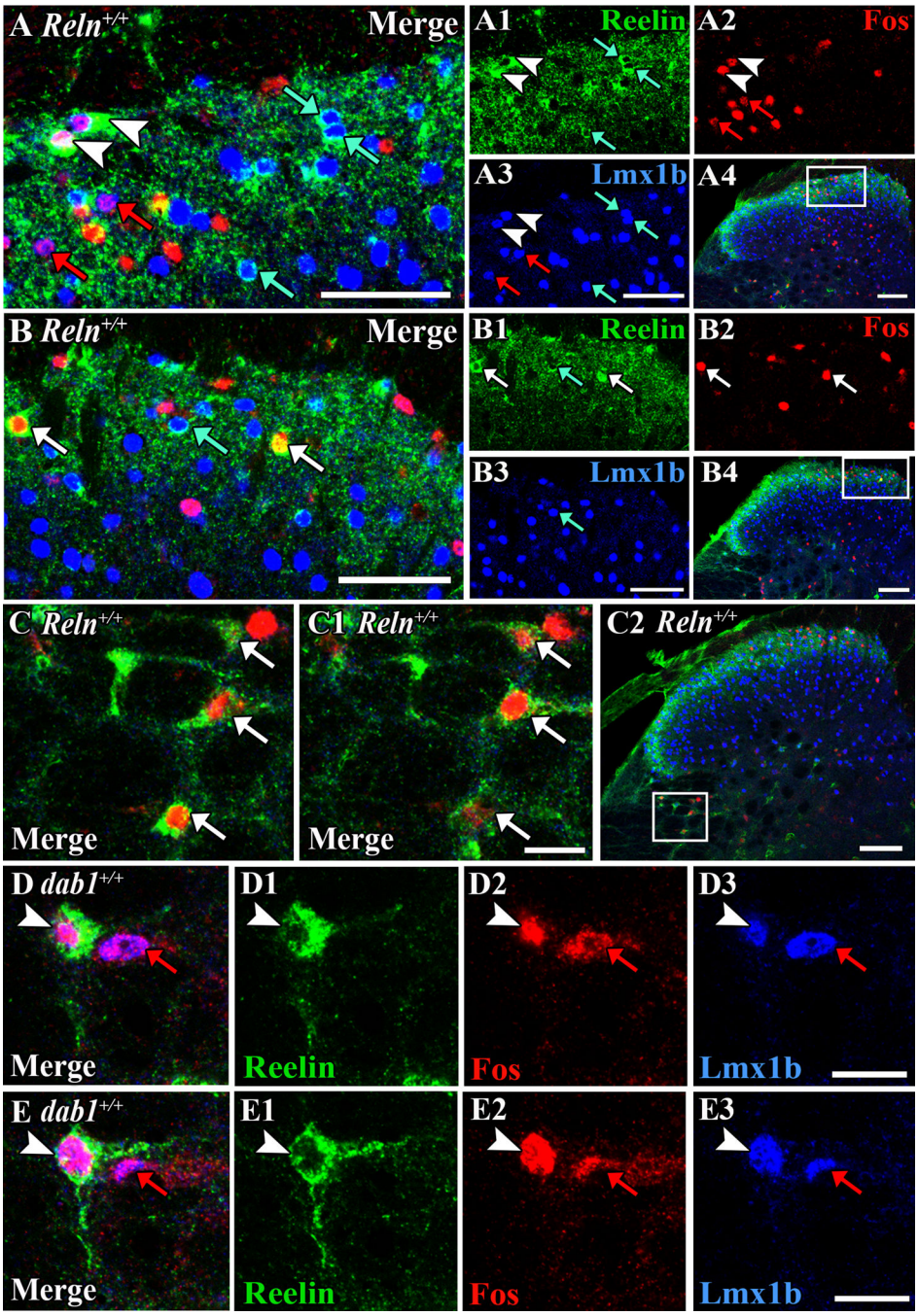

Figure 7. Noxious mechanical stimulation induced Fos expression in Reelin, Lmx1b, and Reelin-Lmx1b neurons

Fos expression (red nuclei) after mechanical stimulation, together with Reelin (green) and Lmx1b (blue)-labeled neurons in laminae I-II (***A-A4***; ***B-B4***) and in the lateral reticulated area of lamina V (***C-C2***; ***D-D3; E-E3***). ***A-A4,*** Boxed area in ***A4*** is enlarged in ***A-A3.*** Two triple-labeled neurons (***A-A3,*** white arrowheads) are found in lamina I along with Lmx1b-Fos neurons (***A, A2–3,*** red arrows) and Reelin-Lmx1b neurons (***A, A1, A3,*** light blue arrows) in *Reln*$^{+/+}$ laminae I-II. ***B-B4,*** Boxed area in ***B4*** is enlarged in ***B-B3*** and illustrates

