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Neurokinin-1-Receptor Expression in the Reelin and Gastrin-Releasing Peptide Neuronal Populations

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Neurokinin-1-Receptor Expression in the Reelin and Gastrin-Releasing Peptide Neuronal Populations

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Physiological Science

by

Sherwin Atighetchi

2024

ABSTRACT OF THE THESIS

Neurokinin-1-Receptor Expression in the Reelin and Gastrin-Releasing Peptide Neuronal Populations

by

Sherwin Atighetchi

Master of Science in Physiological Science

University of California, Los Angeles, 2024

Professor Patricia Emory Phelps, Chair

Reelin signaling has a prominent role in correct neuronal positioning within the spinal cord dorsal horn. Reelin (Reln), its two lipoprotein receptors, and Disabled-1 (Dab1) are highly expressed within laminae I-II on neurons that receive itch stimulation and *dab1*$^{-/-}$ mice display reduced responses to itch-evoking stimuli compared to controls. Gastrin-releasing peptide (GRP) and Neurokinin-1 receptor (NK1R) expressing neurons reportedly function in itch signaling. To understand how mutations in *Reln* or *dab1* affect itch responses, we examined GRP- and NK1R-expressing neurons in *dab1*$^{+/+}$*;GrpeGfp* and *dab1*$^{-/-}$*-GrpeGFP* mice. We identified a few large Reelin-NK1R- and GRP-NK1R-expressing neurons within lamina I, with morphological characteristics of projection neurons in *dab1*$^{-/-}$*;Grp mice*. We also observed Reelin-NK1R-expressing neurons in *dab1*$^{-/-}$*;Grp* mice which were mispositioned between

laminae II-III. These findings, in combination with our previous reports of Dab1-NK1R-expressing neurons, suggest that the Reelin-signaling pathway influences many NK1R-expressing neurons in the superficial dorsal horn.

The thesis of Sherwin Atighetchi is approved.

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John Edward van Veen

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University of California, Los Angeles

2024

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Introduction:

Reelin (Reln), an extracellular matrix protein, binds to its receptors, Apolipoprotein E receptor 2 (Apoer-2) and Very-low-density lipoprotein receptor (Vldlr). After Reelin binds to one or both receptors, the adapter protein Disabled-1 (Dab1) is phosphorylated by Src family kinases. Dab1 phosphorylation initiates a downstream signaling cascade that, during embryonic development, affects the migration and positioning of neurons throughout the central nervous system (Bock et al., 2016). Both Reelin and Dab1 are highly expressed within laminae I-II or superficial dorsal horn of the spinal cord. Similarly, neurons receiving nociceptive input are concentrated within laminae I-II (Iggo et al. 1985).

Previous studies from our lab found that mutations within the Reln-signaling pathway of either Reln or Dab1 mice alter nociceptive processing. Compared with wild-type controls, *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mice display mechanical insensitivity and thermal hyperalgesia (Villeda et al., 2006; Akopians et al., 2008). To confirm these findings, Fos studies were conducted to evaluate neuronal activity within these mice. Following mechanical stimulation, *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mice displayed reduced Fos expression in neurons within laminae I-II and the lateral spinal nucleus compared to wild-type controls. In contrast, heat stimulation induced greater Fos expression in the superficial dorsal horn of *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mice compared to wild-type mice (Wang et al., 2012). These phenotypic findings were confirmed by anatomical studies that found positioning errors in Dab1-expressing neurons within laminae I-II, the lateral reticulated area of lamina V, and the lateral spinal nucleus (LSN), all regions related to pain processing (Villeda et al., 2006; Akopians et al., 2008; Wang et al., 2012). Yvone et al. (2017) used immunofluorescent studies to identify the coexpression of Dab1-labeled neurons with the LIM-homeobox transcription factor 1 beta (Lmx1b), a marker of excitatory glutamatergic neurons. Dab1-Lmx1b-labeled neurons were

detected in the superficial dorsal horn, the lateral reticulated area, and the LSN of *Reln*$^{+/+}$ mice. Additionally, they found fewer Dab1-only and Dab1-Lmx1b cells within *Reln*$^{+/+}$ compared to *Reln*$^{-/-}$ mice in the isolectin B4 (IB4) region of lamina II, together with a dorsal and lateral shift in the IB4 band, and a larger area of laminae I-II in *Reln*$^{+/+}$ compared to *Reln*$^{-/-}$ mice (Yvone et al. 2017). Specifically, there was a 50% reduction in Dab1-Lmx1b neurons observed in lamina II inner dorsal, the lateral reticulated area, and the LSN of the *Reln*$^{-/-}$ mice (Yvone et al. 2017). They also reported that Dab1-Lmx1b neurons in laminae I-II and the lateral reticulated area expressed Fos in response to noxious thermal or mechanical simulation and thus are involved in those nociceptive circuits (Yvone et al. 2017). In 2020, Yvone et al. reported that compared to wild-type mice, the *dab1*$^{-/-}$ mice displayed a reduction in the size of the IB4 area, in addition to more Reelin-Lmx1b neurons within the IB4 band. In summary, these studies found a number of mispositioned Dab1- and Reelin-labeled neurons in the *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mice and proposed that these positioning errors are involved in the alterations observed in their pain processing.

One group of neurons that are integral to nociceptive circuits are the Neurokinin-1 receptor (NK1R)-expressing cells. NK1R is a G-protein coupled receptor found primarily within lamina I and was shown to be a key mediator in nociception (Gautam et al. 2016). Wang et al. (2019) studied the relationship between NK1R-expressing neurons and the Reelin-signaling pathway. They determined that there were more NK1R-expressing neurons present within laminae I-II of *dab1*$^{-/-}$ mice compared to those within *dab1*$^{+/+}$ mice. In addition, some NK1R-expressing neurons also labeled for both Dab1 and Lmx1b, a finding which confirmed their excitatory phenotypes (Wang et al. 2019). Next, they found that NK1R-expressing cells were implicated in thermal hyperalgesia as these cells express Fos in response to noxious heat stimulation (Wang et al., 2019). They also selectively ablated the superficial NK1R-expressing

neurons in *dab1*$^{+/+}$ and *dab1*$^{-/-}$ mice and reported that the ablation eliminated the thermal hyperalgesia previously displayed in *dab1*$^{-/-}$ mice without changing the mechanical insensitivity (Wang et al. 2019). These findings suggest that mispositioned NK1R-expressing neurons contributed to the thermal hyperalgesia displayed by Reelin-signaling mutants, but did not affect the mechanical insensitivity.

In addition to Reelin signaling being involved in nociceptive processing, it is probably involved in pruritogenic processing. Unpublished data from our collaborators showed that *dab1*$^{-/-}$ mice display reduced scratching bouts compared to *dab1*$^{+/+}$ controls. Itch is a somatic sensation regulated via somatosensory pathways. These pathways use various peptides and receptors, such as Gastrin-Releasing Peptide (GRP), a major itch neurotransmitter that is expressed within interneurons of the superficial dorsal horn (Barry et al., 2020). The GRP receptor (GRPR) neurons found in the superficial dorsal horn are activated by GRP released by GRP-labeled neurons and may facilitate the transmission of itch within the spinal cord (Barry et al. 2018, Liu et al. 2011, Sun et al. 2007). Additionally, recent data from our laboratory confirmed that about one-third of the GRP-labeled neurons also express Reelin, but so far, none of them colocalized with Dab1-labeled neurons (Mavilian 2022).

