Title

Reelin and Gastrin-Releasing Peptide Modulate Itch in the Superficial Dorsal Horn of the Spinal Cord

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Publication Date

2022

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Reelin and Gastrin-Releasing Peptide Modulate Itch in the Superficial Dorsal Horn of the Spinal Cord

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

Christine Michelle Mavilian

2022

ABSTRACT OF THE THESIS

Reelin and Gastrin-Releasing Peptide Modulate Itch in the

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Master of Science in Physiological Science

University of California, Los Angeles, 2022

Professor Patricia Emory Phelps, Committee Chair

The Reelin-signaling pathway mediates a vital role in correctly positioning neurons within the dorsal horn of the spinal cord. Reelin binds to its two receptors, Apolipoprotein E receptor 2 (Apoer 2) and Very-low-density receptor (Vldlr) and induces phosphorylation of its intracellular adaptor protein Disabled-1 (Dab1) by Src family kinases. Dab1 phosphorylation activates a downstream signaling cascade that influences neuronal migration and positioning within the central nervous system during embryonic development (D'Arcangelo et.al., 1999).

Previous studies have shown that mutations in Reelin and Dab1 induce alterations in nociceptive processing. *Reeler* and *dab1* mutants display thermal hyperalgesia and decreased sensitivity to mechanical stimulation (Akopians et. al., 2008; Villeda et. al., 2006). In addition to nociceptive processing, the Reelin-signaling pathway may also influence pruritogenic behavior. Itch, like pain, is a major somatic sensation relayed via the somatosensory pathway system

(Jeffrey et. al., 2011). Gastrin-Releasing Peptide (GRP) is a neurotransmitter involved in transmitting itch information and is abundantly expressed in the superficial dorsal horn (Solorzano et al., 2015, Barry et al., 2020). However, the molecular mechanisms downstream of GRP that underlie itching behavior are still elusive. Therefore, the overall aim of this project is to determine whether the Reelin-signaling pathway mediates a role in pruritogenic behavior and whether GRP neurons are implicated in the reduced itching behavior of *dab1*$^{-/-}$ mice. Our preliminary data show that approximately 34% of GRP neurons co-express Reelin and that roughly 16% of Reelin-positive neurons co-express GRP. Unlike Reelin, Dab1-expressing neurons do not colocalize with GRP. We determined that Reelin-Lmx1b-GRP triple-labeled neurons are mispositioned above the IB4 band in *Dab1*$^{-/-}$*-Grp* mice and there are greater total number of GRP cells in *Dab1*$^{-/-}$*-Grp* compared to *Dab1*$^{+/+}$*-Grp* mice . There are also more Reelin-Lmx1b-GRP triple-labeled neurons within the IB4 region of *Dab1*$^{-/-}$*-Grp* mice. Importantly, we discovered an abnormal expansion of the central area above the IB4 band in our mutant mice, which differs from previous findings of a dorsal shift and neuronal compaction of laminae I-II outer. The lateral compaction of the dorsal horn appears to be consistent with previous reports. Future studies will further investigate the relationship between Dab1 and GRP and the abnormal expansion of the central area above the IB4 band.

The thesis of Christine Michelle Mavilian is approved.

Stephanie Correa Van Veen

Carolyn R. Houser

Patricia Emory Phelps, Committee Chair

University of California, Los Angeles

2022

Dedication

I dedicate this thesis to my parents, Susan and Greg, and my brother Christopher for their continuous encouragement in all my educational endeavors.

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Acknowledgements

First, I would like to express my deep gratitude to Dr. Phelps for her unwavering support and leadership over the past several years with this project. I would also like to thank members of my thesis committee Dr. Carolyn Houser and Dr. Stephanie Correa for their critical guidance and evaluation of my thesis.

A special thank you to members of the Phelps lab, Mahlet Mekonnen, for her help with the perfusions and Dab1 experiments, Sherwin Atighetchi, for his help with genotyping, and Samantha Zimmer, for her support as a fellow graduate student in this program.

Thank you to the faculty within the Integrative Biology and Physiology program for helping nurture my scientific curiosity and for allowing me to relay a body of knowledge to support our undergraduate students over the course of their academic studies within the major.

Finally, I wish to thank my parents, brother, and family for their endless sacrifices and for always inspiring me to challenge myself, to set big goals, and to tenaciously achieve them.

INTRODUCTION

Discovery and Characterization of Reelin

The naturally occurring recessive mutant gene *reelin* (*Reln*$^{-/-}$) was first discovered by Falconer (1951). *Reln*$^{-/-}$ mutants were reported to be smaller in size, displayed a reeling gait, and presented abnormal laminar organizations in the cerebral and cerebellar cortices. D'Arcangelo and colleagues (1997) cloned the *Reln*$^{-/-}$ gene and found that it encoded the Reelin protein, and characterized Reelin as an extracellular matrix-like protein that is important in mediating correct neuronal positioning during development.

In addition to identifying the molecular composition of the *Reln*$^{-/-}$ gene, D'Arcangelo et al. (1995) also observed *Reln*$^{-/-}$ mRNA in various regions of the developing central nervous system (CNS). The highest *Reln*$^{-/-}$ mRNA levels were detected in the embryonic stages of development with a decline in expression levels during postnatal and adult stages (D'Arcangelo 1995). In-situ hybridization revealed that *Reln*$^{-/-}$ is expressed by Cajal-Retzius cells in the marginal zone of the forebrain; *Reln*$^{-/-}$ expression is also detected in the olfactory bulb, hippocampus, and cerebellum (D'Arcangelo *et al.* 1995; D'Arcangelo et al. 1997; Ikeda and Terashima, 1997). Furthermore, Reelin transcripts were detected in neurons within the outer lining of the dorsal horn of the spinal cord (Ikeda and Terashima, 1997).

Constituents of the Reelin-signaling cascade were later identified by Howell and colleagues (1997a). The mouse homolog of the *Drosophila* Disabled gene, *mDab1* codes for the protein Disabled-1 (Dab1), and serves as an intracellular adaptor molecule important in neural development (Howell et al. 1997a). Western blot analysis revealed that tyrosine phosphorylation of Dab1 is dependent on Reelin expression and that Reelin and Dab1 expressing cells are located in adjacent neuronal populations (Howell et al., 1997b; Sheldon et al.,1997; Rice et al., 1998). Rice and colleagues (1998) also showed that *Reln*$^{-/-}$ mutants display a 10-fold increase in Dab1

expression compared to wild-type controls; this strengthened earlier findings that Dab1 functions downstream of Reelin.

Mutations in Reelin, Dab1, or both receptors, Apolipoprotein E receptor 2 (Apoer2) and Very-low-density lipoprotein receptor (Vldlr), exhibit similar phenotypic abnormalities and neuroanatomical defects which suggest that they all belong to the same signaling pathway (Howell et al., 1997b; Hiesberger et al., 1999; Trommsdorff et al., 1999). Reelin binds to its two receptors, Apoer2 and Vldlr and induces Dab1 phosphorylation by Src family kinases (D'Arcangelo et al.,1999 Hiesberger et al., 1999). Phosphorylation of Dab1 induces a downstream signaling cascade that influences neuronal migration and positioning within the CNS during embryonic development (D'Arcangelo et.al., 1999). The Reelin-signaling pathway terminates when Dab1 is polyubiquinated and degraded (Arnaud et al., 2003). If Reelin and/or both of its receptors, Apoer2 and Vldlr are absent, Dab1 accumulates in mispositioned neurons (Arnaud et al., 2003; Herz and Chen, 2006). Taken together, these studies helped define the canonical Reelin-signaling pathway.

Contribution of the Reelin Signaling Pathway to Sensory Circuits in the Spinal Cord

While many studies have examined the influence of the canonical Reelin signaling pathway in the developing cerebral cortex, much less is known about the spinal cord. The expression of Reelin and Dab1 in the embryonic spinal cord coincides with regions important in nociceptive processing, which include: laminae I-II of the dorsal horn, the lateral spinal nucleus (LSN) and the lateral lamina V (Kubasak et al., 2004; Villeda et al., 2006; Akopians et al., 2008; Wang et al., 2012; Yvonne et al., 2020). Approximately 20-30% of the neurons within laminae I-II are inhibitory, while the remainder comprise excitatory glutamatergic populations (Todd, 2010).

Roughly 70% of Dab1 neurons and 90% of Reelin neurons co-express LIM-homeobox 1 beta (Lmx1b), a transcription factor that characterizes excitatory glutamatergic interneurons (Yvonne *et al.*, 2017; Yvonne et al., 2020). Lmx1b contributes to the migration of superficial dorsal horn neurons during development (Ding et al., 2004) and is expressed in both postmitotic early-born (dI5) and late-born dorsal neurons (dIL_B) (Lai et al., 2016). A subset of the dI5 and dIL_B neurons are involved in circuits that relay pain and itch (Gross et al., 2002; Lai et al., 2006). These neuronal populations overlap with Reelin and Dab1-positive cells as they migrate together during embryonic development (Yvone et al., unpublished data). Consequently, loss of Reelin and Dab1 result in clear anatomical differences observed by E14.5 (Yvone et al., unpublished data). In the *Reln*$^{-/-}$ spinal cord, the Dab1-Lmx1b neurons migrate further dorsally along the outer rim of the dorsal horn and Reelin-Lmx1b neurons are also sparsely scattered in regions of the deep dorsal horn (Yvone et al., unpublished data).

