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Calretinin and Calbindin neurons are correctly positioned in the superficial dorsal horn of *Reeler* mutants whereas many Gastrin-Releasing Peptide neurons co-express Reelin and may be influenced by Reelin signaling

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

By

Amanda Rose Nguyen

2020

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ABSTRACT OF THE THESIS

Calretinin and Calbindin neurons are correctly positioned in the superficial dorsal horn of *Reeler* mutants whereas many Gastrin-Releasing Peptide neurons co-express Reelin and may be influenced by Reelin signaling

by

Amanda Rose Nguyen

Master of Science in Physiological Science

University of California, Los Angeles, 2020

Professor Patricia Emory Phelps, Committee Chair

The Reelin-signaling pathway is vital for correct neuronal positioning in the central nervous system. Adult mice with the deletion of Reelin or Disabled-1 (Dab1) display an increased threshold for mechanical pain and decreased threshold for thermal pain. Previous studies found anatomical alterations in the pain-processing areas of the dorsal horn, and that a number of these neurons express Reelin or Dab1, implying that they are under the influence of the Reelin-signaling pathway. Wang et al. (2019) reported that Neurokinin-1 receptor-expressing neurons are mispositioned in the superficial dorsal horn and contribute to the hypersensitivity of *Reln*$^{-/-}$ mice to thermal heat. The overall aim of my project is to discover if the Calretinin neurons, implicated in processing mechanical pain, and the generally excitatory Calbindin neurons are

mispositioned in the superficial dorsal horn and likely contribute to mechanical hyposensitivity of *Reln*$^{-/-}$ mice. We found, however, that Calretinin and Calbindin neurons are not mispositioned in the superficial dorsal horn of *Reln*$^{-/-}$ mice. Along with pain, itch is a related major somatosensory sensation. Recently, we determined that *dab1* mutants show reduced scratching behavior compared to wild-type mice, and we hypothesize that mispositioned Gastrin-releasing peptide (GRP)-expressing neurons likely contribute to this phenotype. GRP is well known to be involved in transmitting itch signals, and based on a single cell sequencing study, Haring et al. (2018) reported that Reelin and GRP are highly expressed together in two glutamatergic populations in laminae I-II. Our preliminary data show that 41% of GRP neurons in the superficial dorsal horn express Reelin in wild-type mice. Future studies will determine if GRP neurons also express Dab1 and whether GRP neurons are mispositioned in both *Reln* and *dab1* mutant mice.

The thesis of Amanda Rose Nguyen is approved.

Stephanie Correa Van Veen

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Patricia Emory Phelps, Committee Chair

University of California, Los Angeles

2020

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INTRODUCTION

During embryonic development, the Reelin-signaling pathway plays an important role in correct neuronal positioning within the central nervous system. Reelin, an extracellular matrix protein, binds to the Very-low-density lipoprotein receptor (Vldlr) and Apolipoprotein E receptor 2 (Apoer2). Reelin binding results in tyrosine phosphorylation of a cytoplasmic protein, Disabled 1 (Dab1), by Src-family kinases that then activate downstream signaling events that influence neuronal positioning (Howell et al., 1997; D'Arcangelo et al., 1999; Hiesberger et al., 1999).

If either the *Reelin (Reln)* or *dab1* genes are deleted, both *Reln* and *dab1* mutants have decreased sensitivity to mechanical pain stimulation, together with an increased sensitivity to thermal heat (Akopians et al., 2008; Villeda et al., 2006). Neurons that express Reelin and Dab1 are concentrated in areas of the dorsal horn associated with pain processing: the superficial dorsal horn (laminae I-II), the lateral reticulated area of lamina V, and the lateral spinal nucleus (LSN; Menetrey et al., 1982; Burstein et al., 1987; Kubasak et al., 2004; Villeda et al., 2006; Akopians et al., 2008; Wang et al., 2012). This suggests that the Reelin-signaling pathway influences complex pain-processing circuits in the dorsal horn. In 2017, Yvone et al. found that in $Reln^{+/+}$ mice, 70% of Dab1 neurons in laminae I-II co-express LIM-homeobox 1 beta (Lmx1b), a transcription factor that marks excitatory glutamatergic neurons. In addition, in $Dab1^{+/+}$ mice, Yvone et al. (2020) determined that 90% of Reln neurons co-express Lmx1b. Similar to *Reln* and *dab1* mutants, mice which have Lmx1b conditionally deleted from the spinal cord exhibit mechanical insensitivity and their Reelin-expressing neurons do not migrate correctly into the superficial dorsal horn (Szabo et al., 2015).

We recently reported anatomical alterations of the superficial dorsal horn that contribute to heat hypersensitivity in *Reln* and *dab1* mutants. Wang et al. (2019) detected a significantly

greater number of neurons expressing Neurokinin-1 receptors (NK1Rs) in laminae I-II and fewer NK1R-expressing neurons in the LSN of both *Reln*$^{-/-}$ and *dab1*$^{-/-}$ compared to their respective wild-type controls. Furthermore, ablation of NK1 receptors with intrathecal injections of a substance P analog conjugated to saporin eliminated the hypersensitivity of *dab1* mutants to heat without altering their mechanical insensitivity. These experiments confirmed that misplaced NK1R-expressing neurons are involved in processing thermal pain in *dab1* mutant mice (Wang et al., 2019).