Several investigators also suggested that NK1R-expressing neurons play a role in itch transmission (Bardoni et al. 2019; Sheahan et al. 2020). Bardoni et al. (2019) did not detect double-labeling of Gastrin Releasing Peptide Receptor-eGFP- (GRPR-eGFP) and NK1R-expressing neurons but identified synaptic contacts between the two neuronal populations using electrophysiology. Specifically, using a GRPReGFP-reporter mouse, they showed that GRPR-positive terminals contacted neurons in lamina I that also received numerous NK1R-labeled terminals (Bardoni et al. 2019). GRPR neurons found in laminae I-II are known to

be activated by GRP released by GRP-labeled neurons in order to transfer itch information from the skin to the spinal cord (Solorzano et al. 2015, Liu et al. 2011, Sun et al. 2007). This suggests that itch information is, in part, relayed from GRPR neurons to NK1R-expressing neurons, further implicating NK1R-labeled neurons in the transmission of itch.

Although Bardoni et al. (2019) reported that there were no double-labeled GRPR-eGFP-NK1R-expressing neurons, studies by Sheahan et al. (2020) showed evidence that NK1R-expressing neurons also express GRPR in the superficial and deep dorsal horn. These recent findings suggest a possible role of GRPR-NK1R-coexpressing neurons in mediating itch. In addition, intrathecal injection of GR73,632, a selective NK1R agonist, increased spontaneous scratching bouts, biting bouts, and head grooming responses compared to saline controls (Sheahan et al., 2020). By contrast, intrathecal injections of CP 99,994, an NK1R antagonist, followed by injection of chloroquine, induced fewer biting bouts at the injection site (Sheahan et al., 2020). Based on the results from these experiments, Sheahan et al. (2019) proposed a new model for itch transmission where pruritogenic stimuli on the skin causes superficial dorsal horn-neurons expressing NK1R alone, NK1R and GRPR together, or GRPR alone to synapse onto NK1R-expressing spinal projection neurons in lamina I to transmit the itch signal to the brain. Together, these findings suggest that NK1R-expressing cells may play a central role as interneurons and projection neurons in the transmission of itch.

Currently, the relationship between Reelin and NK1R in the superficial dorsal horn is unclear. In this study, we investigate whether NK1R-expressing neurons also express either Reelin or GRP. We will also determine if mutations in the Reelin-signaling pathway result in the mispositioning of Reelin-NK1R- and GRP-NK1R-expressing neurons within the superficial dorsal horn. These findings should help us better understand the role of NK1R in itch processing.

Material and Methods

Animals

dab1;GRPeGFP mice

Adult *dab1* mice (BALBcByJ-*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 the offspring were genotyped using polymerase chain reaction. *Dab1*$^{+/-}$ mice were intercrossed with *Reln*$^{+/+}$*GRPeGFP*^{trans} mice (UC Davis Mouse Biology Program, details in Mavilian, 2022) to introduce the eGFP reporter into the *dab1* wild-type and mutant mice. *dab1;GRPeGFP*^{trans} mice were carefully bred to ensure that the mice only carry one *GrpeGfp* allele. These mice will be referred to as *dab1;Grp* mice.

Tissue preparation, embedding, and storage:

Adult *dab1*-*Grp* mice were deeply anesthetized before undergoing transcardial perfusion using 4% paraformaldehyde, and the spinal cords were post-fixed for 3 hours. Spinal cords were washed in Millonig's buffer (0.12M) for three 30-minute washes and stored in Millonig's with sodium azide (0.06%). The spinal cords were then dissected and cryoprotected in 30% sucrose made in 0.12M Millonigs phosphate buffer for 2 days. Lumbar enlargement segments were blocked and frozen in Optimum Cutting Temperature (Sakura; REF4583; Torrance, CA) and stored at -80°C until they were sectioned (25-micron thick) using a cryostat and stored in Millonigs buffer with sodium azide (0.06%). Sections were collected at the lower lumbar level (L4-L5).

Immunohistochemical Procedures:

Reelin and *Grp*-positive cells were identified in 25 μm-sections. Initially, Reelin immunofluorescence was carried out using a Tyramide Signal Amplification (TSA) protocol. Free-floating sections were washed in PBS buffer (0.1 M PB; 0.9% NaCl) and then blocked for one hour in 3% normal goat serum in PBS. This was followed by placing the sections in an Avidin-Biotin mixture (1:1; Avidin/Biotin Blocking Kit; ZK1120; Vector Laboratories; Newark, CA). Sections were then incubated overnight with goat anti-Reelin (1:1000; R&D Systems; AF3820; Minneapolis, MN), followed by repeated PBS washes the next day and a 1-hour incubation in biotinylated horse anti-goat IgG (1:800; TSA kit PerkinElmer #NEL745001KT; Shelton, CT). Next, we carried out multiple TNT buffer washes (0.1M Tris-HCl; 0.15M NaCl; 5% Tween) and a 1-hour incubation in streptavidin-conjugated horseradish peroxidase (1:1,000; PerkinElmer #NEL750001EA; Shelton, CT), followed by a 10-minute incubation in Cyanine 5 (Cy5; 1:100; PerkinElmer #NEL745001KT).

NK1R-expressing cells also were labeled using TSA immunohistochemistry. Free-floating sections were washed using TNT buffer and incubated in 1% hydrogen peroxide and 0.1% sodium azide in TNT for 30 minutes. Sections were washed in TNT buffer 3 times and then blocked in TNB followed by Avidin-Biotin. The tissues were incubated overnight in 1:30k Rabbit anti-NK1R (Sigma-Aldrich #S8305; Burlington, MA) in TNB with sequential washes with TNT before being incubated for an hour in donkey anti-rabbit biotin IgG (1:2,000; Jackson ImmunoResearch #131559; Philadelphia, PA) in TNB. Two washes in TNT preceded a 1-hour incubation in streptavidin-conjugated horseradish peroxidase (1:2,000; PerkinElmer #NEL750001EA) and TNB. Sections were incubated in Cyanine 3 (Cy3; 1:150; PerkinElmer #NEL744001KT) for 10 minutes to visualize cells.

Finally, to identify GRP-labeled neurons, sections were washed using 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) before an overnight incubation in chick anti-GFP (1:1000; Aves Labs; GFP-1020; Davis, CA). The next day, sections were washed in PBS, followed by an hour incubation in donkey anti-chick Alexa Fluor 488 (1:500; Invitrogen; 2330673; Eugene, OR) for fluorescence. Each section was mounted onto a glass slide and coverslipped with ProLong Diamond antifade mounting medium before being stored in a 4℃ refrigerator prior to imaging.

Confocal Imaging:

Confocal images of the superficial dorsal horn were obtained using a Zeiss Laser Scanning Microscope (LSM880), with 488 nm, 594 nm, and 640 nm lasers. A 4x2 tile was used to surround the entire superficial dorsal horn, with acquisition parameters of frame size 1024x1024 pixels and 16 bits per pixel. To optimize cell counting, z-stacks were obtained at a confocal slice ~0.6 µm thick. Confocal images were taken at high magnification using a scan speed of 4-5, the pinhole aperture was set at 1 Airy Unit, and a 40x water immersion lens (numerical aperture 1.4) was used. Statistical analysis of the 40X images were performed using the ZEN (Zeiss Efficient Navigation) lite (v. 2.6) imaging software.