Reelin-Fos (***B-B2,*** white arrows) and Reelin-Lmx1b (***B, B1, B3,*** light blue arrows) neurons in *Reln*$^{+/+}$ lamina II. ***C,*** Two serial optical sections of *Reln*$^{+/+}$ (***C-C2***) lateral reticulated area of lamina V show a cluster of several Reelin-Fos neurons (***C, C1***; white arrows). ***D-E, D*** and ***E*** are sequential optical sections of *dab1*$^{+/+}$ lateral reticulated area and depicts a large Reelin-Lmx1b-Fos neuron (***D-D3***; ***E-E3***, white arrowhead) and a large Lmx1b-Fos nucleus (***D-D3; E-E3,*** red arrow) after noxious mechanical stimulation. Scale bars: ***A4; B4; C2,*** 100 μm; ***A; A1–3; B; B1–3,*** 50 μm; ***C-C1; D-D3; E-E3,*** 25 μm.

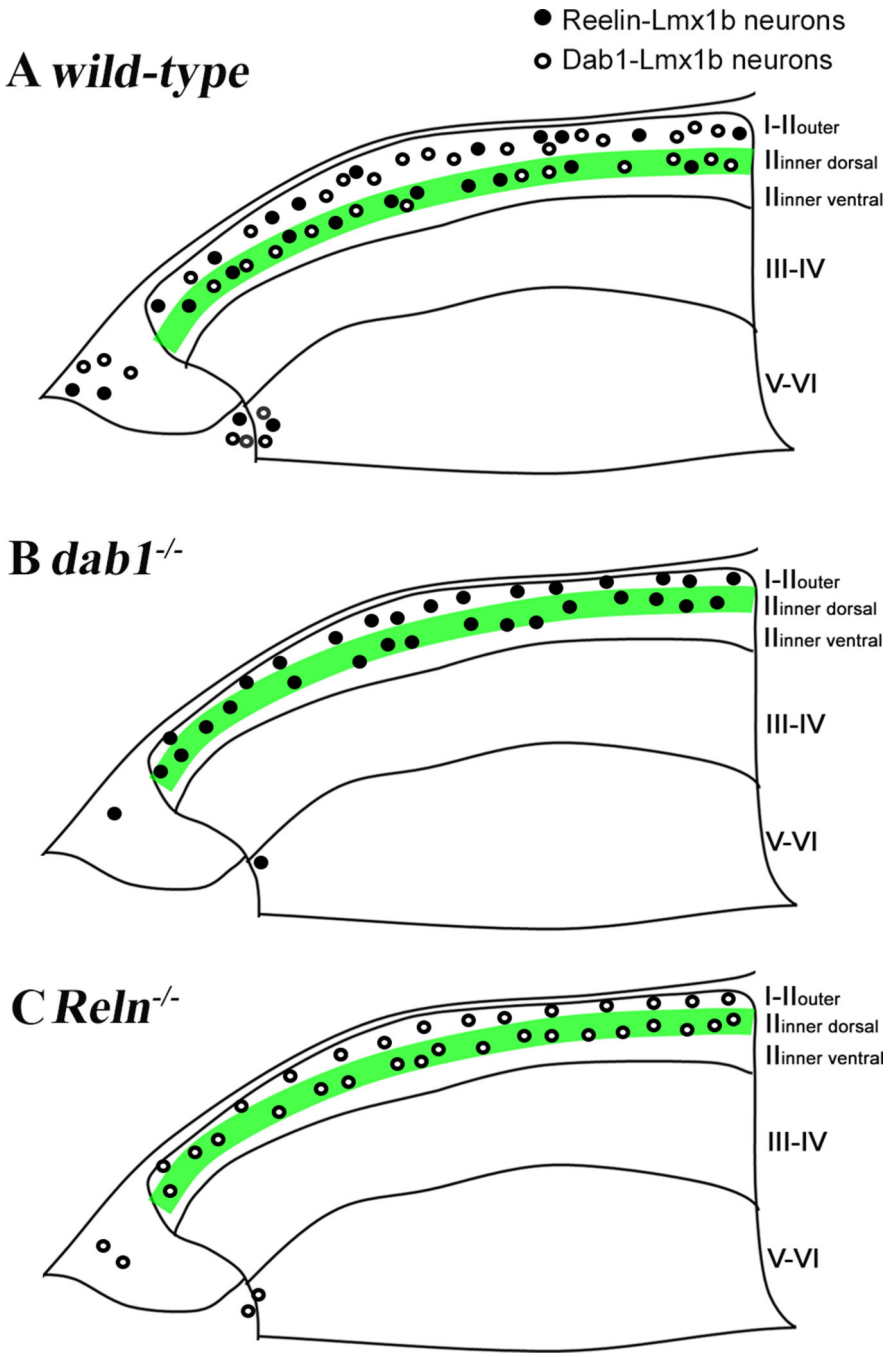

Figure 8. Dorsal horn anatomical abnormalities are similar in *dab1*$^{-/-}$ and *Reln*$^{-/-}$ mice

