Deletion of the Gene Encoding the NMDA Receptor GluN1 Subunit in Schwann Cells Causes Ultrastructural Changes in Remak Bundles and Hypersensitivity in Pain Processing

Coralie Brifault,^{1,2} Haylie Romero,^{1,3} Alicia Van-Enoo,^{1,3} Don Pizzo,² Pardis Azmoon,² HyoJun Kwon,¹ Chanond Nasamran,⁵ Steven L. Gonias,² and Wendy M. Campana^{1,3,4}

¹Department of Anesthesiology, University of California San Diego, La Jolla, California 92093, ²Department of Pathology, University of California San Diego, La Jolla, California 92093, ³Program in Neurosciences, University of California, San Diego, La Jolla, California 92093, ⁴San Diego Veterans Administration Health Care System, San Diego, California 92161, and ⁵Center for Computational Biology and Bioinformatics, Department of Medicine, University of California, San Diego, La Jolla, California 92093

Abnormalities in interactions between sensory neurons and Schwann cells (SCs) may result in heightened pain processing and chronic pain states. We previously reported that SCs express the NMDA receptor (NMDA-R), which activates cell signaling in response to glutamate and specific protein ligands, such as tissue-type plasminogen activator. Herein, we genetically targeted *grin1* encoding the essential GluN1 NMDA-R subunit, conditionally in SCs, to create a novel mouse model in which SCs are NMDA-R-deficient (GluN1[−] mice). These mice demonstrated increased sensitivity to light touch, pinprick, and thermal hyperalgesia in the absence of injury, without associated changes in motor function. Ultrastructural analysis of adult sciatic nerve in GluN1[−] mice revealed increases in the density of A δ fibers and Remak bundles and a decrease in the density of A β fibers, without altered *g*-ratios. Abnormalities in adult Remak bundle ultrastructure were also present including aberrant C-fiber ensheathment, distances between axons, and increased poly-axonal pockets. Developmental and post radial sorting defects contributed to altered nerve fiber densities in adult. Uninjured sciatic nerves in GluN1[−] mice did not demonstrate an increase in neuroinflammatory infiltrates. Transcriptome profiling of dorsal root ganglia (DRGs) revealed 138 differentially regulated genes in GluN1[−] mice. One third of the regulated genes are known to be involved in pain processing, including *spr1a*, *npv*, *fgf3*, *atf3*, and *cckbr*, which were significantly increased. The intraepidermal nerve fiber density (IENFD) was significantly decreased in the skin of GluN1[−] mice. Collectively, these findings demonstrate that SC NMDA-R is essential for normal PNS development and for preventing development of pain states.

Key words: neuropathic pain; NMDA-R; non-myelinating Schwann cells; peripheral nerve

Significance Statement

Chronic unremitting pain is a prevalent medical condition; however, the molecular mechanisms that underlie heightened pain processing remain incompletely understood. Emerging data suggest that abnormalities in Schwann cells (SCs) may cause neuropathic pain. We established a novel mouse model for small fiber neuropathy (SFN) in which *grin1*, the gene that encodes the NMDA receptor (NMDA-R) GluN1 subunit, is deleted in SCs. These mice demonstrate hypersensitivity in pain processing in the absence of nerve injury. Changes in the density of intraepidermal small fibers, the ultrastructure of Remak bundles, and the transcriptome of dorsal root ganglia (DRGs) provide possible explanations for the increase in pain processing. Our results support the hypothesis that abnormalities in communication between sensory nerve fibers and SCs may result in pain states.

Received Mar. 21, 2020; revised Sep. 24, 2020; accepted Oct. 4, 2020.

Author contributions: W.M.C., C.B., H.R., A.V.-E., and S.L.G. designed research; W.M.C., C.B., H.R., A.V.-E., D.P., P.A., H.K., C.N., and S.L.G. performed research; D.P. contributed unpublished reagents/analytic tools; W.M.C., C.B., H.R., A.V.-E., D.P., P.A., H.K., C.N., and S.L.G. analyzed data; W.M.C., C.B., H.R., A.V.-E., and S.L.G. wrote the paper.

This work was supported by the National Institutes of Health (NIH) Grant R01 NS097590 (to W.M.C. and S.L.G.), the Veterans Administration Grant 1101RX002484 (to W.M.C.), and the NIH Grant UL1TR001442 to the Clinical and Translational Science Awards. We thank Ying Jones and the Transmission Electron Microscopy Core Facility for help with

electron microscopy. We also thank Kristen Jepsen, Kate Fischer, and Annina Schmid for their help with RNA-Seq, bioinformatics analysis, and IENFD methodology, respectively.

The authors declare no competing financial interests.