Experimental design, cell counts, and analysis:

There were 6 *dab1*$^{+/+}$*;Grp* and 6 *dab1*$^{-/-}$*;Grp* mice studied. 3 to 6 hemisections per mouse were analyzed, and in total, 50 hemisections were imaged on the confocal. The neurons in these hemisections were then counted and averaged per animal. The 40x confocal images were

analyzed on the ZEN lite imaging software. Laminae I-II (superficial dorsal horn) of each section was outlined, followed by cell counts from the optical slice with the most intense labeling. By scrolling through 0.6 micron z-stacks above and below the reference slice, cells within the reference Z-stack were confirmed. NK1R-expressing neurons were characterized as Reelin- or GRP-positive neurons if they had NK1R expression surrounding the majority of the neuron. Data from the confocal images were analyzed using a 2x6 (genotype x neuronal type) ANOVA statistical analysis with JMP Pro 18 (Cary, NC). The mean per mouse ± the standard error of the mean was determined and then the mean per neuronal type was calculated.

Results

Subsets of NK1R-expressing neurons found in laminae I-II of *dab1*$^{+/+}$*;Grp* and *dab1*$^{-/-}$*;Grp* mouse spinal cords

Unpublished data from our collaboraters showed that *dab1*$^{-/-}$ mice have significantly reduced scratching bouts compared to wild-type mice, and previous studies reported that NK1R-expressing neurons have a role in itch transmission (Bardoni et al. 2019; Sheahan et al. 2020). In addition, some Dab1-labeled neurons were found to express NK1Rs and to be mispositioned in *dab1*$^{-/-}$ compared to *dab1*$^{+/+}$ mice (Wang et al. 2019). Here, we asked if Reelin or GRPeGFP immunoreactive neurons also expressed NK1Rs and if the loss of the Reelin-signaling pathway influences the position of these neurons. Based on analysis of lumbar spinal cord sections, we found Reelin-NK1R-expressing neurons within laminae I and II of both *dab1*$^{+/+}$*;Grp* (Fig. 1A-A4, yellow arrowhead) and *dab1*$^{-/-}$*;Grp* (Fig. 2A, A1, A4, white arrow) mice. In Fig. 2A1, there is a Reelin-NK1R-expressing neuron along the medial edge of the dorsal horn that resembles previously described NK1R projection neurons (Todd et al. 2000; Todd et al. 2005) but notably appears mispositioned medially rather than within lamina I. Evidence of GRP-labeled neurons that express NK1R was also detected in the superficial dorsal horn in both *dab1*$^{+/+}$*;Grp* (Fig. 1, A3-4) and *dab1*$^{-/-}$*;Grp* (Fig. 2A4-5, Fig. 3A1, A4) mice. An NK1R-expressing lamina I neuron has the characteristic NK1Rs around its cell body and processes and is clearly labeled with Gfp (Fig. 3A1, A4).

Subsets of NK1R-expressing neurons are not differentially expressed in *dab1*$^{+/+}$*;Grp* and *dab1*$^{-/-}$*;Grp* mice within laminae I-II

Additional unpublished data from our lab found that Reelin-Lmx1b-GRP neurons are mispositioned within the Isolectin-B4 region (IB4 marks lamina II inner dorsal) of the *dab1*$^{-/-}$*;Grp* mouse lumbar spinal cord compared to their wild-type counterparts (Mavilian, 2022). Based on this finding, we asked whether GRP- or Reelin-GRP-labeled neurons also expressed NK1Rs and, if so, whether these neurons would be mispositioned in Dab1 mutant mice. For these analyses we triple-labeled spinal cord sections for Reelin, NK1R, and GRP from *dab1-Grp* mice. In total, 6 pairs of mice (6 *dab1*$^{+/+}$*;Grp* and 6 *dab1*$^{-/-}$*;Grp*) were used for the study and 3-6 hemisections were analyzed per mouse. We detected GRP-NK1R-expressing neurons in the superficial dorsal horn in wild-type (Fig. 1A-A4, white arrowhead) and mutant mice (Fig. 2A-A4, red arrowhead). Figure 2A1 depicts a large lamina I NK1R-projection-like neuron that expresses GRP in a *dab1*$^{-/-}$*;Grp* mouse. The number of both laminae I-II Reelin-NK1R- (Figs. 1, 2, 3, Table 2) and GRP-NK1R-expressing (Figs. 1, 2, 3, Table 2) neurons did not differ between genotypes. Although we did not detect significant differences in the number of these neurons between genotypes, we did show that ~6% of Reelin neurons in the superficial dorsal horn express NK1Rs in wild-type mice versus ~9.7% of Reelin neurons in mutant mice. We observed a total of 5 Reelin-GRP-NK1R-expressing triple-labeled neurons within the superficial dorsal horn in *dab1*$^{-/-}$*;Grp* mutant mice (Fig. 4A-A2, white arrow). These neurons were not detected within the laminae I-II of our wild-type mouse group, but due to their limited number, they did not differ between genotypes (Fig. 4A-A2, Table 2). Furthermore, no significant changes in the number of total Reelin (Figs. 1, 2, 3, Table 2), total GRP (Figs. 1, 3, Table 2), or Reelin-GRP (Fig. 3, Table 2) neurons were observed between genotypes.

Reelin-NK1R and GRP-NK1R-expressing neurons are found in the deep dorsal horn or lateral spinal nucleus (LSN)

To better understand the distribution of Reelin-NK1R- and GRP-NK1R-expressing neurons, we also examined these neuronal populations in the deep dorsal horn and LSN. In *dab1*$^{+/+}$*;Grp* mice, Reelin-NK1R-expressing neurons were identified within the LSN (Fig. 5B-B1, yellow arrow), whereas in *dab1*$^{-/-}$*;Grp* mice, they were also found near the laminae II-III border (Fig. 2A, A5, white arrow) and in lamina V (Fig. 5B-B1, white arrow) of the spinal cord. Similarly, GRP-NK1R-expressing neurons were found in lamina V in *dab1*$^{-/-}$*;Grp* mice (Fig. 3A, A3, white arrowhead).

GRP neurons are in close contact with NK1Rs

Results from several studies suggested that GRPR-expressing neurons respond to GRP release by GRP-expressing neurons via synaptic connections between the two neuronal populations (Barry et al. 2018, Liu et al. 2011, Sun et al. 2007). Previously, Sheahan et al. (2020) proposed that a subset of spinal dorsal horn NK1R-expressing neurons also expressed Gastrin-Releasing-Peptide Receptor (GRPR). By using dual in-situ hybridization, they determined that ~48% of NK1R-expressing neurons of the superficial dorsal horn expressed GRPR (Sheahan et al. 2020). To further validate their results Sheahan et al. (2020) performed Ca^{2+} imaging of excitatory laminae I-II neurons in the presence of tetrodotoxin and measured Ca^{2+} transients in response to Substance P and GRP. They determined that 12.3% of excitatory superficial dorsal horn neurons were responsive to both Substance P and GRP. Together, these findings indicate that a subset of NK1R-positive neurons express GRPR and are responsive to

GRP. When analyzing our results, we observed that NK1Rs were occasionally in close contact with GRP-expressing neurons within the superficial dorsal horn in both our *dab1*$^{+/+}$*;Grp* (Fig. 6A-A2, yellow arrow, Fig. 6B-B2, white arrowhead) and *dab1*$^{-/-}$*;Grp* (Fig. 6B-B2, yellow arrow and white arrowhead) mice. Figure 3 also depicts a GRP-positive neuron near a lamina III Reelin-NK1R-expressing neuron found in our *dab1*$^{-/-}$*;Grp* mice (Fig. 3A, A5, yellow arrowhead). In our study, we observed these GRP-positive neurons in close contact with NK1Rs across multiple sections from both genotypes of mice.