In addition to the migratory defects, mutations in Reelin and Dab1 result in aberrant phenotypic responses, indicative of altered nociceptive processing. *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mutants display thermal hyperalgesia as observed in the Hargreaves paw withdrawal test (Akopians et. al., 2008; Villeda et. al., 2006). These results were corroborated by Wang and colleagues (2012) who found a reduction in Fos expression in laminae I-II of *Reln*$^{-/-}$ and *dab1*$^{-/-}$ dorsal horns relative to wild-type controls. In the von Frey test for mechanical sensitivity, *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mice displayed significantly reduced sensitivity relative to wild-type controls (Akopians et al., 2008; Villeda et al., 2006). Analogous to *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mutants, mice with a conditional knockout of Lmx1b display reduced mechanical sensitivity, further demonstrating the significance of the Reelin-signaling pathway in the complex pain-processing circuitry (Szabo et al., 2015). Single knockouts of either *Apoer2* and *Vldlr* mice did not result in any thermal hypersensitivity or mechanical insensitivity, while double knockout mice could not be tested due

to low survival levels beyond several weeks after birth (Akopians et al., 2006). Furthermore, formalin testing demonstrated that *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mice displayed reduced responses to chemical pain but no stark contrast was observed in models of visceral and cold-pain (Wang et al., 2012). Together, these findings highlight the specific nociceptive circuits that are linked to the canonical Reelin-signaling pathway.

Overview of the Circuits Involved in Pruritoception

While the contributions of the Reelin-signaling pathway were reported in relation to nociception, its role in itch is still unclear. Itch, a close parallel to pain, is widely defined as an undesirable sensation that is associated with a desire to scratch (Ikoma et al., 2006; Akiyama & Carstens, 2013; Bautista *et al.,* 2014). Although initially categorized as a subset of pain, itch is a distinct disorder, arising from a range of acute and chronic skin conditions (Jeffry et al., 2011). The sensation of itch is the result of pruritoception, the biological processing of pruritogenic stimuli. Pruritoception begins with the activation of receptors that are located on the peripheral terminals of Aδ and C fibers. Depending on the peripheral inputs, itch is classified into chemical and mechanical itch (Chen & Sun, 2020). Chemical itch is further subclassified based on its response to histaminergic or non-histaminergic stimuli (Chen & Sun, 2020). For instance, the H1 receptors respond to histamine and are located on C fibers that express the transient receptor potential cation channel subfamily V member 1 (TRPV1) (Imamachi et al., 2009). By contrast, non-histaminergic stimuli are transmitted via members of the Mas-related G-protein coupled receptor (Mrgpr) family like MrgprA3, an antimalarial drug to which chloroquine binds to, and MrgprD (Liu et al., 2009; Liu et al., 2012). These receptors have important implications in the transmission of chemical itch (Chen & Sun, 2020). Moreover, transient receptor potential channels have both been linked to histamine-dependent and independent pathways (Shim et al.,

2007; Wilson et al., 2011). Following the detection of the pruritic stimulus by the receptors, the central axons of Aδ and C fibers, which stem from the cell bodies within the dorsal root ganglion, synapse onto excitatory and/or inhibitory interneurons located within laminae I, II, and V of the dorsal horn. These interneurons transmit facilitatory or inhibitory signals to precisely regulate pruritoception.

The interneuron populations that mediate the transmission of chemical and mechanical itch are distinct and numerous. Chemical itch is relayed by both inhibitory and excitatory interneuron populations. Among the predominant excitatory spinal neurons include Gastrin Releasing Peptide (GRP) and its receptor (GRPR) (Sun & Chen, 2007). Other excitatory spinal populations involved in itch include natriuretic polypeptide b (Nppb) receptor, Neuromedin b (Nmb) and its receptor (Nmbr), and the Natriuretic peptide receptor A (Npra)-expressing neurons (Mishra & Hoon, 2013, Wan et al., 2014; Zhao et al., 2017). Interestingly, Npra is coexpressed with GRP in a subset of dorsal spinal interneurons (Mishra & Hoon, 2013). It is postulated that Npra acts upstream of GRPR alongside GRP (Chen & Sun, 2020).

While spinal excitatory interneurons act to enhance chemically-induced scratching behaviors, spinal inhibitory interneurons suppress itching. In addition, dysregulation of spinal inhibitory interneurons results in a hyperactive itch circuit (Chen & Sun, 2020; Kardon et al., 2014). This was observed in mice lacking Bhlhb5, a transcription factor transiently expressed in the dorsal horn (Kardon et al., 2014). Mice which lacked Bhlhb5 displayed a significant reduction in two subclasses of somatostatin 2A spinal inhibitory interneurons: galanin and neuronal nitric oxide synthase (Kardon et al., 2014). The remaining two inhibitory interneuron populations, Neuropeptide Y and parvalbumin did not decrease, which suggests that galanin and neuronal nitric oxide synthase function in gating chemical itch (Chen & Sun, 2020; Kardon et al., 2014). Spinal Bhlhb5 neurons also express opioid peptide dynorphin (DYN) (Kardon et al.,

2014). Intradermal and intrathecal injections with kappa opioid receptor (KOR) agonists inhibited itch-induced scratching behaviors, while antagonists to KOR signaling enhanced pruritogen-evoked responses (Kardon et al., 2014). Furthermore, intrathecal injection of a kappa opioid receptor agonist inhibited GRP-induced scratching behavior, which suggests that DYN may either act on GRPR-expressing neurons directly or downstream to regulate itch processing (Kardon et al., 2014). It was later determined that scratching induced by KOR antagonists was suppressed only after ablating GRPR-expressing neurons but not Npra-expressing neurons; this suggests that DYN acts downstream of Npra-expressing neurons and releases either GABA or glycine to regulate the activity of GRPR neurons (Huang et al., 2018).

In addition to pruritogen-evoked scratching behaviors, itch stimulation can be triggered through light touch stimuli, otherwise known as mechanical itch. Unlike chemical itch, mechanical itch is independent of GRPR-positive spinal neuron populations (Bourane et al., 2015). Ablation of NPY-expressing spinal neurons induce spontaneous scratching behavior that persist even after ablating GRPR-positive cells, which suggest that these circuits function independently (Bourane et al., 2015).

The Role of Gastrin Releasing Peptide (GRP) in Itch

Although several cellular populations comprise the circuits which relay pruritoceptive information, GRP has been one of the earliest cellular populations to be linked closely with relaying itch information within the superficial dorsal horn (Solorzano et al., 2015, Barry et al., 2020). GRP and its receptor, GRPR, have been extensively studied within the cerebral cortex, where they mediate an important role in regulating feeding behavior and fear memory (Jeffry et al., 2020). At the spinal level, intrathecal administration of GRPR antagonists has shown to reduce mice scratching behaviors upon injection with chloroquine, compound 48/80, and

protease activated receptor 2 (Sun et al., 2007). Subsequent studies have validated this finding, as ablation of GRPR neurons with saporin conjugated to bombesin, an amphibian homologue to GRP, resulted in reduced scratching responsiveness to an array of pruritogens (Sun et al., 2009).

The role of GRP in itch signaling responses was also examined closely with respect to histaminergic and non-histaminergic itch. Itch behaviors following the administration of histamine-independent pruritogens, like chloroquine, were substantially reduced in GRPR knockout mice, whereas histamine-dependent responses were less altered (Sun et al. 2007; Bardoni et al., 2018). Further support for the spinal role of GRP in histamine-independent itch was shown when a GRPR antagonist delivered intrathecally reduced the neuronal responses to intradermal chloroquine, but not histamine (Akiyama et al., 2014). These findings were countered by a study conducted in 2016, which found that GRP neurons within the superficial dorsal horn seldom expressed Fos, a marker for activated neurons, upon intradermal injection with chloroquine (Bell et al., 2016). However, the study concluded that activated GRP neurons may selectively express Fos, and that there may be some GRP neurons that do not express green-fluorescent protein (GFP) but are still activated by chloroquine (Bell et.al., 2016).

While there was some dispute regarding the role of GRP signaling in non-histaminergic itch, much of the controversy arose from the identification of the distribution of GRP neurons within the superficial dorsal horn. Initial studies utilizing in-situ hybridization, immunohistochemistry, and real time RT-PCR found GRP expression within small and medium-sized dorsal root ganglion (DRG) neurons (Sun et al.,2007; Sun et al., 2009; Liu et al., 2014). These GRP neurons also co-localized with other peptidergic primary afferent markers including calcitonin-gene related peptide (CGRP) and substance P, both of which are implicated in nociceptive functions (Sun et al., 2007; Takanami et al., 2014; Gutierrez-Mecinas et al., 2016). Additionally, approximately 80% of GRP-positive neurons co-expressed TRPV1, a capsaicin

receptor responsible for transmitting thermal and chemical stimuli (Sun et al., 2007; Takanami et al., 2014). Dorsal rhizotomy revealed a significant decrease in the number of GRP-positive fibers in the dorsal horn, further implying that GRP is expressed by primary afferent neurons (Sun et al., 2007).

Despite these findings, more evidence has pointed towards the characterization of GRP as an excitatory dorsal horn interneuron. mRNA for GRP has been identified within the superficial dorsal horn of the transgenic GRP-eGFP mouse line, in which enhanced green fluorescent protein is expressed under the GRP promoter region (Gutierrez-Mecinas et al., 2016). In-situ hybridization has revealed that these eGFP-positive cells are localized mainly within lamina II of the dorsal horn and over 90% express GRP mRNA (Solorzano et al, 2015). A large population of these GRP neurons are glutamatergic as they co-express vesicular glutamate transporter 2 (VGLUT2), a marker for excitatory interneurons within the superficial dorsal horn (Gutierrez-Mecinas et al., 2016). The lack of colocalization between GRP and Pax2, a transcription factor for GABAergic inhibitory neurons, further suggests that GRP neurons are excitatory (Gutierrez-Mecinas et al., 2016).