Correspondence should be addressed to Wendy M. Campana at wcampana@ucsd.edu.

<https://doi.org/10.1523/JNEUROSCI.0663-20.2020>

Copyright © 2020 the authors

Introduction

In small fiber neuropathy (SFN), damage to or impairment of myelinated A δ and unmyelinated C-fibers is associated with neuropathic pain, which can be severe and debilitating (Sopacua et al., 2019). Diagnosis of SFN requires skin biopsies to evaluate intraepidermal nerve fiber density (IENFD) and quantitative sensory testing (Devigili et al., 2019). Decreases in IENFD are associated with pain (Devigili et al., 2008; Gasparotti et al., 2017). However, disparities between peripheral nerve pathology and pain severity exist in many neuropathic pain patients (Shillo et al., 2019). In addition, a large portion of SFN cases are idiopathic. Thus, understanding the molecular and cellular mechanisms underlying SFN remains an important goal.

In the PNS, Schwann cells (SCs) provide myelination and ensheathment of axons, which is essential for maintaining axonal integrity (Shy et al., 2002; Harty and Monk, 2017). Myelinating SCs wrap membrane sheaths around large (A β) and medium diameter (A δ) axons, whereas non-myelinating SCs ensheath multiple small-diameter C-fibers in the absence of myelin (Jessen and Mirsky, 2005). Clusters of C-fibers in association with a single SC are called Remak bundles; these C-fibers constitute 80% of the axons in the human peripheral nerve (Griffin and Thompson, 2008). Abnormalities in SCs may be associated with neuropathic pain states, especially following peripheral nerve injury (for review, see Campana, 2007; Harty and Monk, 2017). For example, conditional deletion of the gene encoding LDL receptor-related protein-1 (LRP1) in SCs exacerbates pain-related behaviors in mice after partial sciatic nerve ligation (Orita et al., 2013). Deletion of the GABA-B receptor in myelinating SCs causes hyperalgesia and tactile allodynia even in the absence of nerve injury (Faroni et al., 2014).

In addition to its primary role in mediating excitatory synaptic transmission in neurons (Chen and Kukley, 2020), the NMDA receptor (NMDA-R) has been identified in myelinating and non-myelinating SCs (Kinkelin et al., 2000; Christensen et al., 2016). We demonstrated that SCs in primary culture express multiple NMDA-R subunits, including GluN1, GluN2b, GluN2c, and GluN3b (Campana et al., 2017). In response to glutamate, SCs demonstrate robust cell signaling, including activation of ERK1/2 and Akt (Campana et al., 2017). SCs also require the NMDA-R to respond to protein ligands that activate cell signaling, such as tissue-type plasminogen activator, activated α_2 -macroglobulin, and matrix metalloproteinase-9 (Mantuano et al., 2015). Understanding how SC NMDA-R regulates PNS physiology is an important objective.

In this study, we deleted the gene encoding the essential GluN1 NMDA-R subunit, *grin1*, conditionally in SCs, by expressing Cre recombinase under the control of the myelin protein zero (P0) promoter (Feltri et al., 2002). In the resulting SC GluN1-deficient (GluN1 $^{-}$) mice, myelination of sciatic nerves was unchanged; however, the density of A δ fibers and C-fibers in Remak bundles was increased while the density of A β fibers was decreased. In adult GluN1 $^{-}$ mice, major ultrastructural abnormalities in Remak bundles were observed, including an increase in the incidence of polyaxonal pockets, large variations in the distance between C-fibers, and irregular ensheathment of C-fibers. The increase in abundance of Remak bundles and the abnormalities observed in adult nerves were partially explained by ultrastructural analysis of sciatic nerves during development. GluN1 $^{-}$ mice demonstrated increased mechanical and thermal sensitivity and reduced IENFD. These signs are

reminiscent of those observed in SFN and fibromyalgia (Oaklander, 2016). *grin1* deletion in SCs significantly modified the transcriptome profile of dorsal root ganglia (DRGs), which house sensory neuron cell bodies. A large cohort of the regulated genes are associated with pain processing. These results identify SC NMDA-R as a major regulator not only of SC function but also, pain states. The GluN1 $^{-}$ mouse model offers the potential to contribute to our understanding of SFN and fibromyalgia.

Materials and Methods

Animals

Transgenic mice that are homozygous for floxed *grin1* (*grin1*^{fl/fl}; B6.129S4-*grin1*^{tm2Sll/J}, The Jackson Laboratory) were crossed with P0-Cre mice (strain B6N.FVB-Tg(Mpz-cre)26Mes/J) that express Cre recombinase under the control of the P0 promoter in the C57BL/6J background (Feltri et al., 1999, 2002). In this strain of P0-Cre mice, Cre is embedded in the complete endogenous mouse P0 regulatory domain and thus, there is a high level of specificity for SCs (Feltri et al., 1999, 2002). Age matched male littermates that were *grin1*^{flox/flox} and either P0-Cre-negative (GluN1 $^{+}$) or P0-Cre-positive (GluN1 $^{-}$) were compared. All animal experiments were approved by the Institutional Animal Care and Use Committee at University of California San Diego.