Discussion

Although the role of NK1R-expressing neurons is well-studied in nociceptive processing, much less is known about the role of NK1R-expressing neurons in processing itch-evoking stimuli. Here, we identified a few Reelin- and GRP-positive neurons that express NK1Rs within the lumbar superficial dorsal horn, and found examples of these neurons that have characteristics of large projection neurons. We also found that these Reelin-NK1R- and GRP-NK1R-expressing neurons were present in both *dab1*$^{+/+}$*;Grp* and *dab1*$^{-/-}$*;Grp* mice, although their expression did not differ between genotypes. In addition, we confirmed that the overall counts of total Reelin, total GRP, and Reelin-GRP neurons found in laminae I-II did not differ between genotypes. Notably, triple-labeled Reelin-GRP-NK1R-expressing neurons were rare and only observed in *dab1*$^{-/-}$*;Grp* mice. A few Reelin-NK1R- and GRP-NK1R-expressing neurons were also present in the deep dorsal horn. Finally, GRP-positive neurons appeared to be in close contact with NK1R-expressing processes in both wildtype and mutant *dab1;Grp* mice.

A small subset of Reelin-labeled neurons express NK1Rs in both *dab1*$^{+/+}$*;Grp* and *dab1*$^{-/-}$*;Grp* mice

Our previous work reported the presence of Dab1-NK1R-expressing neurons within the superficial dorsal horn and that both *Reln*$^{-/-}$ and *dab1*$^{-/-}$ mice had more neurons expressing NK1Rs within laminae I-II than their wild-type counterparts (Wang et al., 2019). In the current study, we noticed only a trend of more Reelin neurons expressing NK1Rs in *dab1*$^{+/+}$*;Grp* compared to *dab1*$^{-/-}$*;Grp* mice (6% versus 9.7% of Reelin neurons express NK1Rs). Although not significant, these findings align with those of Wang et al. (2019) that found more NK1R-expressing neurons in laminae I-II of *dab1*$^{-/-}$ than in *dab1*$^{+/+}$ mice. Perhaps some of the

mispositioned NK1R-expressing neurons found in *dab1*$^{-/-}$ mice (Wang et al. 2019) may be Reelin-positive, although further testing would need to be conducted to confirm this.

Large lamina I NK1R-expressing projection neurons were reported previously within the lumber spinal cord of rats (Todd et al. 2000; Todd et al. 2002; Todd et al. 2005; Todd 2010). These neurons were similar in shape and size to the NK1R-expressing neurons observed within our mice along the rim of lamina I and were also present in both *dab1*$^{+/+}$*;Grp* and *dab1*$^{-/-}$*;Grp* mice. Interestingly, we found that some of these large lamina I NK1R-positive neurons expressed Reelin while others were reported to express Dab1 (Wang et al. 2019). Together, these findings indicate that some of the large NK1R-projection neurons present in lamina I are under the influence of the Reelin-signaling pathway. Future tests should confirm if these are independent subsets of large projection neurons.

Our previous studies have demonstrated that 90% of Reelin-labeled neurons in laminae I-II coexpress LIM-homeobox transcription factor 1-beta (Lmx1b) and confirmed that most Reelin-expressing neurons have a glutamatergic phenotype (Yvone et al. 2020). In addition, Reelin-Lmx1b cells constitute a separate neuronal population from previously reported Dab1-Lmx1b cells. Yvone et al. (2020) also determined that Reelin-Lmx1b neurons were mispositioned in laminae I-II inner dorsal of *dab1* mutant mice as more Reelin-Lmx1b neurons were found in this area than in *dab1*$^{+/+}$ mice. These findings, along with our observation of large lamina I Reelin-NK1R-expressing projection neurons, suggest that the Reelin-NK1R-expressing neurons most likely have a glutamatergic phenotype. To confirm this and determine if these Reelin-NK1R-expressing neurons may be similarly mispositioned in our *dab1*$^{-/-}$*;Grp* mouse line, future experiments are needed.

Reelin-GRP-expressing neurons are correctly positioned in laminae I-II

Unpublished findings (Mavilian 2022) from our lab reported that the number of total Reelin, total GRP, and Reelin-GRP neurons in the superficial dorsal horn were similar between both wildtype and mutant *dab1;Grp* mice. We have confirmed these findings and further determined that Reelin-NK1R and GRP-NK1R-expressing neurons also did not differ between genotypes. In the unpublished study cited above, total GRP neurons were greater in *dab1*$^{-/-}$*;Grp* than in *dab1*$^{+/+}$*;Grp* mice when analyzing the region laminae I-II outer, or the area above the IB4 band. Because NK1R-expressing neurons are found primarily within lamina I of the lumbar spinal cord (Gautam et al. 2016), future experiments could investigate whether Reelin-NK1R- and GRP-NK1R-expressing neurons are mispositioned in *dab1*$^{-/-}$*;Grp* mice above the IB4 band compared to the wildtype controls.

***dab1;Grp* mutant mice have a few mispositioned NK1R-expressing neurons near the lamina II-III border.**

Villeda et al. (2006) reported mispositioned Dab1-NK1R- expressing neurons between lamina II and lamina III in *Reln*$^{-/-}$ mice, and Akopians et al. (2008) found similarly located NK1R-expressing neurons in *dab1*$^{-/-}$ and *Scrambler* (Dab1 hypomorph) mice. Wang et al. (2019) also showed that more Dab1-NK1R-expressing neurons were detected in *Reln*$^{-/-}$ than in *Reln*$^{+/+}$ mice and that some of these mispositioned neurons expressed Fos after heat stimulation. Here, we report the presence of a few Reelin-NK1R-expressing neurons between lamina II and III in our *dab1*$^{-/-}$*;Grp* mice and note that they resemble misplaced Dab1-NK1R-expressing neurons in *Reln*$^{-/-}$ along with those neurons reported by Villeda et al. (2006) and Akopians et al. (2008). This

finding suggests a small number of Reelin-NK1R- and Dab1-NK1R-expressing cells are mispositioned in *dab1* and *Reln* mutants, respectively.