Additionally, we found that *dab1*$^{-/-}$ mice have significantly reduced itch behavior compared to wild type mice (Wang et al., unpublished data). After the pruritogens histamine and chloroquine were injected, total scratching bouts were quantified. The *dab1*$^{-/-}$ mice displayed significantly fewer scratching bouts compared to *dab1*$^{+/+}$ mice. Gastrin-releasing peptide (GRP) is a well-known neurotransmitter involved in transmitting itch information and is produced by a population of excitatory interneurons in the superficial dorsal horn. Pagani et al. (2019) estimated that approximately 83% of GRP-expressing neurons in the dorsal horn co-express Lmx1b. In addition, Haring et al. (2018) used single-cell RNA sequencing of neurons in the mouse dorsal horn and reported that Reelin and GRP are highly co-expressed in two glutamatergic neuron populations, Glut8 and Glut9.

The overall aim of this project is to identify additional mispositioned cells that contribute to reduced mechanical sensitivity or itch in our *Reln*$^{-/-}$ or *dab1*$^{-/-}$ mice. Specifically, related to mechanical pain, we examined two populations of superficial dorsal horn interneurons that express the calcium binding proteins, Calretinin and Calbindin. Calretinin neurons in the superficial dorsal horn are implicated in mechanical allodynia (Peirs et al., 2015), and 85% of these neurons are reportedly excitatory (Smith et al., 2016). We asked if these Calretinin-positive

neurons belong to a subset of Lmx1b-expressing neurons and if they are mispositioned in *Reln* mutants. Calbindin neurons in the superficial dorsal horn are also predominantly glutamatergic (Todd, 2010). Because *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mice both have reduced sensitivity to mechanical stimuli, we hypothesized that these two excitatory calcium-binding neuronal populations may be misplaced within the superficial dorsal horn or the LSN and therefore contribute to their sensory defects. In addition, we began an investigation of GRP neurons that could be related to reduced itch behavior in *dab1* mutant mice. We obtained preliminary data to determine the percentage of GRP-eGFP neurons that co-express Reelin. Future studies will investigate whether GRP cells are mispositioned within the superficial dorsal horn or LSN of *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mice compared to wild-type controls.

MATERIALS AND METHODS

Animals

Reln mice

Adult *Reln* (B6C3Fe-ala-*Reln*^{rl}) mice were originally obtained from Jackson Laboratory, and a breeding colony was established at UCLA. Offspring were genotyped according to D'Arcangelo et al. (1996).

GRP-eGFP mice

GRP-eGFP (STOCK Tg(GRP-EGFP)DV197Gsat/Mmucd) mice and genotyping protocol were obtained from Mutant Mouse Regional Resource Center at UC Davis (#010444-UCD), and a breeding colony was established at UCLA. The initial analyses to determine if GRP-eGFP neurons co-express Reelin was conducted on two male GRP-eGFP transgenic mice.

Tissue preparation and immunohistochemical procedures

Adult *Reln* and *GRP-eGFP* mice were anesthetized and perfused transcardially with 4% paraformaldehyde and post-fixed for 3 hours. *Reln* spinal cords were dissected and cryoprotected in 30% sucrose made in phosphate buffer for 2 days and *GRP-eGFP* spinal cords for 5 days rather than 2 due to COVID-19. Lumbar segments were blocked and frozen in Optimum Cutting Temperature (Sakura) and stored at -80°C until cryostat sectioning.

Immunofluorescence experiments were conducted on 25 μm-thick, free-floating lumbar 4-5 spinal cord sections. Primary antibodies included rabbit anti-Calretinin (1:80,000; Swant; 7697), rabbit anti-Calbindin (1:100,000; Swant; CB38), guinea pig anti-Lmx1b (1:20,000; kind gift of Drs. Muller and Birchmeier; Muller et al., 2002), goat anti-Reelin (1:1000; R&D Systems; AF3820), and chick anti-GFP (1:1000; Aves Labs; GFP-1020). We used Tyramide Signal Amplification Plus kits (PerkinElmer) with the following secondary antibodies: donkey anti-rabbit IgG to label Calretinin (TSA kit PerkinElmer #NEL745001KT) and Calbindin (PerkinElmer #NEL745001KT); and donkey anti-guinea pig to label Lmx1b (PerkinElmer #NEL744B001KT). Isolectin B4 (IB4) was visualized using a biotinylated IB4 conjugate (1:200; Vector, B-1205). Donkey anti-chick Alexa Fluor 488 (#703-545-155) was used to detect GFP.

A Zeiss Laser Scanning Microscope (LSM800) with 488 nm, 561 nm, and 640 nm lasers was used to obtain confocal images of the superficial dorsal horn. Low and high magnification confocal images with the pinhole aperture set to 1 Airy unit were obtained, using a 10X objective (numerical aperture 0.45) and 40X oil immersion lens (numerical aperture 1.4) respectively. The 40X images were used for statistical analyses. ZEN (Zeiss Efficient Navigation) lite (v. 2.6) imaging software was used for analysis.

Statistical analyses

To identify positioning errors we first quantified the number of Calretinin neurons that were Calretinin-positive only, Lmx1b-positive only, and Calretinin-Lmx1b neurons within IB4 (lamina II inner dorsal), above IB4 (laminae I-II outer) and in the LSN per hemisection. We then averaged the number of cells by cell type and area in four to six hemisections of each *Reln* mouse (n=10; 5 *Reln*$^{+/+}$, 5 *Reln*$^{-/-}$ mice). Neurons were counted from only one optical slice per hemisection, but we examined the cells throughout the Z-stack to confirm their cellular identity. The averages of Calretinin-, Lmx1b-, and Calretinin-Lmx1b-labeled neurons were compared by genotype and area for each cell type with a 2x2 repeated measures ANOVA and post hoc *t*-tests.