Primary mouse SC cultures

Primary cultures of mouse SCs were isolated as previously described (Wang et al., 2013) with minor modifications. Adult mice were deeply anesthetized with isoflurane and euthanized by cervical dislocation. Sciatic nerve fragments (1.5 cm) were surgically resected, rinsed, placed in complete medium consisting of DMEM, 10% fetal bovine serum (FBS; Invitrogen), 2 μ M forskolin, and 10 ng/ml neuregulin- β -1 (R&D Systems; catalog #9875-NR) and incubated at 37°C in a CO₂ incubator for a week. The medium was changed once every two days. At 7 d, nerve fragments were digested with a mixture of collagenase (0.2%, Stemcell Technologies) and 0.2% Dispase (Stemcell Technologies) at 37°C. After centrifugation, cell pellets were re-suspended in complete medium. Cells were plated in poly-L-lysine-coated T25-flasks (10 μ g/ml, Sigma-Aldrich) at a density of 5×10^5 cells/flask.

RNA isolation and RT-qPCR

Adult GluN1 $^{+}$ and GluN1 $^{-}$ mice were euthanized and uninjured sciatic nerves and DRGs were harvested. Sciatic nerves were stripped of epineurium. Nerves from two equivalent mice were pooled to generate one replicate ($n=4$ /experiment). Total RNA was extracted in Trizol and purified using the Nucleospin RNA kit (Macherey-Nagel). The same kit was used to purify total RNA from cultured SCs. RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). RT-qPCR was performed using TaqMan gene expression products and an AB Step one Plus Real-Time PCR System (Applied Biosystems). The relative change in gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. GAPDH mRNA was measured as a standard. The primer-probe sets used included: *gapdh* (Mm99999915_g1), *grin1* (Mm00433790_m1), *grin2b* (Mm00433820_m1), *lrp1* (Mm00464608-m1), *sox10* (Mm00569909_m1), *sprr1a* (Mm01962902_s1), *npv* (Mm01410146_m1), and *atf3* (Mm00476033_m1), *tnfa* (Mm00443258_m1) and *ccl2* (Mm00441242_m1).

Immunoblot analysis

Proteins from sciatic nerves and DRGs were extracted in RIPA buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail and phosphatase inhibitor (Roche Diagnostics). Equal amounts of cellular protein were loaded onto Protean TGX gels (Bio-Rad) and electro-transferred to PVDF membranes (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, and 0.1% Tween 20 (TBS-T buffer) and incubated with the following primary antibodies:

anti-GluN1 (1:1000; Cell Signaling Technology, catalog #5704S) and anti-GAPDH (1:5000; SIGMA, catalog #G9545). Primary antibodies were detected with HRP-conjugated species-specific secondary antibodies (Cell Signaling Technology). Conjugated antibodies were detected with the ECL reagent, Prosignal (Prometheus), and the Azure C300 imaging system. Densitometry analysis was performed using ImageJ software.

Immunohistochemistry (IHC) and image analysis

Animals were deeply anesthetized with isoflurane and subjected to intracardiac perfusion with fresh PBS followed by 4% paraformaldehyde. For IHC, sciatic nerve tissues were paraffin embedded. Cross sections (4 μ m) were prepared (at least three from each harvested tissue) and stained with antibodies for myelin P0 (Abcam, catalog #ab183868), GFAP (Dako, catalog #Z0334), CD11b (Abcam, catalog #ab133357), and Sox10 (Cell Marque, catalog #ab383A). Nerve sections on slides were stained using a Ventana Discovery Ultra (Ventana Medical Systems). Antigen retrieval was performed using CC1 (Tris based; pH 8.5) for 40 min at 95°C. Slides were incubated with the primary antibody for 30 min at 37°C. The secondary antibody (HRP-coupled goat anti-rabbit; OmniMap system; Ventana, catalog #760-4311) was incubated on the sections for 12 min at 37°C and visualized using diaminobenzidine as a chromogen followed by hematoxylin as a counterstain. Slides were rinsed, dehydrated through alcohol and xylene and cover slipped. Light microscopy was performed using a Leica DFC420 microscope with Leica Imaging Software 2.8.1 (Leica Biosystems).