NK1R-positive processes may contact GRP-positive neurons

It is unclear, based on the literature, whether or not NK1R-expressing neurons may also express Gastrin Releasing Peptide Receptor (GRPR). Previously, Bardoni et al. (2019) did not detect double-labeling of GRPR-positive and NK1R-expressing neurons, although they did show physiological evidence of synaptic contacts between these two populations. In contrast, Sheahan et al. (2020) suggested that NK1R and GRPR were coexpressed within a subset of superficial dorsal horn neurons. They were able to show that in the superficial dorsal horn 48% of *Tacr1*-labeled cells (gene encoding NK1R), coexpressed *Grpr* by using double in-situ hybridization. In our study, we observed possible interactions between NK1Rs and GRP-positive neurons within the superficial dorsal horn. Reportedly, GRPR-expressing neurons are only expressed within the superficial dorsal horn, where they directly respond to GRP released by GRP neurons (Sun et al. 2007; Bardoni et al. 2019; Sheahan et al. 2020). Therefore, the presence of possible interactions between NK1Rs and GRP-positive neurons would warrant further investigation.

In this study we have established that a subset of large Reelin-NK1R-expressing neurons are present within laminae I-II of *dab1;Grp* mice. We also identified Reelin-NK1R-expressing neurons mispositioned between laminae II and III in *dab1*$^{-/-}$*;Grp* mice, which resemble previously reported NK1R-expressing neurons in Reelin-signaling pathway mutants. Together

with our previous findings on Dab1-NK1R-expressing neurons, the correct positioning of many NK1R-expressing neurons appears to be controlled by the Reelin-signaling pathway.

Figures + Tables

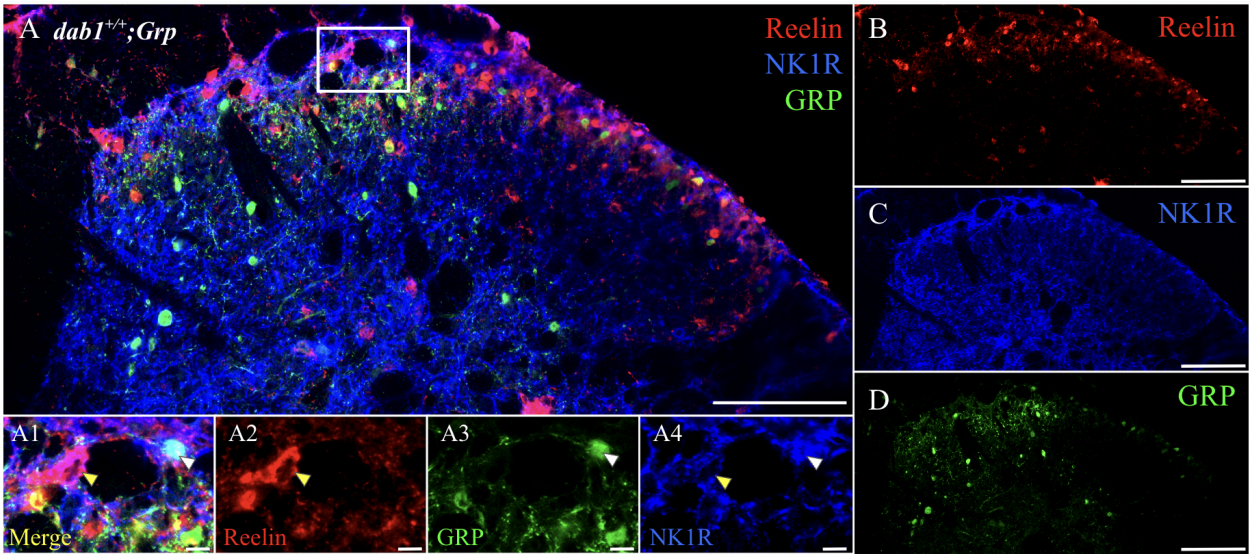

Figure 1: Neurokinin-1 receptors surround Reelin or Gastrin-releasing peptide-expressing neurons in laminae I-II of a *dab1*$^{+/+}$*;Grp* mouse.

Spinal cord midline is oriented to the left, and dorsal is toward the top in this and subsequent figures. (A) Merged confocal slice of a lumbar dorsal horn hemisection from an adult female *dab1*$^{+/+}$*;Grp* mouse labeled with anti-Reelin (red), anti-GRPeGFP (green), and anti-NK1R (blue). Single channel images show Reelin- (B), NK1R- (C), and GRP-expression (D) in laminae I-II. A1-A4 depicts an enlargement of the box in A. A1 shows all three channels merged: GRP-NK1R-expressing neuron (white arrowhead) and a Reelin-NK1R-expressing neuron (yellow arrowhead). Single channel of Reelin-labeled cells (A2, yellow arrowhead), GRP-labeled (A3, white arrowhead), and NK1R-expressing cells (A4, white and yellow arrowheads). Scale bars: A-D= 100µm; A1-A4 =10µm.

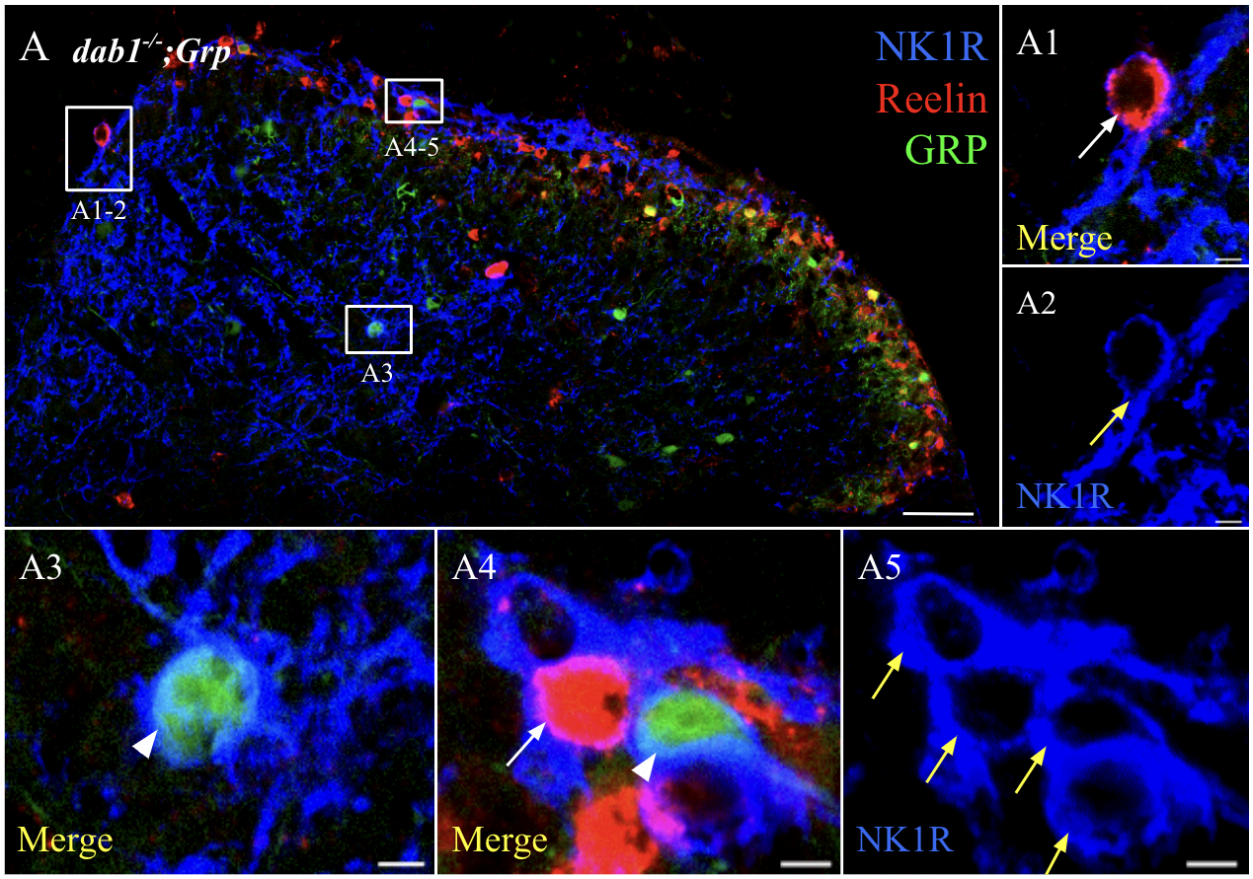

Figure 2: Large Reelin-NK1R-expressing neuron and a rare GRP-NK1R-expressing neuron found in lamina I and V of a *dab1*$^{-/-}$*;Grp* mouse.