We then conducted similar analyses of the Calbindin neurons. Calbindin-positive only, Lmx1b-positive only, and Calbindin-Lmx1b neurons were counted within IB4 (lamina II inner dorsal), above IB4 (laminae I-II outer) and in the LSN in each hemisection. We analyzed four to six hemisections from each *Reln* mouse and derived the means of each cell type/area (n=6; 3 *Reln*$^{+/+}$, 3 *Reln*$^{-/-}$ mice). Neurons were counted from only one optical slice per section, but the neurons were examined throughout the Z-stack to confirm the cellular identity. The averages of Calbindin-, Lmx1b-, Calbindin-Lmx1b-labeled neurons were compared by genotype and area for each cell type with a 2x2 repeated measures ANOVA and post hoc *t*-tests.

To estimate the percentage of GRP neurons that co-express Reelin, we counted Reelin-, GRP-, and Reelin-GRP-labeled neurons in six hemisections of each *GRP-eGFP* mouse (n=2 GRP-eGFP transgenic wild-type mice). One confocal slice of a Z-stack of laminae I-II (measured 120 μm from the dorsal border of grey and white matter) was analyzed per hemisection, and then averaged per mouse. Mean percentages of Reelin-GRP neurons out of total Reelin or total GRP neurons were calculated.

RESULTS

Calretinin neurons are positioned correctly in the superficial dorsal horn of *Reln*$^{-/-}$ mice

Wang et al. (2019) identified mispositioned NK1R-expressing neurons in the superficial dorsal horn that contributed to heat hypersensitivity in our mutants, and here we ask if there are specific anatomical alterations in the dorsal horn that could explain their mechanical insensitivity. We examined Calretinin neurons first because they are implicated in mechanical allodynia and many Calretinin neurons express the Lmx1b transcription factor and thus are glutamatergic. Figure 1 illustrates the optical slices of *Reln*$^{+/+}$ and *Reln*$^{-/-}$ superficial dorsal horns used to quantify Calretinin and Lmx1b above and within the IB4 region. Obvious differences are not seen in the distribution of Calretinin and Lmx1b neurons between genotypes.

We next quantified the Calretinin and Lmx1b-expressing neurons in laminae I-IIouter and found that the numbers of Calretinin-only and Calretinin-Lmx1b cells did not differ between *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice (Table 1). There were, however, significantly more Lmx1b-only cells in *Reln*$^{-/-}$ compared to *Reln*$^{+/+}$ mice per hemisection (*Reln*$^{+/+}$ $\bar{x}$ = 56±4 Lmx1b cells/hemisection; *Reln*$^{-/-}$ $\bar{x}$ = 68±6 Lmx1b cells/hemisection) as well as more total Lmx1b-labeled neurons in *Reln*$^{-/-}$ than *Reln*$^{+/+}$ mice (*Reln*$^{+/+}$ $\bar{x}$ = 59±4 Lmx1b cells/hemisection; *Reln*$^{-/-}$ $\bar{x}$ = 72±6 Lmx1b cells/hemisection, Table 1).

Then we analyzed the Calretinin and Lmx1b cell populations in lamina II inner dorsal, or the IB4 region, of *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice. Previous studies showed that the area of the IB4 band did not differ between either *Reln*$^{+/+}$ and *Reln*$^{-/-}$ or *dab1*$^{+/+}$ and *dab1*$^{-/-}$ mice, yet more Dab1- and Reelin-labeled neurons, respectively, were found in the IB4 region of both mutants (Yvone et al., 2017, 2020). The numbers of Calretinin-only, Lmx1b-only, and Calretinin-Lmx1b labeled neurons in lamina II inner dorsal, however, did not differ between *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice (Table

2). Here we found only a trend of more Lmx1b-only cells in the IB4 region in *Reln*$^{-/-}$ compared to *Reln*$^{+/+}$ mice (Table 2).

Although we conclude that Calretinin neurons are not mispositioned in *Reln*$^{-/-}$ mice, the pattern of nonpeptidergic primary afferent terminals marked by IB4 was consistently different between genotypes. The IB4 band appeared to be located more dorsally in *Reln*$^{-/-}$ compared to *Reln*$^{+/+}$ mice and at high magnification the afferents were more condensed in the *Reln*$^{-/-}$ (Fig. 1 B1, B3) compared to more punctate in the *Reln*$^{+/+}$ mice (Fig. 1 A1, A3).

Calbindin neurons are positioned correctly in the superficial dorsal horn of *Reln*$^{-/-}$ mice

We suspected that mispositioned excitatory neurons contribute to the mechanical hyposensitivity of our mutants and therefore we analyzed the Calbindin neurons that are predominantly glutamatergic in laminae I-II (Todd, 2010). Figure 2 shows examples of optical slices of *Reln*$^{+/+}$ and *Reln*$^{-/-}$ superficial dorsal horns used to quantify Calbindin and Lmx1b above and within the IB4 region. We quantified the number of Calbindin-only, Lmx1b-only, and Calbindin-Lmx1b neurons in laminae I-IIouter and found no significant differences between the number of Calbindin-only, Lmx1b-only, and Calbindin-Lmx1b neurons between *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice (Table 3).

Then we analyzed Calbindin, Lmx1b, and double-labeled cell populations in lamina II inner dorsal, the IB4 band of *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice (Fig. 2). Again, there were no significant differences in the numbers of Calbindin-only, Lmx1b-only, and Calbindin-Lmx1b cells between *Reln*$^{+/+}$ and *Reln*$^{-/-}$ in lamina II inner dorsal (Table 4). Consistent with the number of Lmx1b-only cells in laminae II inner dorsal identified in Calretinin-Lmx1b experiments, the Calbindin analysis showed only a trend of more Lmx1b-labeled neurons in *Reln*$^{-/-}$ ($\bar{x}$ = 33 Lmx1b-only

neurons per hemisection) than *Reln*$^{+/+}$ ($\bar{x}$ = 27 Lmx1b-only neurons per hemisection). This may be explained by our small sample size. Due to our negative findings, we stopped this analysis at three mice per genotype. We did, however, find differences in the neuronal distribution in the afferent IB4 terminals similar to those reported above, with the IB4 afferents located more dorsally and appearing more condensed in *Reln*$^{-/-}$ (Fig. 2 B1, B3) compared to *Reln*$^{+/+}$ mice (Fig. 2 A1, A3).