Solorzano et al. (2015) also studied the interaction between TRPV1, a population of primary afferents that are critical for the detection of pruritic stimuli and GRP. They hypothesized that if GRP neurons were involved in the primary afferent transduction of pruritic stimuli, then they would be co-expressed with TRPV1 and would also be ablated upon administration of capsaicin (Solorzano et al., 2015). As anticipated, intrathecal injection with capsaicin ablated the TRPV1 primary afferents in the lumbar dorsal horn (Solorzano et al., 2015). Importantly, intrathecal injection of capsaicin neither altered the magnitude nor distribution of GRP-positive cells within the superficial dorsal horn, verifying that GRP is not derived from primary afferent receptors (Solorzano et al., 2015). These findings were

strengthened by Fleming et al. (2012) and Mishra et al. (2012) who determined that GRP expression was only expressed by interneurons derived from the dorsal spinal cord. The dorsal spinal cord displayed abundant levels of GRP mRNA distribution and immunohistochemistry revealed high expression levels of GRP (Fleming et al., 2012). By contrast, GRP mRNA expression was seldom detected in the DRG and the dorsal root axons (Fleming et al., 2012). GRP neurons were restricted to lamina II of the dorsal horn, but dorsal to the PKC**γ** layer which delineates lamina II inner ventral (Albisetti et al., 2019). These GRP-labeled neurons also partially overlapped with Isolectin B4 (IB4), a marker for primary afferents within lamina II inner dorsal (Albisetti et al., 2019).

Although the controversy surrounding the characterization of GRP has been resolved, the colocalization between GRP neurons and nociceptive afferent neurons such as CGRP, substance P, and TRPV1 has raised new questions concerning the role of GRP in the transmission of itch, and possibly, pain signals (Sun et al., 2007; Takanami et al., 2014; Gutierrez-Mecinas et al., 2014). Several studies have shown that ablation of spinal GRP neurons does not alter responses to pain stimuli (Albisetti et al., 2019; Barry et al., 2020). By contrast, a study conducted by Sun et al. (2017) found a specific subset of GRP neurons that receive synaptic input from pain and itch primary afferent neurons. Upon activation, these GRP neurons generated both pain and itch responses, with pain responses being intensity dependent (Sun et al., 2017). Higher capsaicin doses decreased pain responses while medium range activation yielded stronger pain transmission (Sun et al., 2017). Ablation of GRP neurons significantly impacted strong pain responses, while itch remained relatively unaltered (Sun et al., 2017).

Preliminary Evidence of Reelin Signaling in Pruritoceptive Transmission

The ambiguity surrounding the molecular mechanisms by which GRP transmits itch information, and its potential role with pain, has thus raised our interest in exploring its relationship to Reelin. In addition to nociceptive dysfunctions, we have unpublished data that show our *dab1*$^{-/-}$ mice display significantly reduced itching behaviors. We thus suspect that the Reelin-signaling pathway may also influence pruritogenic behavior via GRP neurons. Like Reelin and Dab1, approximately 83% of GRP neurons coexpress Lmx1b (Pagani et al., 2019). RNA sequencing has revealed the co-expression of Reelin and GRP in two glutamatergic populations, Glut8 and Glut9 (Häring et al., 2018). The Glut8 and Glut9 clusters express Neuromedin U Receptor 2 (Nmur2) and Neuropeptide FF (Npff), respectively (Bell et al., 2020). These neurons are localized within the superficial dorsal horn and are implicated in nociceptive functions, further suggesting a linkage to the Reelin-signaling pathway (Bell et al., 2020).

The overall aim of this project is to determine whether the Reelin-signaling pathway plays a role in pruritogenic behavior and whether or not GRP neurons are implicated in the reduced itching behavior of *dab1*$^{-/-}$ mice. We first characterized the distribution of GRP neurons in *Grp* mice and determined whether Reelin neurons overlapped with GRP-expressing neurons. Since Dab1 is a critical constituent of the canonical Reelin-signaling pathway, we then asked whether Dab1-expressing neurons also co-express GRP. Because our *dab1*$^{-/-}$ mice exhibited reduced scratching bouts compared to wild-type controls, we wondered if these excitatory neuronal populations were mispositioned within the superficial dorsal horn of our *Dab1*$^{-/-}$*-Grp* and *Reln*$^{-/-}$*-Grp* mice. Finally, we analyzed the anatomical positioning errors in the superficial dorsal horn between *Dab1*$^{+/+}$*-Grp* and *Dab1*$^{-/-}$*-Grp* mice.

MATERIALS AND METHODS

Animals

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 bred at UCLA. Two male *Grp-eGFP* transgenic mice were used for initial analyses to determine if GRPeGFP neurons co-express Reelin. Three additional male *Grp-eGFP* transgenic mice (#010444-UCD) were obtained from Dr. Allan Basbaum's laboratory at UC San Francisco. Statistical analyses were conducted on n=5 mice. They will be referred to as *Grp* mice.

$Reln^{-/-}$; Grp-eGFP mice

Adult *$Reln^{-/-}$* (B6C3Fe-ala-*Relnrl*) 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). The *Grp* mice were intercrossed with the *$Reln^{-/-}$* mice to introduce the GFP reporter into the Reelin-signaling pathway wild-type and mutant mice. They will be referred to as *$Reln^{-/-}$-Grp* and *$Reln^{+/+}$-Grp* mice.

$Reln^{rl\text{-}Orl}$; Grp-eGFP mice

Adult *$Reln^{rl\text{-}Orl}$* mice (>95% Balb/C) were originally a gift from Dr. André Goffinet (University of Louvain, Louvain-la-Neuve, Belgium) and a breeding colony was established at UCLA. The Reeler Orleans *($Reln^{rl\text{-}Orl}$)* mutation causes the loss of Reelin secretion (de Bergeyck et al., 1997). The *Grp* mouse line did not survive during the pandemic, but it was successfully bred with *$Reln^{+/+}$* mice. We therefore intercrossed *$Reln^{rl\text{-}Orl}$* mice with *Reln-Grp* mice and used the wildtype

offspring to introduce the GRP-GFP reporter into our *dab1* mice. They are referred to as *Reln*$^{rl\text{-}Orl\ +/+}$*-Grp* mice.

Dab1$^{-/-}$; *Grp-eGFP mice*

Adult *dab1*$^{-/-}$ mice (Balb/CByJ-*dab1*$^{-/-}$*)* were gifts from Dr. J. Cooper (Fred Hutchinson Cancer Research Center, Seattle, WN) and Dr. B. Howell (SUNY Upstate Medical University, Syracuse, NY). A breeding colony was established at UCLA and offspring were genotyped using polymerase chain reaction as described by Birch et al. (2003). Male *dab1*$^{+/-}$ mice were intercrossed with female *Reln*$^{rl\text{-}Orl\ +/+}$*-Grp* and *Reln*$^{+/+}$*-Grp* mice to establish the experimental *Dab1-Grp* mouse line. Genotyping was conducted for *Dab1*, *Reln*, and *Reln* $^{rl\text{-}Orl}$ genes to ensure that only wild-type *Reln* and *Reln*$^{rl\text{-}Orl}$ genes were introduced into the Dab1 colony. Breeding within the *Dab1*$^{+/-}$*-Grp* mouse line was restricted to one transgenic and one wild-type parent for the GRP gene to ensure that offspring did not contain two transgenic alleles.

Tissue Preparation and Immunohistochemical Procedures

Adult, 2.5-month male *Grp, Reln-Grp,* and *Dab1-Grp* mice were anesthetized and perfused transcardially with 4% paraformaldehyde and post-fixed for 3 hours. Postfixed tissues were washed in 0.12 M Millonig's buffer three times for a duration of 30 minutes each and stored overnight in Millonig's buffer with 0.06% azide. The spinal cords were then dissected and cryoprotected in 30% sucrose made in phosphate buffer for 2 days. Lumbar segments were blocked and frozen in Optimum Cutting Temperature and stored at -80°C until cryostat sectioning.

To determine Reelin immunofluorescence, we used a Tyramide Signal Amplification (TSA) kit (PerkinElmer). The Reelin-positive cells in the L4-L5 spinal cord were detected in transverse 25μm-thick cryostat sections in a 2-day experiment. Free floating sections were washed in a PBS buffer (0.1 M PB; 0.9% NaCl) and blocked for 1 hour in 3% normal goat serum in PBS followed by Avidin-Biotin. Sections were incubated overnight with goat anti-Reelin (Table 1), followed by repeated washes in PBS and a 1-hr incubation with biotinylated horse anti-goat IgG (1:800; Vector Laboratories (Burlingame, CA); BA-9500). Multiple washes with TNT (0.1M Tris-HCl; 0.15M NaCl; 5% Tween) preceded a 1-hr incubation period with streptavidin-conjugated horseradish peroxidase (1:500; PerkinElmer #NEL750001EA) in TSA specific blocking buffer (TNB; 0.1M Tris-HCl; 0.15M NaCl; 0.5% Blocking reagent; PerkinElmer #FP1020), followed by a 10-minute incubation with Cyanine 5 (Cy5; 1:100; PerkinElmer #NEL745001KT).

To identify GRP-labeled neurons, sections were incubated with 1% hydrogen peroxide and 0.1% sodium azide in PBS (0.1 M PB; 0.9% NaCl) followed by blocking in normal donkey serum (10% NDS + 0.1% TX in PBS). Sections were then incubated for 24 hours with chick anti-GFP (Table 1) and washed in PBS, followed by a 1-hour incubation with donkey anti-chick Alexa Fluor 488 (Jackson Immunoresearch; #703-545-155).

To detect whether Reelin and GRP co-expressing cells express Lmx1b, sections were washed with 1% hydrogen peroxide and 0.1% sodium azide in PBS (0.1 M PB; 0.9% NaCl) followed by blocking in normal donkey serum (5% NDS + 0.1% TX in PBS). A standard TSA immunofluorescence protocol was then used as described above. The tissues were incubated with recycled guinea-pig anti-Lmx1b (Table 1) overnight followed by multiple washes with PBS. A 1-hr incubation with biotinylated donkey anti-guinea pig IgG (1:1000; Jackson Immunoresearch; #706-065-148) preceded multiple washes with TNT. Tissues were then incubated in streptavidin-

conjugated horseradish peroxidase (1:500; PerkinElmer #NEL750001EA) in TSA specific blocking buffer (TNB; 0.1M Tris-HCl; 0.15M NaCl; 0.5% Blocking reagent; PerkinElmer #FP1020), followed by a 10-minute wash in Cyanine 3 (Cy3; 1:150; PerkinElmer #NEL744001KT).