A, Schematic of the dorsal horn in wild-type mice. Both Reelin-Lmx1b and Dab1-Lmx1b-expressing neurons are seen in laminae I-II outer and lamina II inner dorsal. Double-labeled cells are also present in the lateral reticulated area of lamina V and LSN. ***B,*** The area of laminae I-II outer in *dab1*$^{-/-}$ mice was reduced compared to wild-type mice, but the number of Reelin-Lmx1b neurons did not differ. The *dab1*$^{-/-}$ IB4 area (lamina II inner dorsal) contained more Reelin-Lmx1b neurons than wild-type mice, but the IB4 areas did not differ. Fewer Reelin-Lmx1b neurons were found in the lateral reticulated area and LSN of *dab1*$^{-/-}$

versus wild-type mice. ***C,*** The area of laminae I-II outer in *Reln*$^{-/-}$ mice was also reduced compared to wild-type mice, yet the number of Dab1-Lmx1b neurons did not differ. More Dab1-Lmx1b neurons in lamina II inner dorsal and fewer Dab1-Lmx1b neurons in lateral lamina V and LSN were found in *Reln*$^{-/-}$ versus wild-type mice (Table 3 and Yvone *et al.*, 2017).

Table 1:

List of primary antibodies used in this study

Primary antisera	Source; Catalog #	Host species	Working dilutions
cFos (Ab-5)	Calbiochem (EMD Biosciences Inc., La Jolla, CA); PC38	Rabbit	1:20,000 (TSA)
Disabled-1 (Dab1)	Gift from Dr. Brian Howell (Howell *et al.*, 1997); B3	Rabbit	1:5,000 (TSA)
Green Fluorescent Protein (GFP)	Aves Labs (Tigard, OR); GFP-1020	Chicken	1:1,000 (IF)
Isolectin B4 (IB4; Biotinylated Griffonia (Bandeiraea) Simplicifolia Lectin I)	Vector (Burlingame, CA); B-1205	IB4 conjugate	1:200 (IF)
LIM homeobox transcription factor 1 beta (Lmx1b)	Gift from Drs. Müller and Birchmeier (Müller *et al.*, 2002)	Guinea pig	1:20,000 (TSA)
Neuronal Nuclei (NeuN)	EMD Millipore (Billerica, MA); MAB377	Mouse	1:900 (IF)
Paired box 2 (Pax2)	Zymed Laboratories (Thermo Fisher Scientific, Rockford, IL); 71–6000	Rabbit	1:10,000 (TSA)
Reelin (G10)	EMD Millipore (Billerica, MA); MAB5364	Mouse	1:1,000 (TSA)
Reelin	R&D Systems (Minneapolis, MN); AF3820	Goat	1:1,000 (TSA)

Table 2:

Reelin-Lmx1b neurons in the laminae I-II inner dorsal are increased in *dab1* mutants versus wild-type mice

Genotype/Cell type	*dab1* $^{+/+}$	*dab1* $^{-/-}$	p-value
Reelin-only	5±1.7	4±0.8	n.s.
Lmx1b-only	101±9	101±9.8	n.s.
Reelin-Lmx1b	20±1.6	31±1.6	0.004
Total Reelin	25±3.3	35±1.9	0.04
Total Lmx1b	121±8.8	131±10.1	n.s.

Means±SEM shown per hemisection, combining the numbers within and above the IB4 layer from *dab1*$^{+/+}$ and *dab1*$^{-/-}$ mice, 4 mice/genotype, 3 hemisections/mouse. **Analyses were carried out on a single optical section for each hemisection.**

Table 3:

Single-labeled Dab1 neurons are increased in *Reln*$^{-/-}$ compared to *Reln*$^{+/+}$ laminae I-II inner dorsal

Genotype/Cell type	*Reln* $^{+/+}$	*Reln* $^{-/-}$	p-value
Dab1-only	10±0.7	12±0.6	0.03
Lmx1b-only	112±10.3	106±6.4	n.s.
Dab1-Lmx1b	28±2.8	30±2.5	n.s.
Total Dab1	37±3	43±2.5	n.s.
Total Lmx1b	140±8.6	137±8.9	n.s.

Means±SEM expressed per hemisection, combining the numbers within and above the IB4 layer from *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice, 4 mice/genotype, 6 hemisections/mouse. **Analyses were carried out on a single optical section for each hemisection.**