Calretinin and Calbindin neurons are positioned correctly in the LSN of *Reln*$^{-/-}$ mice

Because the overall number of neurons in the lateral spinal nucleus (LSN) are reduced by 50% in *Reln*$^{-/-}$ and *dab1*$^{-/-}$ compared to wild-type mice (Yvone et al., 2017), we analyzed the number of Calretinin-only, Lmx1b-only, and Calretinin-Lmx1b neurons in the LSN. On average, there were the same number of Calretinin-only, Lmx1b-only, and Calretinin-Lmx1b neurons in the LSN between genotypes (*Reln*$^{+/+}$ $\bar{x}$ = 0.83 Calretinin-only neuron/hemisection, $\bar{x}$ = 1.32 Lmx1b-only neuron/hemisection, $\bar{x}$ = 0.42 Calretinin-Lmx1b neuron/hemisection; *Reln*$^{-/-}$ $\bar{x}$ = 0.48 Calretinin-only neuron/hemisection, $\bar{x}$ = 1.07 Lmx1b-only neuron/hemisection, $\bar{x}$ = 0.10 Calretinin-Lmx1b neuron/hemisection). In addition, we report that there were no differences in the number of Calbindin-only, Lmx1b-only, and Calbindin-Lmx1b neurons in the LSN between *Reln*$^{+/+}$ ($\bar{x}$ = 1 Calbindin-only neuron/hemisection, $\bar{x}$ = 1.33 Lmx1b-only neuron/hemisection, $\bar{x}$ = 0.50 Calbindin-Lmx1b neuron/hemisection) and *Reln*$^{-/-}$ mice ($\bar{x}$ = 0.06 Calbindin-only neuron/hemisection, $\bar{x}$ = 0.37 Lmx1b-only neuron/hemisection, $\bar{x}$ = 0.17 Calbindin-Lmx1b neuron/hemisection). In sum, we found that Calretinin and Calbindin neurons are not mispositioned in the LSN of *Reln*$^{-/-}$ mice.

Distribution of GRP neurons in the dorsal horn

We previously found that our *dab1* mutant mice have reduced scratching behavior and Gastrin-releasing peptide (GRP) is a well-known transmitter associated with processing itch in the dorsal horn. We first characterized the distribution of GRP neurons in the dorsal horn of the GRP-eGFP mouse. This has been an area of dispute in the field. It was first reported that dorsal root ganglion is the source of GRP (Sun and Chen, 2007; Sun et al., 2009), and then Fleming et al. (2012) and Mishra et al. (2012) reported that the dorsal horn is the source. To resolve the controversy, Albisetti et al. (2019) reported that GRP neurons were restricted to lamina II of the superficial dorsal horn, dorsal to the PKCγ neuron layer which defines lamina II inner ventral, and partially overlapping with the IB4 area. As shown in Figure 3A, we found that GRP-expressing neurons are distributed medially and laterally throughout laminae I-II but are mainly concentrated in the lateral half of lamina II. In addition, a few GRP neurons are detected in the deep dorsal horn but none were found in the LSN.

Reelin-positive neurons make up a subpopulation of GRP neurons

Haring et al. (2019) reported that GRP is highly co-expressed with Reelin in their glutamatergic groups 8 and 9. We used the GRP-eGFP mice to determine what percentage of GRP-expressing neurons co-express Reelin in lower lumbar spinal cord. Reelin-GRP neurons are distributed medially and laterally throughout laminae I-II (Fig. 3B). We found that 40.72% of GRP-positive neurons co-express Reelin ($\bar{x}$ = 6 Reelin-GRP neurons/hemisection; $\bar{x}$ = 14 GRP neurons/hemisection). Additionally, 24.11% of Reelin-positive neurons co-express GRP ($\bar{x}$ = 24 Reelin neurons/hemisection). In Figure 3B, the yellow arrows mark examples of Reelin-GRP

neurons. The insets show a large Reelin-labeled cell in lamina I, and a GRP-only and Reelin-GRP neuron in lamina II (Fig. 3 B1-B3).

DISCUSSION

Recently we found mispositioned Neurokinin-1 receptor expressing neurons in the superficial dorsal horn that contribute to heat hypersensitivity of *Reln*$^{-/-}$ mice (Wang et al., 2019). To identify mispositioned cells in the superficial dorsal horn that likely contribute to the reduced mechanical pain phenotype of *Reln*$^{-/-}$ mice, we colocalized cells that express Calretinin, Calbindin, and Lmx1b in the superficial dorsal horn. In the Calretinin-Lmx1b experiments, we found more Lmx1b-only cells in laminae I-IIouter in *Reln*$^{-/-}$ compared to *Reln*$^{+/+}$ mice, but the number of Calretinin-expressing neurons in laminae I-II outer or II inner dorsal did not vary between genotypes. Similarly, when we examined Calbindin neurons in *Reln*$^{-/-}$ mice, they also did not differ from *Reln*$^{+/+}$ mice. In our next set of experiments, we plan to determine whether mispositioned GRP neurons contribute to reduced itch in *dab1* mutants. Our first goal was to determine the percentage of GRP neurons that express Reelin in GRP-eGFP mice. We found that 41% of GRP neurons in wild-type mice express Reelin. Conversely, 24% of the Reelin-expressing neurons co-express GRP. As we know that 90% of the Reelin-expressing neurons in the superficial dorsal horn are glutamatergic and mispositioned, we suspect that some GRP cells are mispositioned.