(A) A merged confocal slice of a dorsal horn hemisection from an adult male *dab1*$^{-/-}$*;Grp* mouse. Reelin (red), GRPeGFP (green), and NK1R (blue) are all expressed, primarily in the superficial dorsal horn. Enlargement of box A1-2 illustrates a large Reelin-NK1R-expressing neuron (white arrow) along the medial dorsal horn. A2 Single channel of A1 showing the region of high NK1R immunoreactivity (yellow arrow). A3 GRP-NK1R-expressing neuron (white arrowhead) found in the deep dorsal horn of a mutant mouse. A4 Lamina I Reelin (white arrow) and GRP (white arrowhead) neurons both express NK1Rs and are adjacent to each other. A5 single channel of A4 depicts regions of high NK1R expression (yellow arrows) of cell profiles within lamina I. Scale bars: A = 100µm; A1-A5 = 10µm.

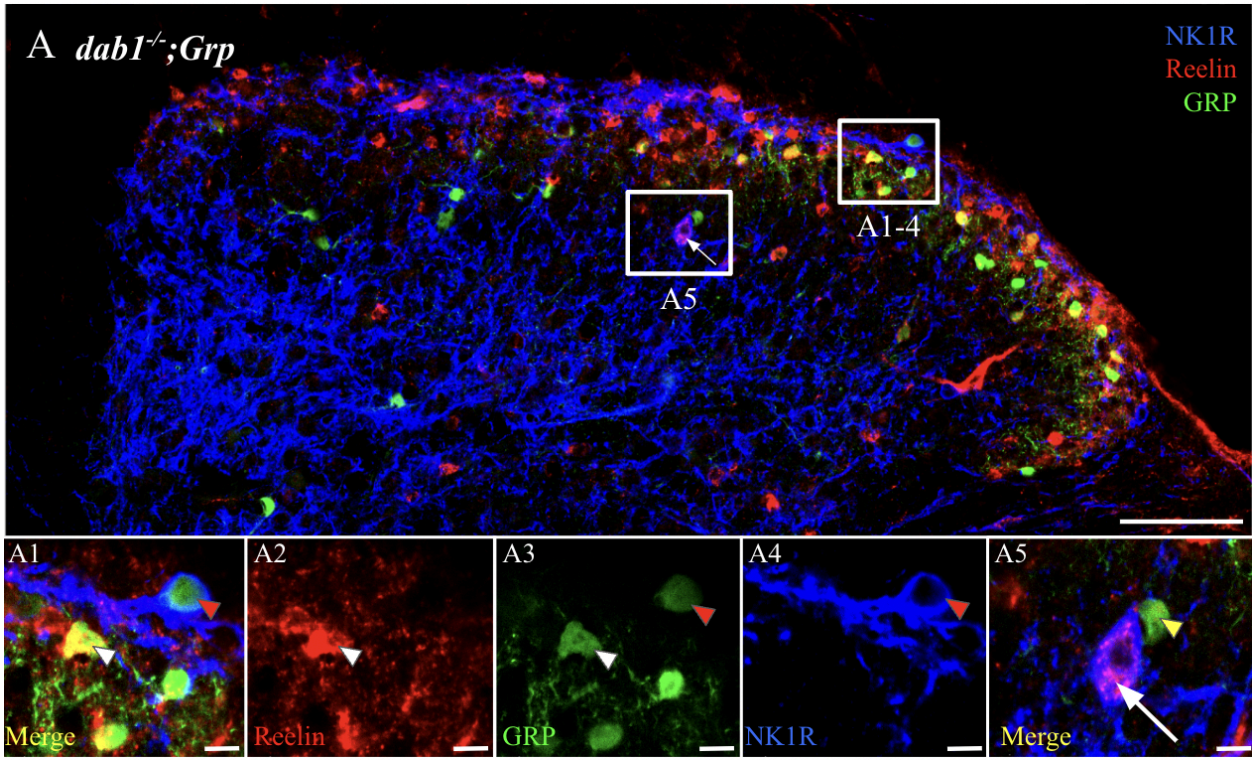

Figure 3: Neurokinin-1 receptors (NK1Rs) are expressed in Reelin and GRP cells of laminae I-II, and a few are found in the deep dorsal horn from a *dab1*$^{-/-}$*;Grp* mouse.

(A) Confocal slice of a dorsal horn section from an adult female, *dab1*$^{-/-}$*;Grp* mouse shows high expression of Reelin (red), GRPeGFP (green), and NK1R (blue) immunoreactivity in laminae I-II. Box in A is enlarged in A1-A4. A double-labeled Reelin-GRP neuron (A1-3, white arrowhead) was found near a GRP-positive cell surrounded by NK1Rs (red arrowhead in A1, 3-4). A2-4 shows the single channel of Reelin (white arrowhead), GRPeGFP-labeled cells (red and white arrowheads), and an NK1R-expressing neuron (red arrowhead) with characteristics of NK1R projection neurons in lamina I. A5 shows a mispositioned Reelin-NK1R (white arrow) neuron found near the laminae II-III border near a GRP-positive neuron (yellow arrowhead). Scale bars: A = 100µm; A1-A5 = 10µm.