Grp and Reln-Grp tissue sections were used for Dab1 staining. To locate 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. A 48-hour incubation with rabbit anti-Dab1 (Table 1) preceded several washes in PBST and TNT followed by a 1-hour incubation with donkey anti-rabbit IgG (1:1,000; Jackson Immunoresearch #711-065-152) in TNB. Multiple washes with TNT preceded a 1-hour incubation with streptavidin-conjugated horseradish peroxidase in TNB and a 5-min incubation with TSA Plus Cy5 (1:100; PerkinElmer #NEL745001KT).

To examine locations of Reelin, Lmx1b, and GRP-expressing neurons within and above the IB4 region, IB4 was visualized using a biotinylated IB4 conjugate (Table 1). Following immunohistochemical procedures, triple labeled sections were mounted and coverslipped using Flouro-Gel with Tris Buffer (Electron Microscopy Sciences; 17985-11) and quadruple labeled sections were mounted and coverslipped with ProLong™ Diamond Antifade Mountant (Thermo Fisher Scientific; P36970) mounting media.

Imaging and Statistical Analyses

Sections were photographed using a Zeiss Laser Scanning Microscope (LSM880). Confocal images of the superficial dorsal horn were obtained with 488 nm, 594nm, and 640 nm lasers for triple-labeled images and 488 nm and 640 nm for double-labeled images. The 405 nm laser was used to image IB4 as a terminal marker for lamina II inner dorsal. Acquisition parameters were

set at a frame size of 1024x1024 pixels with 16 bits per pixel. A 4x2 tile parameter was utilized to capture the entire superficial dorsal horn, with images taken bidirectionally at a scan speed of 4-5. Hemisections were imaged using a 40X water immersion lens (numerical aperture 1.2) for high magnification images with the pinhole aperture set to 1 Airy Unit. A single image capturing Dab1 neurons and GRP processes was taken at 63X oil immersion lens (numerical aperture 1.4). To ensure proper identification of cells, the mediolateral area of laminae I-II per hemisection was scanned with a z-separation of 0.6 µm.

The 40X images were used for statistical analysis using the ZEN (Zeiss Efficient Navigation) lite (v. 2.6) imaging software. A single optical section with the highest signal was selected as the reference point for analyzing cells within the superficial dorsal horn. The optical section selected for analysis varied within the z-series. Laminae I-II were measured between the gray and white dorsal border as delineated by Yvone et al. (2020). Since we only analyzed a single optical section with a thin z-separation, we likely underestimated the total number of neurons in the section. Images were analyzed using the LSM Image browser, and then transferred to Photoshop for final assembly and adjustment of brightness and contrast.

To determine colocalization, Reelin, Lmx1b, and GRP channels were viewed together and then separately by channel. Colocalization was determined by cytoplasmic Reelin encircling the Lmx1b-positive nucleus and/or GRP-labeled cytoplasm. The remaining optical slices were utilized to confirm the neuronal profiles. For the Reelin-GRP and Dab1-GRP dataset, we counted labeled neurons in 5 hemisections per mouse in the *Grp* male mice (n=5 mice). For the Reelin-Lmx1b-GRP-IB4 dataset, both the IB4-positive area and the region above IB4 (laminae I-II outer) were outlined. Cells immunoreactive for Reelin, Lmx1b, GRP, Reelin-Lmx1b, Reelin-GRP, Lmx1b-GRP, and Reelin-Lmx1b-GRP were counted in 5-6 hemisections in the *Dab1* $^{+/+}$-*Grp* and *Dab1*$^{-/-}$-*Grp* male mice (n=5 mice/genotype). The means for Reelin-, Lmx1b-, GRP-

only, Reelin-Lmx1b, Reelin-GRP, Lmx1b-GRP, and Reelin-Lmx1b-GRP neurons were compared by genotype and area for each cell type by a repeated measures 2x2 for analysis of variance (ANOVA) model. The means of total Reelin, total Lmx1b, total GRP, and total neurons within and above the IB4 region, and mean percentages were also compared by genotype and/or area using ANOVA. Means ± standard errors are reported.

RESULTS

Mediolateral distribution of GRP in the dorsal horn

Our unpublished data shows that our *dab1*$^{-/-}$ mice display significantly reduced scratching bouts and previous studies have found that Gastrin-Releasing Peptide is the major itch neurotransmitter within the dorsal horn (Solorzano et al., 2015, Barry et al., 2020). Using immunohistochemistry, we first mapped the distribution of GRP within the dorsal horn of *Grp* mice (i.e., *Grp-eGFP* mice). We did not find evidence of GRP in the dorsal root ganglion, or their axons as reported by Solorzano and colleagues (2015). As shown in Figure 1A, we found GRP-expressing neurons scattered medially and laterally across the superficial dorsal horn, but mainly concentrated in lateral lamina II. A few GRP-positive cells are scattered in the medial deep dorsal horn, but no GRP-expressing neurons are located within the LSN.

GRP-expressing neurons colocalize with Reelin

RNA sequencing data revealed that Reelin and GRP are coexpressed in two glutamatergic populations Glut8 and Glut9 (Häring et al., 2018) and our previous study showed that approximately 90% of Reelin cells express the transcription factor Lmx1b, consistent with a glutamatergic phenotype (Yvone et al., 2017). In addition, a large population of GRP neurons are reported to be glutamatergic and co-express the excitatory marker VGLUT2 and Lmx1b

(Gutierrez-Mecinas et al., 2016; Pagani et al., 2019). Because a majority of Reelin- and GRP-expressing neurons belong to the same two glutamatergic subsets, we asked whether the GRP cells in the *Grp* mice co-express Reelin. As shown in Figure 1B, arrowheads delineate cells that are co-labeled with Reelin and GRP. Double-labeled Reelin-GRP neurons appeared to be scattered both medially and laterally in the superficial dorsal horn (Fig. 1). We observed that 34% of GRP cells express Reelin and approximately 16% of Reelin cells express GRP ($\bar{x}$ = 5 Reelin-GRP neurons/hemisection; n=5 mice). The enlargements depict a Reelin-only, a GRP-only, and a Reelin-GRP cell in lamina II (Figure 1; B1-B3). These findings confirm previous single-cell sequencing data that Reelin and GRP belong to the same subset of glutamatergic interneurons within the superficial dorsal horn (Häring et al., 2018).

Dab1 neurons do not comprise a subset of GRP neurons

Because our *dab1*$^{-/-}$ mice exhibited a decrease in scratching bouts in response to chloroquine and histamine (unpublished data), we hypothesized that one of the constituents of the Reelin-signaling pathway, namely Dab1, may also comprise a subset of the GRP interneurons within the superficial dorsal horn of our *Grp* mice. Previous studies found that 83% of GRP-expressing neurons coexpress Lmx1b (Pagani et al., 2019) and 70% of Dab1 neurons colocalize with Lmx1b (Yvonne et al., 2017), suggesting that some of the Lmx1b-expressing Dab1-positive cells may belong to a specific glutamatergic subset of interneurons that overlaps with GRP-expressing neurons. With double-immunolabeling, we showed that GRP-labeled neurons are adjacent to but do not colocalize with Dab1 neurons in the superficial dorsal horn (Fig. 2A). The enlargements in Figure 2A1-A3 depict a large GRP neuron marked by a white arrow and a Dab1 neuron marked by a black arrow. Interestingly, GRP-labeled processes appear to line the surface of the Dab1 neuron (Figure 2B). The enlargement in Figure 2B1 depicts a Dab1 neuron marked by a

black arrow and GRP neurons are marked by white arrows. Importantly, the results from the *Grp* mice show that GRP-expressing neurons and Dab1 neurons occupy distinct glutamatergic subsets that do not overlap with one another.

Reelin-Lmx1b-GRP neurons are mispositioned in the *Dab1*$^{-/-}$-*Grp* laminae I-II outer and IB4 regions

To determine whether alterations in the Reelin-signaling pathway would give rise to neuroanatomical defects previously found in *dab1*$^{-/-}$ mice (Yvonne et al., 2017), we first examined the area of the superficial dorsal horn in *Dab1*$^{+/+}$-*Grp* and *Dab1*$^{-/-}$-*Grp* mice. Specifically, we measured the area of laminae I-II outer, which receives peptidergic input and the IB4 region, which is marked by non-peptidergic primary afferents within lamina II inner dorsal (Albisetti et al., 2019). Unlike the substantial decrease in laminae I-II outer observed in *dab1*$^{-/-}$ mice (Yvonne et al., 2017) there was no difference in the area of laminae I-II outer across genotypes (*Dab1*$^{+/+}$-*Grp* 21928 ± 1870 μm^2; *Dab1KO* $^{-/-}$-*Grp* 22176 ± 1870 μm^2; p = 0.9274). Although they do not differ statistically, *Dab1*$^{-/-}$-*Grp* mice appear to have a slightly larger area of laminae I-II outer than their wild-type controls. Consistent with our previous results, we found that the IB4 area in did not vary between genotypes (*Dab1*$^{+/+}$-*Grp* 16579 ± 1070 μm^2; *Dab1*$^{-/-}$-*Grp* 19075 ± 1070 μm^2; p = 0.1379).