Migration of Calretinin and Calbindin neurons are likely not influenced by the Reelin-signaling pathway

There are greater numbers of both Reelin-Lmx1b and Dab1-Lmx1b neurons in the IB4 terminal zones of *dab1*$^{-/-}$ and *Reln*$^{-/-}$ mice (Yvone et al., 2020). The Calretinin-Lmx1b and Calbindin-Lmx1b neurons, however, likely belong to a separate subset of Lmx1b-expressing neurons because they do not co-express either Reelin or Dab1 and were not mispositioned (Yvone et al, 2020). Yvone et al. (2020) also found that the number of Lmx1b-only cells in laminae I-II outer and lamina II inner dorsal does not significantly vary between genotypes. It is important to note that the population of Lmx1b-only cells examined in Yvone et al. (2020) differs from those in this study. The Lmx1b-only cells in Yvone et al. (2020) are those that do not co-express Reelin or Dab1, while the Lmx1b-only cells in this study are those that do not co-express Calretinin or Calbindin.

Dab1 neurons are mispositioned in *Reln*$^{-/-}$ mice, and Yvone et al. (2017) reported that Dab1 neurons do not express Calretinin, and only few Dab1-Lmx1b neurons express Calbindin. This suggests that, although the neurons that express these calcium-binding proteins could contribute to the mechanical hyposensitivity phenotype in *Reln*$^{-/-}$ mice, the Reelin-signaling pathway does not likely influence the migration and final positions of Calretinin- and Calbindin-positive neurons. This reasoning is consistent with our finding similar positioning of these neurons in *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice.

Alterations in the afferent input of Calretinin and Calbindin neurons may contribute to mechanical hyposensitivity of *Reln*$^{+/+}$ mice

Calretinin and Calbindin cells receive nonpeptidergic nociceptive input as they are surrounded by the IB4 marker in laminae II inner dorsal (Peirs et al., 2015). In this study the IB4 afferents appeared more concentrated in *Reln*$^{-/-}$ compared to *Reln*$^{+/+}$ mice. The extra Reelin and Dab1-positive cells located in the IB4 band may influence afferent density and possibly alter connectivity. Perhaps Calretinin and Calbindin neurons receive a different pattern of nonpeptidergic nociceptive input in *Reln*$^{-/-}$ compared to *Reln*$^{+/+}$ mice, and may alter their responses to mechanical pain stimulation in *Reln*$^{-/-}$ mice.

Calretinin and Calbindin are largely restricted to glutamatergic neurons in the dorsal horn (Todd, 2010), yet only a small percentage of Calretinin and Calbindin neurons co-expressed Lmx1b in adults (Szabo et al., 2015). This suggests that adult Calretinin and Calbindin-positive neurons we analyzed make up only a small subset of Lmx1b-expressing neurons. It is also a possibility that Calretinin and Calbindin neurons are among the many glutamatergic cells that turn off Lmx1b expression during development (Szabo et al., 2015).

Nearly 41% of GRP neurons express Reelin

As we previously found our *dab1* mutant mice have reduced scratching behavior in response to itch stimulation, and have more Reelin-expressing neurons in lamina II inner dorsal compared to wild-type, we wondered if some of the mispositioned Reelin-positive cells co-express GRP. Our preliminary data found that 41% of GRP-positive neurons co-express Reelin and thus it is likely that the Reelin-GRP-expressing cells may be affected in our *dab1* mutants.

Future studies will determine if some or all of the colocalized Reelin-GRP neurons are mispositioned in *dab1* mutants.

In summary, the mechanisms that contribute to the mechanical and itch insensitivity of *Reln* mutants are still not well understood. So far, we have shown that the cell bodies of two populations of dorsal horn neurons involved in mechanical pain, Calretinin and Calbindin, are correctly positioned, but they may not receive normal synaptic input. On the other hand, we now know that some GRP cells express Reelin and because Reelin-expressing neurons are mispositioned (Yvone et al., 2020) they likely contribute to the reduced scratching behavior seen in our *dab1* mutants.

FIGURES

Figure 1: Representative example of a confocal slice of lumbar superficial dorsal horn used to quantify Calretinin (CR) and Lmx1b neurons

Dorsal horns from *Reln*$^{+/+}$ (A, A1-3; n=5) and *Reln*$^{-/-}$ (B, B1-3; n=5) mice are shown oriented with lateral to the left and dorsal towards the top in these and subsequent sections. Calretinin (CR)-labeled cells are red and Lmx1b nuclei are blue. The IB4 afferent terminals (green band) mark laminae II inner dorsal in all images. (A, B) Merged images of CR, Lmx1b, and IB4 in the dorsal horn. Insets A1-A3 and B1-B3 show the enlargement of the boxed area in A and B. A1 and B1 represent Calretinin in the IB4 region, A2 and B2 illustrate Lmx1b in the IB4 region, and A3 and B3 show the three channels merged. A white arrow marks a single-labeled Calretinin cell, the black arrow a single-labeled Lmx1b cell, and a yellow arrow a double-labeled CR-Lmx1b positive neuron. Scale bars: A, B, 50 μm; A1-A3, B1-B3 = 20 μm