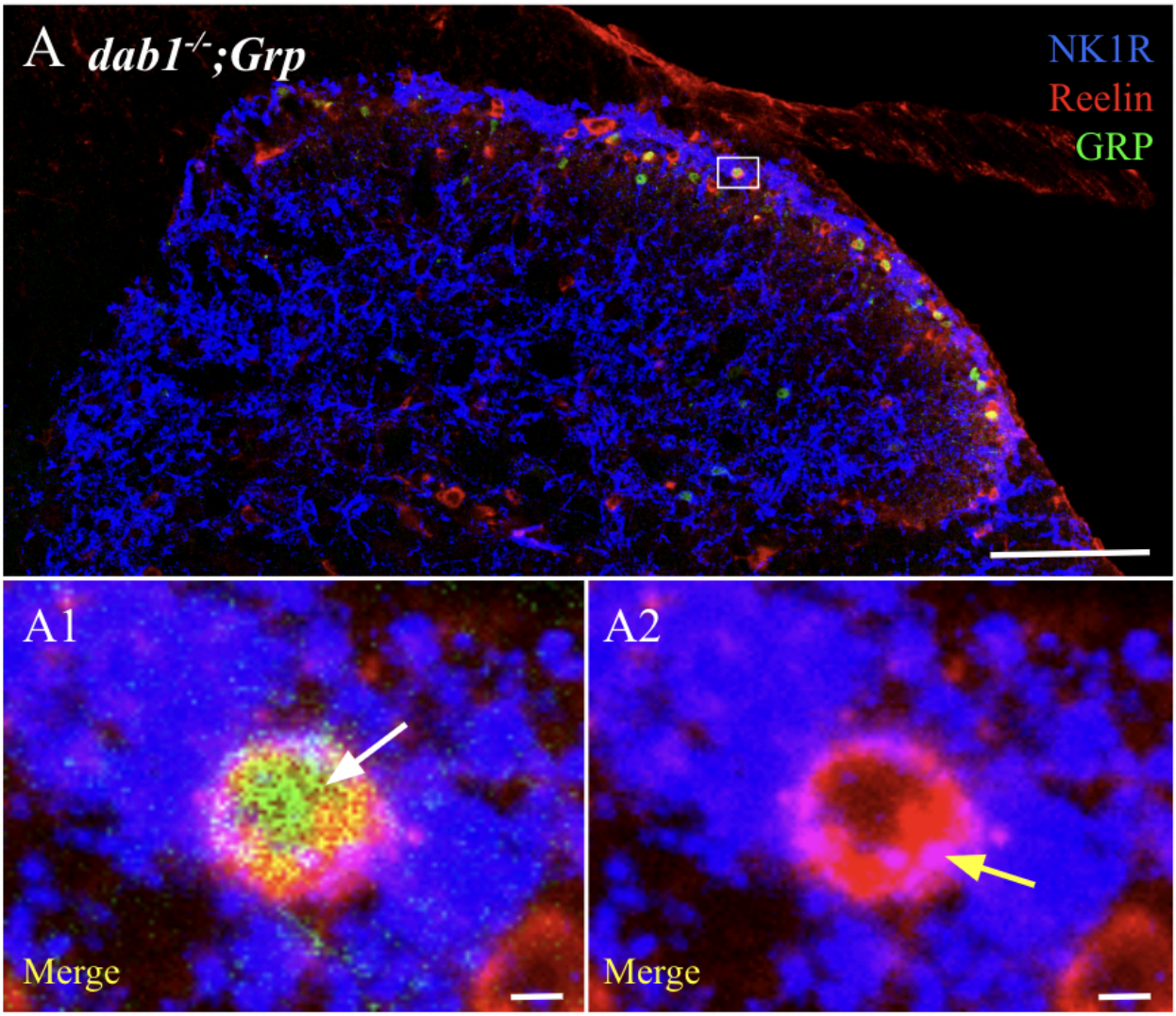

Figure 4: A rare Reelin-GRP-NK1R-coexpressing neuron in laminae I-II of a *dab1*$^{-/-}$*;Grp* mouse.

(A) A 0.6µm thick confocal slice of a coronal dorsal horn section from an adult male, *dab1;Grp* mutant mouse shows a Reelin-GRP-NK1R-expressing triple-labeled neuron. The box in A is enlarged in A1-2. A1 depicts a Reelin-GRP-NK1R-expressing cell (white arrow). A2 shows the same cell but with GRPeGFP removed to better visualize the Reelin-labeled neuron surrounded by the expression NK1Rs (yellow arrow). Scale bars: A = 100µm; A1-A2 = 10µm.

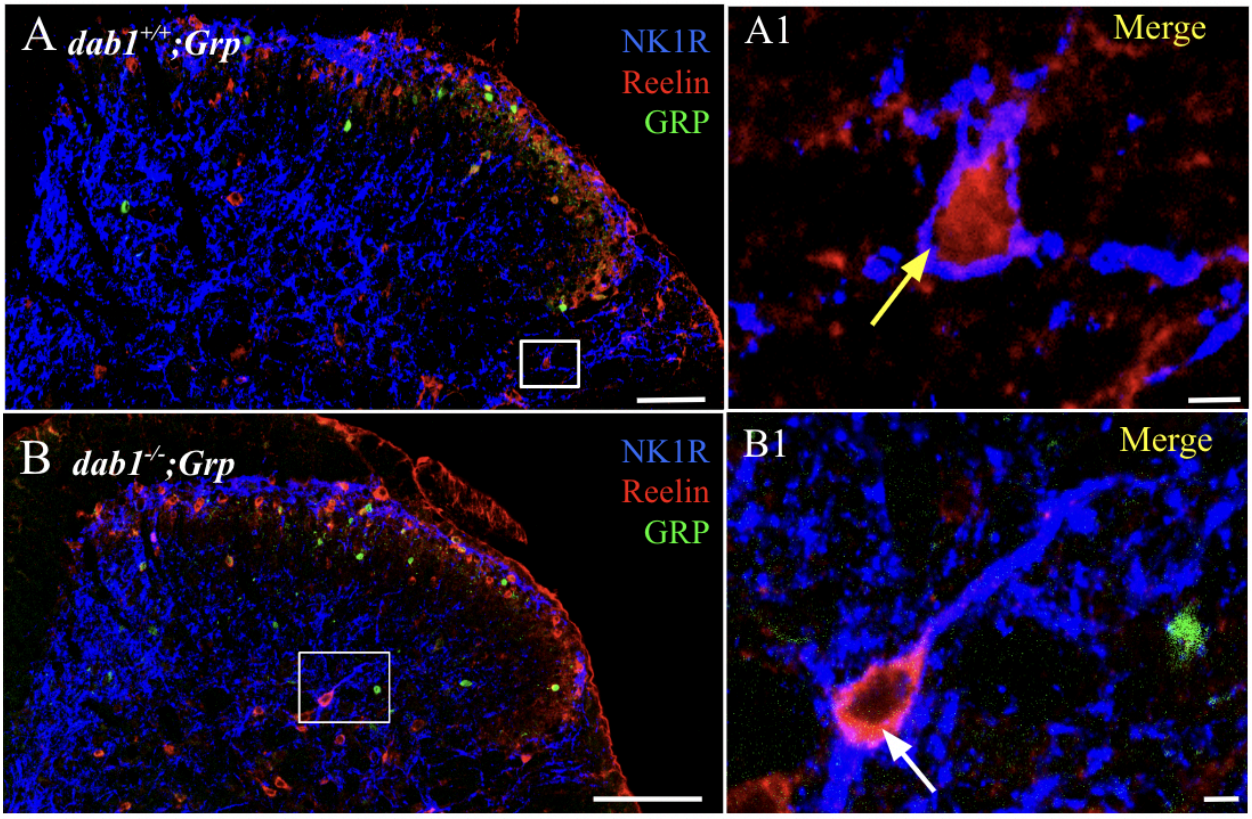

Figure 5: Reelin-NK1R-expressing neurons found in the lateral spinal nucleus of *dab1*$^{+/+}$*;Grp* and in lamina V of a *dab1*$^{-/-}$*;Grp* mouse.