We next examined the distribution of Reelin, Lmx1b, GRP single-, double-, and triple-labeled neurons within laminae I-II outer (Fig. 3). Although the total number of triple-labeled Reelin-Lmx1b-GRP neurons is small, we found more triple-labeled neurons in *Dab1*$^{-/-}$-*Grp* laminae I-II outer (2 ± 0.3) than found in *Dab1*$^{+/+}$-*Grp* mice (0.7 ± 0.3, p = .002; Figs. 3,4, Table 2). Most comparisons between single and double-labeled neurons did not vary between genotypes (Table 2). However, the laminae I-II outer region of *Dab1*$^{-/-}$-*Grp* mice also contained

fewer Reelin-only neurons (7 ± 0.6) compared to *Dab1*$^{+/+}$*-Grp* mice (9 ± 0.6; p = .04; Table 2). The total number of GRP neurons was greater in the mutant (4.1 ± 0.5) than in the wild-type mice (2.5 ± 0.5; p = 0.04; Table 2). We also found that Reelin-Lmx1b-GRP neurons comprised on average 4% of total Reelin neurons in *Dab1*$^{+/+}$*-Grp* mice (4.1% ± 1.4%) and roughly 12% of total Reelin neurons in *Dab1*$^{-/-}$*-Grp* mice above the IB4 region (11.6% ± 1.4%.; p = 0.0018; Table 4). Triple-labeled neurons constituted roughly 2% and 4% of the total Lmx1b neurons in wild type (1.5% ± 0.6 %) and mutant mice (3.8% ± 0.6%; p = 0.0114, Table 4), respectively. Finally, Reelin-Lmx1b-GRP neurons comprised an average of 32% of total GRP neurons in *Dab1*$^{+/+}$*-Grp (*32% ± 6.6%) and 54% of total GRP neurons in *Dab1*$^{-/-}$*-Grp* mice (54 % ± 6.6%; p = 0.0361; Table 4). Our data shows that while the laminae I-II outer areas above IB4 do not differ between genotypes, *Dab1*$^{-/-}$*-Grp* mice had more triple labeled neurons in this region compared with *Dab1*$^{+/+}$*-Grp* mice.

We also analyzed the distribution of Reelin, Lmx1b, and GRP-expressing neurons within the IB4 region. We again found more triple-labeled Reelin-Lmx1b-GRP neurons in the IB4 region of *Dab1*$^{-/-}$*-Grp (*2 ± 0.3) than in *Dab1*$^{+/+}$*-Grp* mice (0.8 ± 0.3; p = 0.01; Figs. 3,4; Table 3). Reelin-Lmx1b-GRP neurons comprised an average of 12% of total Reelin neurons in *Dab1*$^{+/+}$*-Grp* (11.7 % ± 1.4) and 23% in *Dab1*$^{-/-}$*-Grp* mice (22.7% ± 1.4%; p = <.0001; Table 4). Triple-labeled neurons comprised roughly 3% and 6% of total Lmx1b neurons in wild type (3.2% ± 0.6 %) and mutant mice (6% ± 0.6 %, p = 0.0049; Table 4), respectively. Finally, Reelin-Lmx1b-GRP neurons comprised a mean of 27% of total GRP neurons in *Dab1*$^{+/+}$*-Grp* (27% ± 6.6%) and 51% in *Dab1*$^{-/-}$*-Grp* mice (51% ± 6.6%, p = 0.0221; Table 4). There were no differences in the number of single- or double-labeled neurons across genotypes (Table 3). Together, these results demonstrate that while the area of the IB4 band does not differ across

genotypes, *Dab1*$^{-/-}$*-Grp* mice still had more triple-labeled neurons in this region than *Dab1*$^{+/+}$*-Grp* mice.

Dab1$^{-/-}$*-Grp* mice exhibit a displaced IB4 region

As previously reported by Yvone et al. (2017, 2020), the IB4 regions of *dab1*$^{-/-}$ and *Reln*$^{-/-}$ mice are shifted dorsally and show a neuronal compaction of laminae I-II outer. Here, we asked whether this anatomical shift is also observed in the *Dab1*$^{-/-}$*-Grp* mice compared to their wild-type controls (Fig. 5). We found that while the areas of both the IB4 region and laminae I-II outer did not differ between genotypes, surprisingly, there is a slight increase in the size of both the IB4 and laminae I-II outer regions in the *Dab1*$^{-/-}$*-Grp* mice as compared to their wild-type controls (Fig. 5). Upon closer examination, we observed a substantial displacement of the IB4 region more ventrally, specifically in the central region of the dorsal horn, as marked by the white arrow (Fig. 5B1) that contrasts with reports of a dorsal IB4 shift and neuronal compaction in laminae I-II outer in *dab1*$^{-/-}$ and *Reln*$^{-/-}$ mice (Yvone et al., 2017; Yvone et al., 2020). Interestingly, we observed no aberrant displacement of the IB4 region in *Reln*$^{-/-}$*-Grp* mice (data not shown).

DISCUSSION

In this study, we identified a distinct subset of GRP neurons that also express Reelin and Lmx1b in the superficial dorsal horn in *Grp* mice and established that on average, 30% of GRP neurons co-express Reelin and Lmx1b in the superficial dorsal horn of *Dab1*$^{+/+}$*-Grp* mice and 53% in *Dab1*$^{-/-}$*-Grp* mice. These findings confirm previous reports that some GRP neurons belong to a glutamatergic subset of dorsal horn interneurons that express Reelin and Lmx1b (Gutierrez-Mecinas et al., 2016; Häring et al., 2018; Pagani et al., 2019). We mapped out the distribution of

GRP neurons above and within the IB4 region relative to Reelin and Lmx1b in *Dab1*$^{+/+}$-*Grp* and *Dab1*$^{-/-}$-*Grp* mice and observed more Reelin-only neurons in *Dab1*$^{+/+}$-*Grp* than in the *Dab1*$^{-/-}$-*Grp* mice in laminae I-II outer but a greater total number of GRP neurons in the mutants than in the wild-type controls. Importantly, the number of Reelin-Lmx1b-GRP neurons was greater in laminae I-II outer and within the IB4 band of *Dab1*$^{-/-}$-*Grp* mice compared to *Dab1*$^{+/+}$-*Grp* mice, suggesting that some of the GRP neurons are mispositioned. By contrast, GRP-expressing neurons do not colocalize with Dab1 but have processes that interact with the Dab1 neurons. Interestingly, our *Dab1*$^{-/-}$-*Grp* mice did not exhibit the characteristic central IB4 dorsal shift, and central neuronal compaction of laminae I-II outer that was observed in *dab1*$^{-/-}$ and *Reln*$^{-/-}$ mice (Yvone et al., 2017; Yvone et al., 2020). Together, these findings highlight the anatomical defects that likely correlate with the itch abnormalities observed in our *dab1*$^{-/-}$ mice.

Migration of GRP neurons is likely influenced by the loss of the Reelin-signaling pathway

There is substantial evidence to show that disruption to the dorsal horn circuits may lead to aberrant nociceptive and pruriceptive processing (Sun et al., 2007; Takanami et al., 2014; Gutierrez-Mecinas et al., 2014). The Reelin-Lmx1b-GRP neurons of our *Dab1*$^{-/-}$-*Grp* mice are numerous within the IB4 region. Yvone et al. (2020) also found more Reelin-Lmx1b neurons in *dab1*$^{-/-}$ mice and more Dab1-Lmx1b neurons within the IB4 region of *Reln*$^{-/-}$ mice. Because there was no colocalization between GRP and Dab1, we suspect that the terminal-like staining we observed may represent evidence of presynaptic contact between GRP and Dab1 neurons, and that changes in the positioning of either or both neuronal populations may alter connections in the itch circuity.

GRP-expressing neurons are largely implicated in the processing of histamine-independent itch (Sun et al. 2007; Akiyama et al., 2014; Bardoni et al., 2018). The *dab1*$^{-/-}$ mice

exhibit attenuated itch responses to both histamine and chloroquine (unpublished data). We believe that the mispositioned Reelin-Lmx1b-GRP neurons are implicated in the reduced itch responses to chloroquine. However, other markers for histaminergic itch, such as Neuromedin B, may also participate in the itch circuit within *dab1*$^{-/-}$ mice (Zhao et al., 2014; Wan et al., 2017). Our previous reports show that Reelin-Lmx1b and Dab1-Lmx1b neurons belong to separate populations, but sustain similar migratory defects (Yvone et al., 2020). It is possible that the Dab1-Lmx1b neurons may co-express a peptide that participates in the processing of histaminergic itch. Future studies could determine if the Dab1-labeled neurons co-express peptides.

Ectopic Distribution of the IB4 band may contribute to altered pruriceptive processing

Yvone et al. (2020) reported that *dab1*$^{-/-}$ and *Reln*$^{-/-}$ mice exhibited similar anatomical abnormalities in the superficial dorsal horn. Surprisingly, our results differ from that report, and from the preliminary data on the *Reln-Grp* mice. Villeda et al. (2006) found that CGRP and TRPV1-positive afferents were ectopically positioned in the cervical segment of *Reln*$^{-/-}$ mice, an abnormal finding that resembles the shifted IB4 band in the current study. It will, therefore, be of interest to examine the distribution of the CGRP and TRPV1 primary afferents in Dab1-GRP mice. We will examine whether or not the IB4 band is also mispositioned in the female *Dab1*$^{-/-}$-*Grp* mice we have processed and test if another primary afferent marker, Neurokinin-1 (NK1R) receptor, which is implicated in nociception, is also mispositioned (Villeda et al., 2006). We also wondered if the observed anatomical error may reflect the dorsal horn of the *Reln*$^{rl\text{-}Orl}$ mice we used to transfer the GRPeGFP reporter into the *dab1* mouse line. Thus, future experiments should analyze the IB4 distribution within the superficial dorsal horn of *Reln*$^{rl\text{-}Orl}$ mice.

In conclusion, the mechanisms that contribute to the altered itch sensitivity of *dab1*$^{-/-}$ mice are still not well understood. Thus far, we observed an increase in the triple-labeled Reelin-Lmx1b-GRP neurons in the IB4 region and laminae I-II outer, despite the small number of these triple-labeled neurons present within the superficial dorsal horn. While we know that these triple-labeled neurons likely sustain migratory defects that contribute to the aberrant phenotype in *dab1*$^{-/-}$ mice, future analyses will examine the source of these mispositioned neurons, which most likely arise from lamina II inner ventral or the deep dorsal horn.