Figure 1

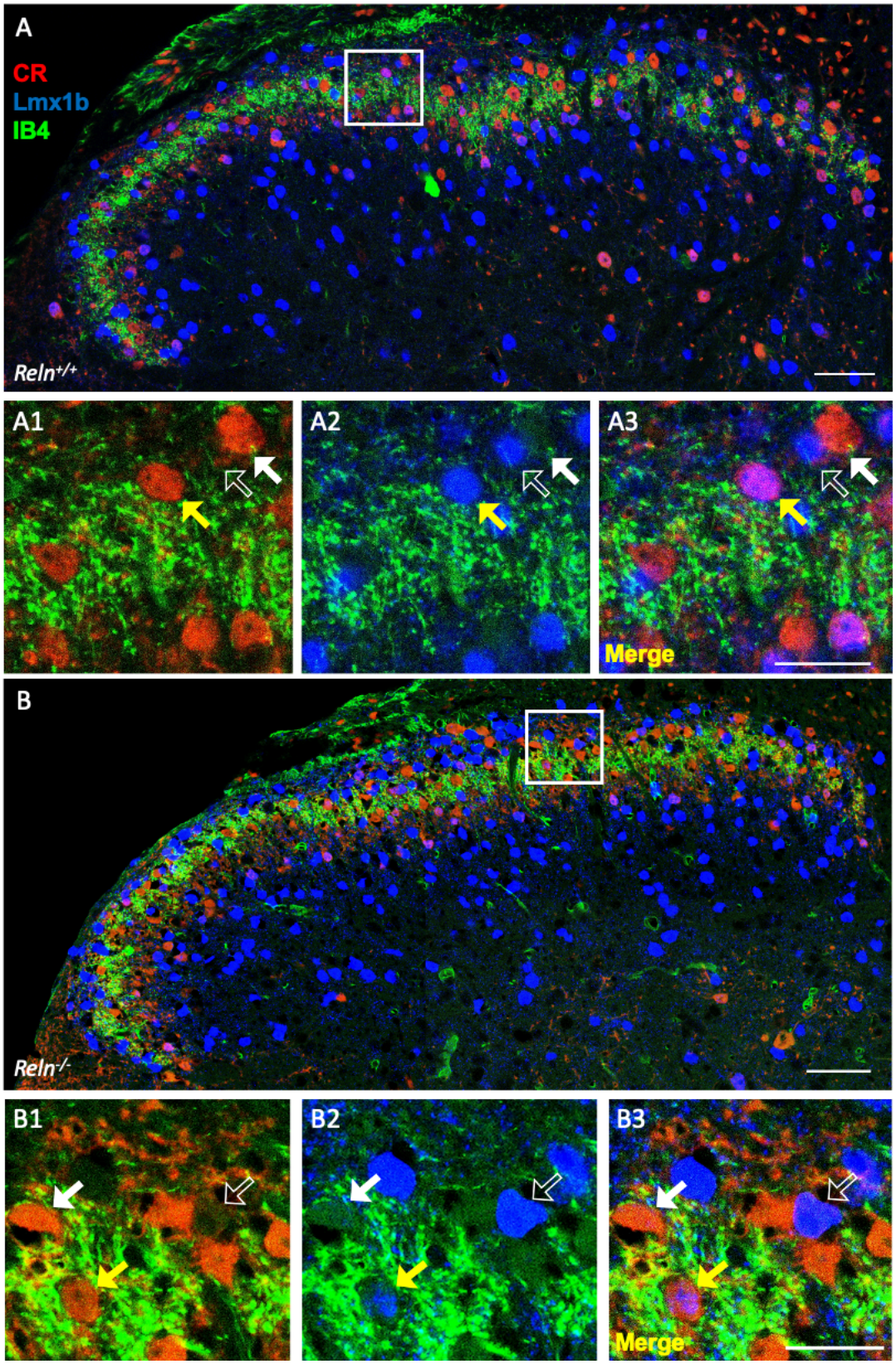


Figure 2: Calbindin and Lmx1b neurons do not vary in *Reln*$^{+/+}$ and *Reln*$^{-/-}$ lumbar superficial dorsal horn

Representative confocal slices show Calbindin (red), Lmx1b (blue), and IB4 (green) in *Reln*$^{+/+}$ (A; n=3) and *Reln*$^{-/-}$ (B; n=3) dorsal horns. The boxed areas in A and B are enlarged in A1-A3 and B1-B3. A1 and B1 show Calbindin in the IB4 band, A2 and B2 show Lmx1b and IB4, and A3 and B3 show the three channels merged. A white arrow marks a single-labeled Calbindin cell, the black arrow a single-labeled Lmx1b cell, and a yellow arrow a double-labeled Calbindin-Lmx1b positive neuron. There are no obvious differences in the distribution of Calbindin and Lmx1b neurons between genotypes. Scale bars: A, B, 50 μm; A1-A3, B1-B3 = 20μm