(A) A confocal slice of a dorsal horn section from an adult male *dab1*$^{+/+}$*;Grp* mouse shows expression of Reelin (red), GRP-GFP (green), and NK1Rs (blue). A1 shows an enlargement of the box in A. A1 is the merged channel with a large Reelin-labeled neuron in the lateral spinal nucleus of the spinal cord that is surrounded by NK1Rs (yellow arrow). (B) A confocal slice of the dorsal horn of a *dab1*$^{-/-}$*;Grp* mouse showing a large lamina V Reelin-NK1R-expressing cell. An enlargement of the box in B is shown in B1. The white arrow marks what is a large Reelin-NK1R neuron in lamina V that may be out of position. Scale bars: A = 100µm; A1-A2 = 10µm.

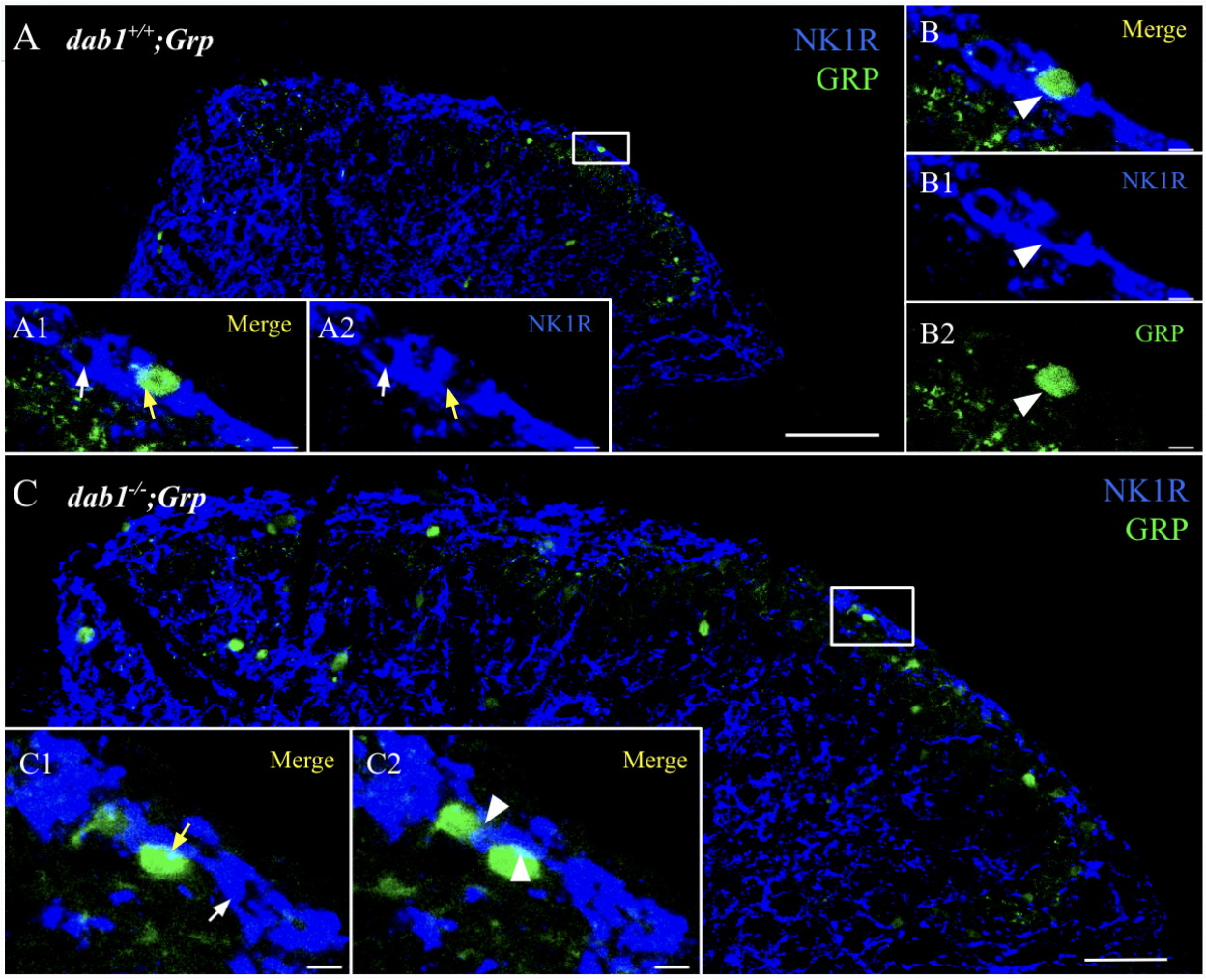

Figure 6: GRP-positive neurons in lamina I are surrounded by NK1Rs in both *dab1*$^{+/+}$*;Grp* and *dab1*$^{-/-}$*;Grp* mice.

(A) Confocal slice of a dorsal horn section from an adult male *dab1*$^{+/+}$*;Grp* mouse depicts GRPeGFP (green) and NK1R (blue) immunoreactivity along the outer rim of the superficial dorsal horn. A1-A2 Enlargement of the box in A displays GRP immunoreactivity surrounded by NK1Rs in lamina I (A1) and NK1Rs expression alone (A2). GRP-positive neurons are in close contact with NK1Rs (A1). White arrows in A1-A2 indicate an NK1R-rich region, while the yellow arrow points to the region of contact between the GRP-positive neurons and NK1Rs. (B) A different slice of the same GRP-positive neuron shown in A identified further interactions

between NK1Rs and the GRP-positive neuron (white arrowhead). (C) Confocal slice of a dorsal horn section from an adult male, *dab1*$^{-/-}$*;Grp* mouse shows GRP (green) and NK1R (blue) immunoreactivity. Contact between a GRP-positive neuron and NK1Rs is illustrated (C1). C2 displays a different slice of the Z-stack shown in C1. An additional GRP-labeled cell in contact with NK1Rs is detected (C2, white arrowhead). Scale bars: A,C = 100μm; A1-A2, B-B2, C1-C2 = 10μm.

TABLE 1: List of sources and concentrations for primary antibodies

Primary Antisera	**Source; Catalog #**	**Host Species**	**Dilutions**
GFP	Aves Labs; GFP-1020	Chick	1:1000
NK1R	Sigma-Aldrich; S8305	Rabbit	1:30,000 (TSA)
Reelin	R&D Systems; AF3820	Goat	1:1,000 (TSA)

TABLE 2: Cell counts in the superficial dorsal horn do not differ between *dab1*$^{+/+}$*-Grp* and *dab1*$^{-/-}$*-Grp* mice

Cell type	***dab1*$^{+/+}$*;Grp***	***dab1*$^{-/-}$*;Grp***	**p-value**
Total Reelin	17.7±0.8	20.2±0.8	n.s. (.98)
Total GRP	10.±0.8	9.3±0.8	n.s. (.27)
Reelin-GRP	2.5±0.8	3.5±0.8	n.s. (.81)
Reelin-NK1R	1.2±0.8	2±0.8	n.s. (.76)
GRP-NK1R	0.03±0.8	0.42±0.8	n.s. (.63)
Reelin-GRP-NK1R	0±0.8	0.2±0.8	n.s. (.56)

Table 2: Averages from the mean±SEM of Reelin-, GRP-, Reelin-GRP-, Reelin-NK1R-, GRP-NK1R, and Reelin-GRP-NK1R-expressing neurons per hemisection of the superficial dorsal horn in *dab1;Grp* mice. 6 mice/genotype and 3-6 hemisections per mouse were analyzed.

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