FIGURES

Figure 1: GRP neurons co-express Reelin in the lumbar superficial dorsal horn.

(A) A single confocal image of the dorsal horn section from a 2.5-month-old adult transgenic male *Grp* mouse. GRP expressing neurons are dispersed across laminae I-II with a few scattered GRP cells in the deep dorsal horn. (B) Merged image of GRP neurons co-labeled with Reelin. Enlargement of the box in B is shown in B1-B3; B1 depicts merged channels, B2 shows GRP only, and B3 shows Reelin-only. A black arrow marks a single Reelin cell, a white arrow marks a large single GRP cell, and the yellow arrowhead marks double-labeled Reelin-GRP cells. Orientation of the dorsal horn is medial to the left and dorsal towards the top in this and subsequent figures. Scale bars: A, B = 100 μm; B1-B3 = 5 μm.

Figure 1

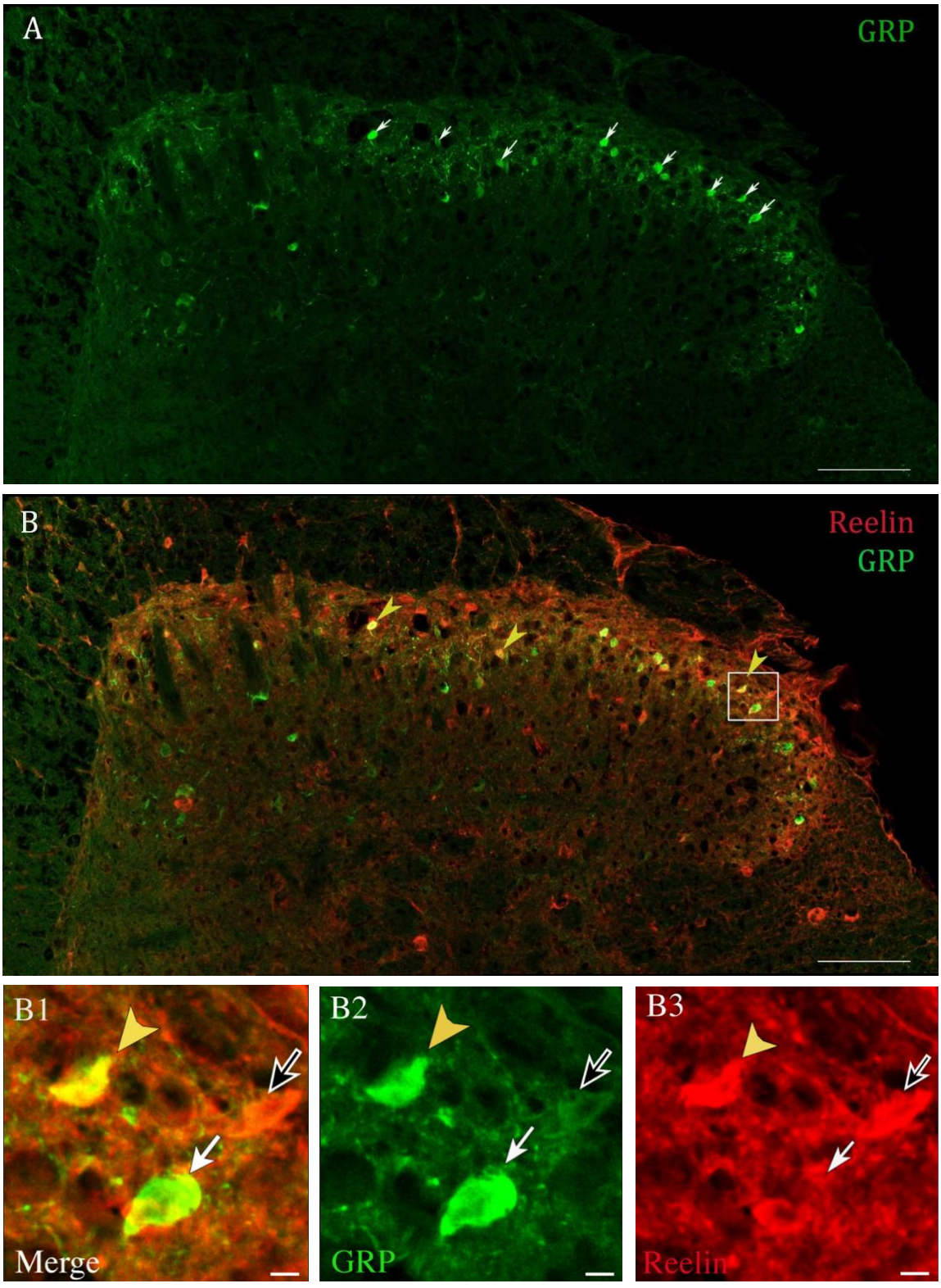


Figure 2: GRP neurons do not colocalize with Dab1 in the lumbar superficial dorsal horn.

(A) Representative confocal slice depicts the expression of Dab1 (red) and GRP (green) in a 2.5-month-old transgenic male *Grp* mouse. The boxed region in A is enlarged in A1-A3. A1 depicts a merged channel, A2 displays GRP only, and A3 shows Dab1 only. A black arrow marks a single-labeled Dab1 neuron, and a white arrow marks a large single GRP cell. (B) Merged image depicting GRP processes closely associated with Dab1 neurons taken at a magnification of 63X. Enlargement of the box in B is shown in B1. Black arrows mark single-labeled Dab1 neurons and white arrows mark GRP neurons. Scale Bars: A, B = 100 μm; A1-A3 = 5 μm; B1 = 20 μm.

Figure 2

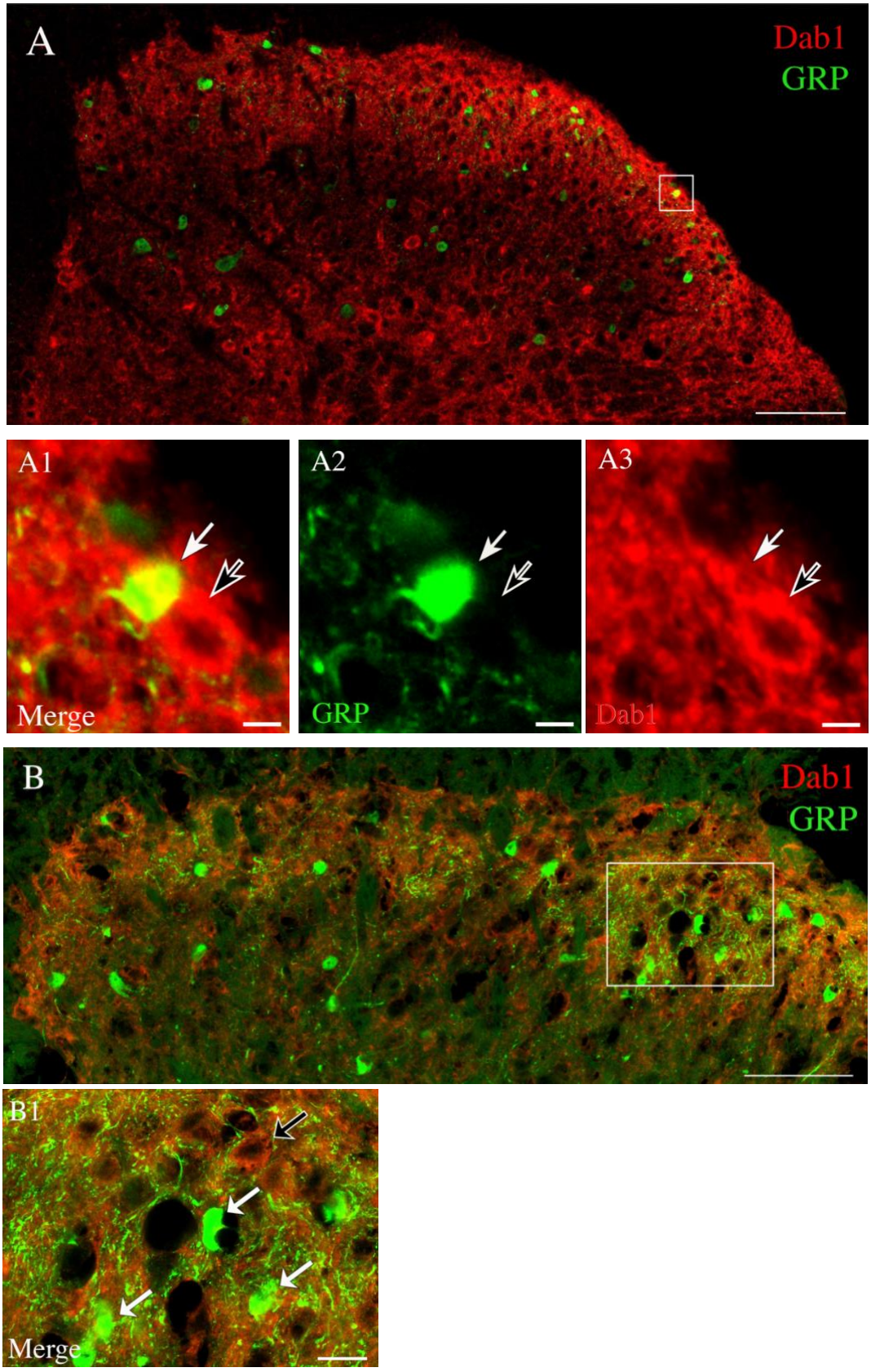


Figure 3: *Dab1*$^{+/+}$*-Grp* type mice exhibit triple-labeled neurons within the IB4 region

(A) Immunofluorescence labeling illustrates Reelin (red), GRP (green), and Lmx1b (blue) neurons within the outlined IB4 (purple) band, which marks lamina II inner dorsal, in the *Dab1*$^{+/+}$*-Grp* mouse. There are very few triple-labeled neurons in the lamina II inner dorsal *Dab1*$^{+/+}$*-Grp* mouse. The enlargement of the region boxed in A is shown in A1 with merged channels. A2 depicts GRP only, A3 depicts Reelin only, and A4 shows Lmx1b only. Yellow arrowheads indicate a triple-labeled Reelin-Lmx1b-GRP neuron. White arrowheads depict a double-labeled Reelin-Lmx1b neuron and white arrows mark single-labeled Lmx1b neurons. Scale bars: A =100 μm; A1-A4 = 5 μm.