Figure 2

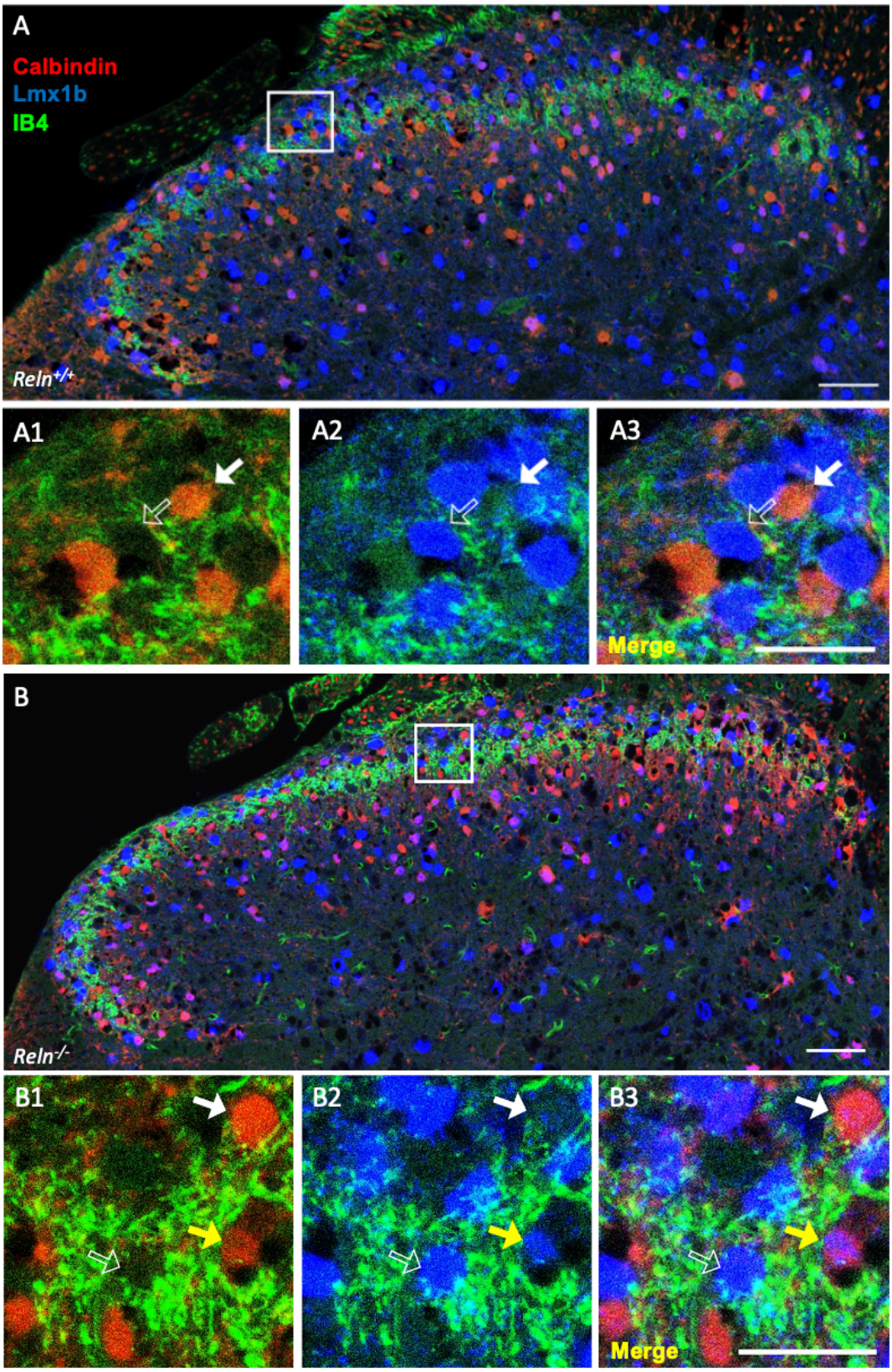
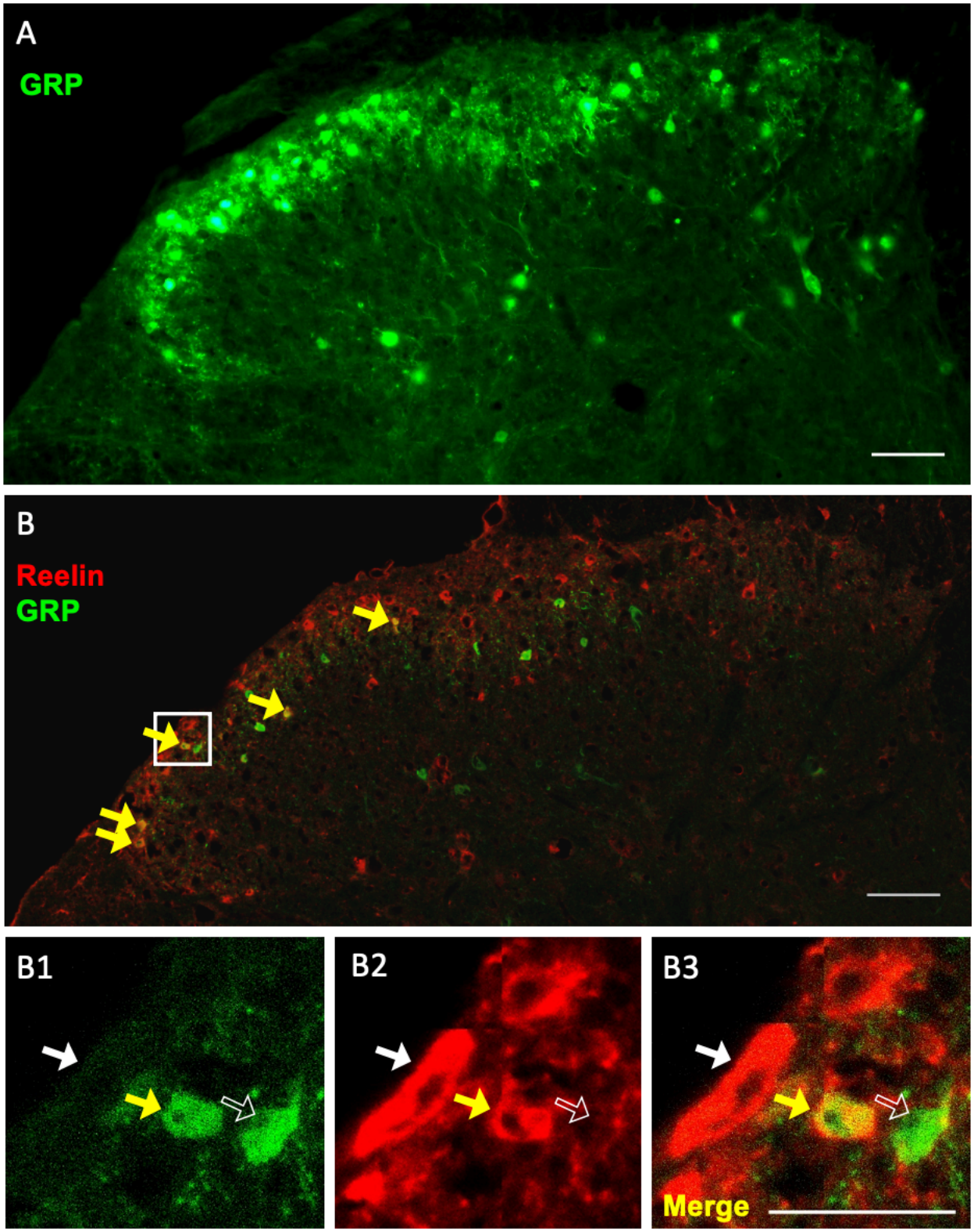