Figure 3

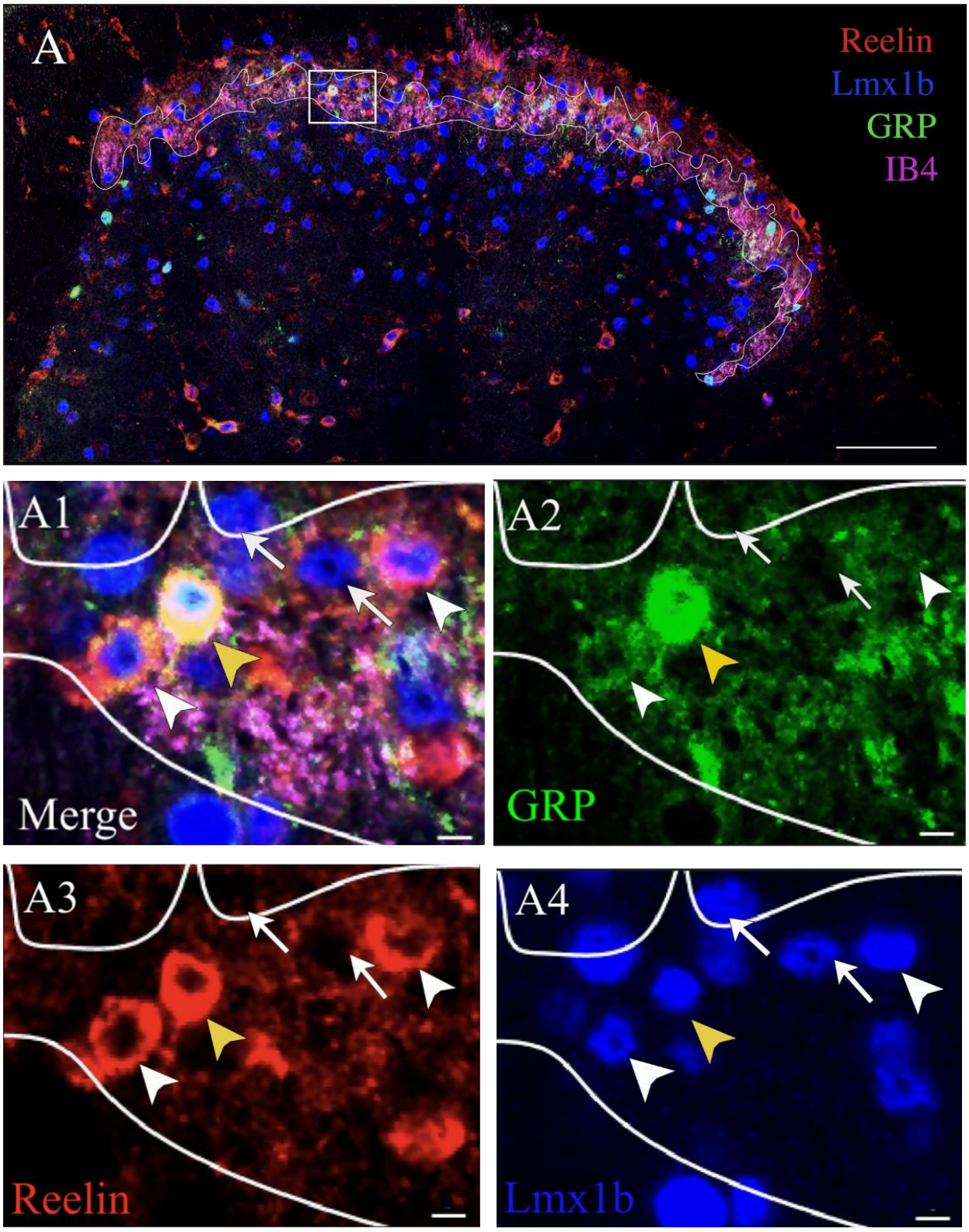


Figure 4: *Dab1*$^{-/-}$*-Grp* mice exhibit triple-labeled neurons within and above the IB4 region

(A) A single confocal slice depicting Reelin (red), GRP (green), and Lmx1b (blue) neurons within and above the outlined IB4 (purple) band in the *Dab1*$^{-/-}$*-Grp* mouse. The IB4 region appears to be displaced ventrally in the central region of the superficial dorsal horn. The enlargement of the region boxed in A is shown in A1 with merged channels. A2 depicts GRP-only, A3 depicts Reelin-only, and A4 shows Lmx1b-only. Yellow arrowheads indicate triple-labeled Reelin-Lmx1b-GRP neurons. White arrowheads depict a double-labeled Reelin-Lmx1b neuron and white arrows mark single-labeled Lmx1b neurons. Scale bars: A = 100 μm; A1-A4 = 10 μm.

Figure 4

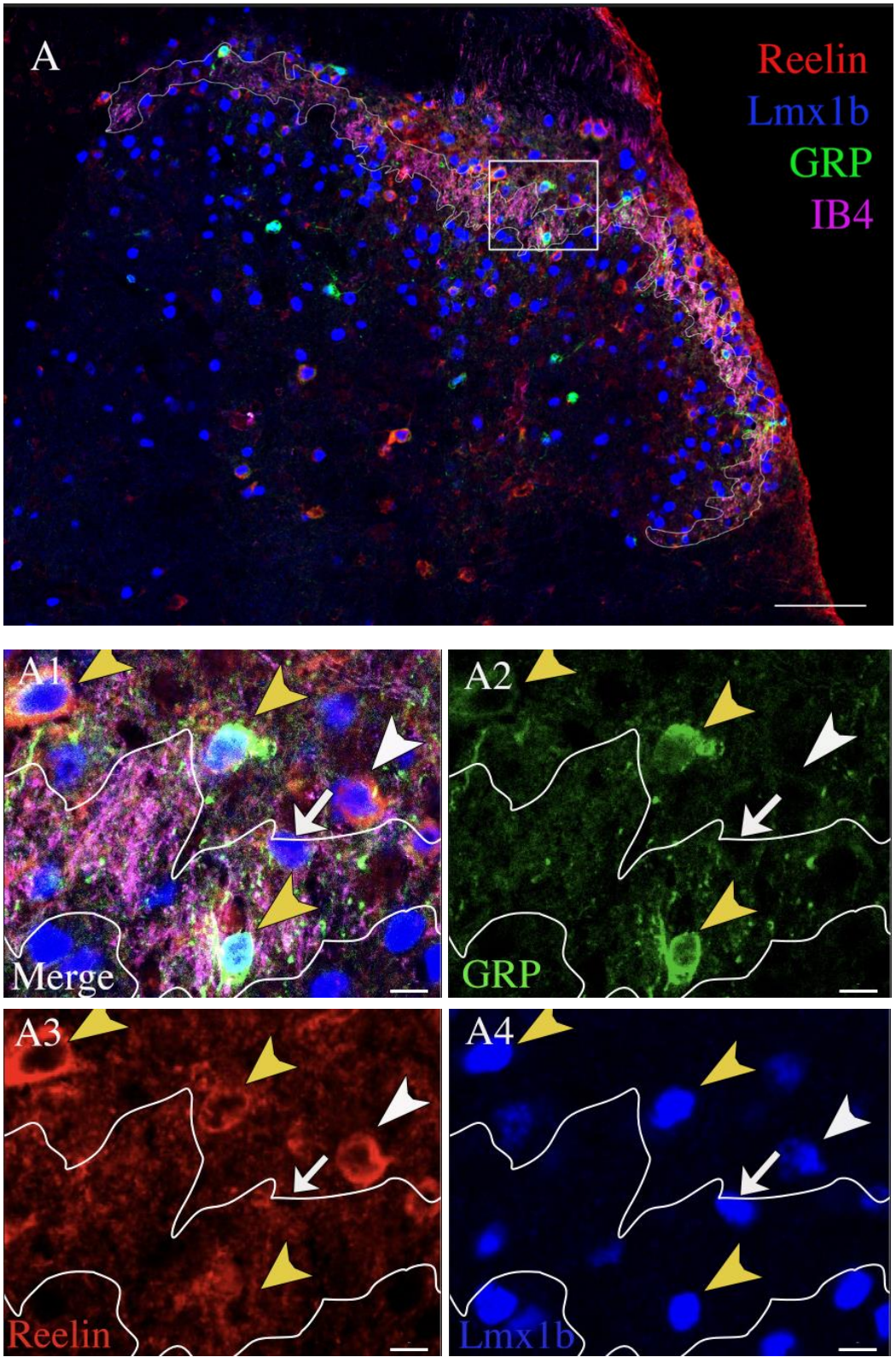
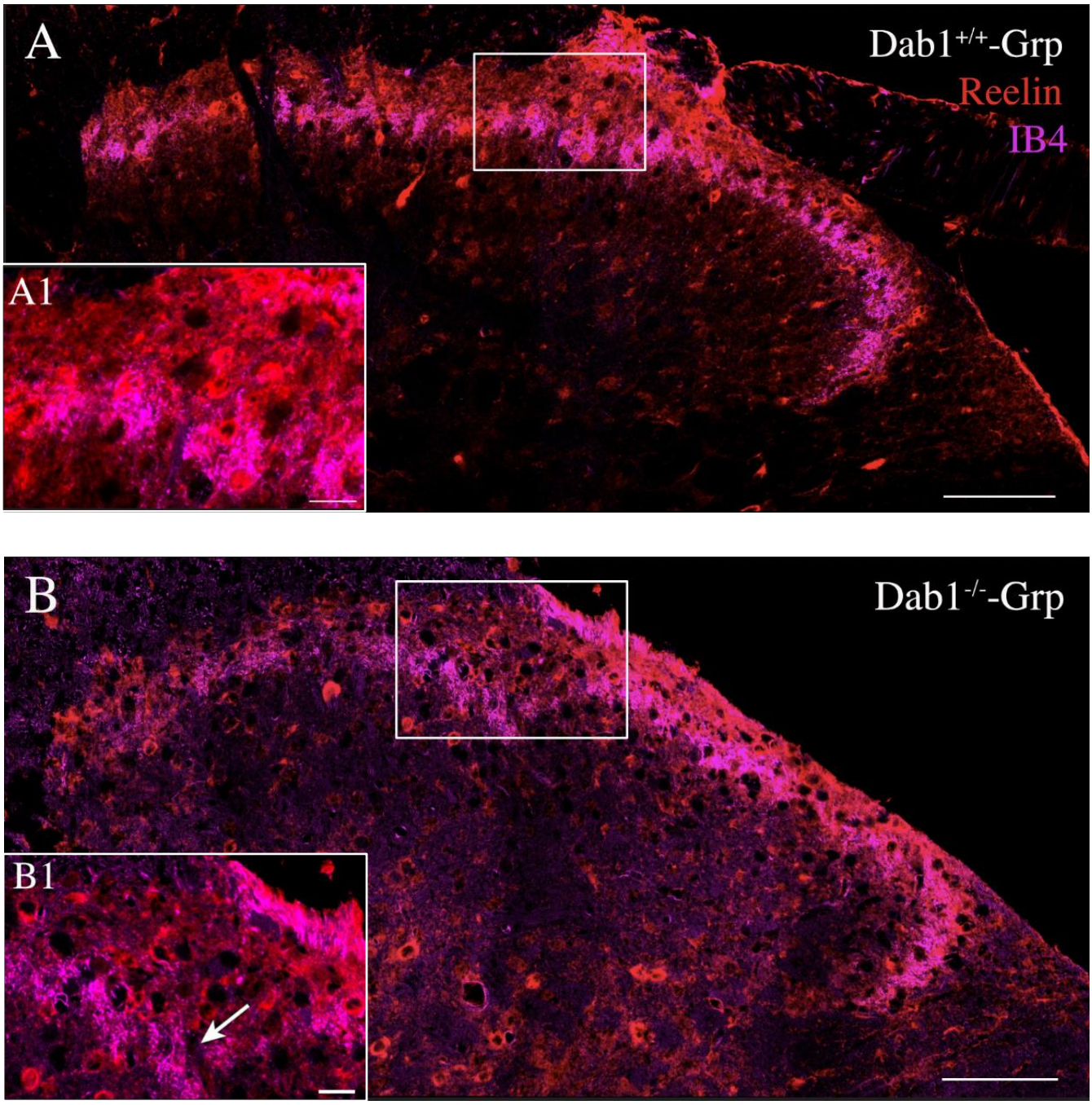


Figure 5: *Dab1*$^{-/-}$*-Grp* mice exhibit a displaced IB4 band.

(A) A *Dab1*$^{+/+}$*-Grp* mouse is labeled with Reelin (red) and IB4 (purple) band. There is a distinct, uniform distribution of IB4 terminal staining that delineates lamina II inner ventral. Box in A is enlarged in A1. (B) A hemisection depicting Reelin and IB4 from a *Dab1*$^{-/-}$*-Grp* mouse. The IB4 terminal zone does not differ in area between *Dab1*$^{+/+}$*-Grp* and *Dab1*$^{-/-}$*-Grp* mice. The area of laminae I-II outer is slightly larger in *Dab1*$^{-/-}$*-Grp* compared to *Dab1*$^{+/+}$*-Grp* mice. Figure B shows a ventral displacement of the IB4 region within the central region of the dorsal horn and a dip in the IB4 band. Enlargement of the displacement boxed in B is shown in B1 with a white arrow marking the region of the IB4 band dip. There is some variation in the shape of the displacement, but the laminae I-II outer region appears to be larger in all *Dab1*$^{-/-}$*-Grp* mice compared to *Dab1*$^{+/+}$*-Grp*. Scale bars: A-B = 100 μm; A1, B1, = 20 μm.

Figure 5



TABLES

TABLE 1: List of primary antibodies used in this study.

Primary Antisera	**Source; Catalog #**	**Host Species**	**Working Dilutions**
Reelin	R&D Systems; AF3820	Goat	1: 500 (TSA)
GRPeGFP	Aves Labs; GFP-1020	Chick	1:1000
LIM-homeobox transcription factor 1-beta (Lmx1b)	Gift from Drs. Müller and Birchmeier (Müller et al., 2002)	Guinea-Pig	1:20,000 (TSA)
Isolectin B4 ((IB4; Biotinylated Griffonia (Bandeiraea) Simplicifolia Lectin I)	Vector (Burlingame, CA); B-1205	IB4 Conjugate	1:350
Disabled-1 (Dab1)	Gift from Dr. Brian Howell (Howell et al., 1997); B3	Rabbit	1:5,000 (TSA)

TABLE 2: **Reelin-Lmx1b-GRP neurons above the IB4 region are increased in *Dab1*$^{-/-}$*-Grp* versus *Dab1*$^{+/+}$*-Grp* mice.** Means ± SEM shown per hemisection, above the IB4 layer from *Dab1*$^{+/+}$*-Grp* and *Dab1*$^{-/-}$*-Grp* mice, 5 mice/genotype, 5-6 hemisections/mouse. Analyses were carried out on a single optical section for each hemisection. *p < 0.05, **p < 0.01.

Genotype/Cell Type	*Dab1*$^{+/+}$*-GRP*	*Dab1*$^{-/-}$*-GRP*	p-value
Reelin-only	9 ± 0.6	7 ± 0.6	.04*
Lmx1b-only	40 ± 3.4	43 ± 3.4	n.s (.53)
GRP-only	0.3 ± 0.1	0.6 ± 0.1	n.s. (.21)
Reelin-GRP	0 ± 0.05	0 ± 0.05	n.s. (.10)
Reelin-Lmx1b	9 ± 0.8	9 ± 0.8	n.s. (.93)
Lmx1b-GRP	1 ± 0.4	1 ± 0.4	n.s. (.78)
Reelin-Lmx1b-GRP	0.7 ± 0.3	2 ± 0.3	.002**
Total Reelin	19 ± 1.1	18 ± 1.1	n.s (.81)
Total Lmx1b	51 ± 4.3	56 ± 4.3	n.s. (.51)
Total GRP	2.5 ± 0.5	4.1 ± 0.5	.04*
Total Cells	60 ± 4.1	63.2 ± 4.1	n.s. (.64)

TABLE 3: **Reelin-Lmx1b-GRP neurons in the IB4 region are increased in *Dab1*$^{-/-}$*-Grp* versus *Dab1*$^{+/+}$*-Grp* mice.** Means ± SEM shown per hemisection, within the IB4 layer from *Dab1*$^{+/+}$*-Grp* and *Dab1*$^{-/-}$*-Grp* mice, 5 mice/genotype, 5-6 hemisections/mouse. Analyses were carried out on a single optical section for each hemisection. *$p < 0.05$

Genotype/Cell Type	***Dab1*$^{+/+}$*-GRP***	***Dab1*$^{-/-}$*-GRP***	**p-value**
Reelin-only	3 ± 0.6	3 ± 0.6	n.s. (.84)
Lmx1b-only	20 ± 3.4	27 ± 3.4	n.s. (.17)
GRP-only	0.5 ± 0.1	0.4 ± 0.1	n.s. (.57)
Reelin-GRP	0 ± 0.05	0 ± 0.05	n.s. (.25)
Reelin-Lmx1b	4 ± 0.8	4 ± 0.8	n.s. (.69)
Lmx1b-GRP	2 ± 0.4	1 ± 0.4	n.s. (.92)
Reelin-Lmx1b-GRP	0.8 ± 0.3	2 ± 0.3	.01*
Total Reelin	7 ± 1.1	9 ± 1.1	n.s. (.36)
Total Lmx1b	27 ± 4.3	35 ± 4.3	n.s. (.23)
Total GRP	3.5 ± 0.5	3.7 ± 0.5	n.s. (.78)
Total Cells	30 ± 4.1	37± 4.1	n.s. (.22)

TABLE 4: **The mean percentage of Reelin-Lmx1b-GRP neurons out of total Reelin, total Lmx1b, and total GRP are greater in *Dab1*$^{-/-}$*-Grp* mice.** Mean percentages ± SEM shown per hemisection, within the IB4 layer from *Dab1*$^{+/+}$*-Grp* and *Dab1*$^{-/-}$*-Grp* mice, 5 mice/genotype, 5-6 hemisections/mouse. *p < 0.05, **p < 0.01, *** p< 0.001.

Genotype / Area	% Reelin-Lmx1b-GRP out of Total Reelin	p-value	% Reelin-Lmx1b-GRP out of Total Lmx1b	p-value	% Reelin-Lmx1b-GRP out of Total GRP	p-value
Dab1$^{+/+}$*-GRP*; IB4	11.7% ± 1.4%	0.0001***	3.2% ± 0.6%	0.0049**	27% ± 6.6%	0.0221**
Dab1$^{-/-}$*-GRP;* IB4	22.7 % ± 1.4%	0.0001***	6.0 ± 0.6%	0.0049**	51% ± 6.6%	0.0221**
Dab1$^{+/+}$*-GRP*; above IB4	4.1% ± 1.4%	0.0018 **	1.5 ± 0.6%	0.0114**	32% ± 6.6%	0.0361**
Dab1$^{-/-}$*-GRP;* above IB4	11.6% ± 1.4%	0.0018 **	3.8 ± 0.6%	0.0114**	54% ± 6.6%	0.0361**

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