Figure 3: Approximately 40% of GRP-eGFP neurons co-express Reelin

(A) A 25 μm section from the dorsal horn of an adult GRP-eGFP (green) transgenic mouse. GRP-expressing neurons are distributed throughout laminae I-II and a few are located in the deep dorsal horn. (B) A single confocal slice of the dorsal horn from a GRP-eGFP mouse is colabeled with Reelin (red). Enlargement of the boxed area in B shown in B1-B3: B1 shows GRP only, B2 shows Reelin only, and B3 has the two channels merged. A white arrow labels a large single-labeled Reelin cell, the black arrow marks a single-labeled GRP cell, and the yellow arrows mark double-labeled Reelin-GRP cells. The majority of GRP neurons are located in the lateral portion of the dorsal horn, while Reelin-GRP neurons are dispersed across laminae I-II. Scale bars: A, B = 50 μm; B1-B3 = 20 μm

Figure 3



TABLES

Table 1: Calretinin-Lmx1b neurons in lamina I-II outer do not differ between *Reln*$^{+/+}$ vs. *Reln*$^{-/-}$ mice

Genotype/Cell type	*Reln*$^{+/+}$	*Reln*$^{-/-}$	p-value
Calretinin-only	14±0.9	16±1.9	0.56
Lmx1b-only	56±3.7	68±5.7	0.0034*
Calretinin-Lmx1b	3±1.0	4±1.0	0.70
Total Calretinin	16±0.8	20±1.7	0.45
Total Lmx1b	59±3.8	72±6.4	0.015*

Single-labeled Lmx1b neurons are increased in lamina I-II outer of *Reln*$^{-/-}$ compared to *Reln*$^{+/+}$ mice. Means±SEM shown per hemisection, 5 mice/genotype, 3-6 hemisections/mouse. Analyses were carried out on one confocal slice/hemisection. Statistical significance marked by asterisks.

Table 2: Calretinin-Lmx1b neurons in laminae II inner dorsal do not differ between *Reln*$^{+/+}$ vs. *Reln*$^{-/-}$ mice

Genotype/Cell type	*Reln*$^{+/+}$	*Reln*$^{-/-}$	p-value
Calretinin-only	35±4.1	36±3.6	0.68
Lmx1b-only	28±2.0	32±3.0	0.15
Calretinin-Lmx1b	4±1.7	4±1.0	0.78
Total Calretinin	40±2.6	40±3.0	1.00
Total Lmx1b	33±3.0	36±3.6	0.29

Calretinin-only, Lmx1b-only, and Calretinin-Lmx1b neurons are positioned correctly in laminae II inner dorsal of *Reln*$^{-/-}$ compared to *Reln*$^{+/+}$ mice. Means±SEM shown per hemisection, 5 mice/genotype, 3-6 hemisections/mouse. Analyses were carried out on one confocal slice/hemisection.

Table 3: Calbindin-Lmx1b neurons in lamina I-II outer do not differ between *Reln*$^{+/+}$ vs. *Reln*$^{-/-}$ mice

Genotype/Cell type	*Reln*$^{+/+}$	*Reln*$^{-/-}$	p-value
Calbindin-only	14±1.4	13±2.6	0.78
Lmx1b-only	49±7.2	45±3.6	0.39
Calbindin-Lmx1b	9±1.4	9±1.8	0.89
Total Calbindin	23±2.8	22±2.9	0.62
Total Lmx1b	57±8.6	54±4.7	0.30

Calbindin-only, Lmx1b-only, and Calbindin-Lmx1b neurons are positioned correctly in lamina I-II outer of *Reln*$^{-/-}$ compared to *Reln*$^{+/+}$ mice. Means±SEM shown per hemisection, 3 mice/genotype, 6 hemisections/mouse. Analyses were carried out on one confocal slice/hemisection.

Table 4: Calbindin-Lmx1b neurons in laminae II inner dorsal do not differ between *Reln*$^{+/+}$ vs. *Reln*$^{-/-}$ mice

Genotype/Cell type	*Reln*$^{+/+}$	*Reln*$^{-/-}$	p-value
Calbindin-only	19±1.3	18±1.2	0.90
Lmx1b-only	27±3.2	33±0.3	0.12
Calbindin-Lmx1b	7±0.2	9±2.2	1.00
Total Calbindin	26±1.6	27±1.1	0.59
Total Lmx1b	34±3.2	42±2.0	0.12

Calbindin-only, Lmx1b-only, and Calbindin-Lmx1b neurons are positioned correctly in laminae II inner dorsal of *Reln*$^{-/-}$ compared to *Reln*$^{+/+}$ mice. Means±SEM shown per hemisection, 3 mice/genotype, 6 hemisections/mouse. Analyses were carried out on one confocal slice/hemisection.

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