Immunofluorescence (IF) microscopy was performed to examine cultured mouse SCs fixed in 4% PFA. Slides were incubated with primary antibody directed against p75^{NTR} (rabbit monoclonal 1:1000, Cell Signaling, catalog #8238) for 16 h at 4°. Species-specific secondary antibody was applied (Alexa Fluor 488-conjugated donkey anti-rabbit IgG; Invitrogen, catalog #A21207). Sections were mounted and viewed using an inverted fluorescent microscope (DMI8; Leica Biosystem). Control nerve sections were treated with secondary antibody only. At least three sections from each tissue were analyzed.

Assessment of motor and sensory function

Mice were acclimated to the behavior testing facility for at least 30 min. For experiments using von Frey hairs and insect pins, animals were placed on a mesh stand. For thermal testing, animals were placed on a Plexiglas platform with a radiant heat source. For all sensory and motor testing, each animal was tested on three independent days. Results were averaged and subjected to statistical analysis. All experiments were performed by an investigator blinded to mouse identity.

von Frey test

Mechanical sensitivity was tested by applying 0.04- to 4-g von Frey filaments (Stoelting) to the plantar surface of the ipsilateral hind paw. Filaments were presented in a consecutive fashion, either ascending or descending, using the up-down method as previously described (Dixon, 1980; Chaplan et al., 1994) and modified for mouse (Poplawski et al., 2018). The filament that caused paw withdrawal 50% of the time [the 50% paw withdrawal threshold (PWT)] was determined.

Pinprick test

Response to mechanical stimuli was tested by gently applying an Austerlitz insect pin (size 000; FST) to the plantar surface of the hind paw as previously described (Poplawski et al., 2018). The pin was applied to two areas, the most lateral toe and the mid-lateral area of the paw, with at least a 5-min interval. A response was considered positive when the animal briskly removed its paw, and the test was graded 1 for this area. If the application did not elicit a positive response, the grade was 0. The saphenous territory (medial paw) of the same paw was tested as a positive control, which always elicited a positive response. Each area was tested twice and the results of the two tests were averaged.

Hargreaves test

Responses to thermal stimuli were tested on both hind paws as previously described (Hargreaves et al., 1988; Poplawski et al., 2018) using the Basile plantar test apparatus (Ugo Basile). A constant intensity radiant

heat source (30 I.R.) was aimed at the midplantar area of the hind paw with a cutoff at 10 s to avoid any possible burning injury. The time from heat source activation until paw withdrawal was automatically recorded. Each paw was tested three times with 10-min intervals and the results of the three tests were averaged.

Rotarod

Motor function testing was performed using an accelerating Rotarod (Ugo Basile). The Rotarod speed was increased from four to 40 rotations per minute over a 300-s time period. The latency to fall (sec) reflects the average of three different trials each day.

Transmission electron microscopy

Sciatic nerves from adult, postnatal day (P)5 and P15 GluN1[−] and littermate control GluN1⁺ mice were processed for plastic embedding. Adult mice were deeply anesthetized with isoflurane and subjected to intracardiac perfusion with fresh 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS) followed by glutaraldehyde (2.5%) in PBS. Nerve tissue (5 mm) was then collected 15 mm distal from the DRG. P5 mice were euthanized and nerve tissue (2 mm) was collected 2 mm distal from the DRG. P15 mice were euthanized and nerve tissue (5 mm) was collected 3–5 mm distal from the DRG. Collected nerves were immersed in modified Karnovsky's fixative (2% glutaraldehyde in 0.10 M sodium cacodylate buffer), and further postfixed in 1% OsO₄ in 0.1 M cacodylate buffer for 1 h. Tissues were stained with 2% uranyl acetate for 1 h, dehydrated in serially increasing series of ethanol (50–100%) while remaining on ice. Tissues were then subjected to one wash with 100% ethanol and two washes with acetone, then embedded in Durcupan epoxy resin. Sections were cut at 60 nm on a Leica UCT ultramicrotome, and picked up on 300 mesh copper grids. Sections were poststained with 2% uranyl acetate for 5 min and Sato's lead stain for 1 min. Grids were viewed using a JEOL 1200EX II (JEOL) transmission electron microscope and photographed using a Gatan digital camera (Gatan).

Quantitative analyses of TEM images

In transverse semithin sections of P5 sciatic nerve, myelinated nerve fibers, not-myelinated sorted axons, unsorted axon bundles, and total sorted axons were counted (10–13 thin sections per sciatic nerve; $n = 3$ mice/cohort). In P15 mice, myelinated nerve fibers and not-myelinated axon bundles (early Remak bundles) were counted (nine thin sections per mouse; $n = 3$ mice/cohort). In adult mice, myelinated axons, Remak bundles, and total axons in Remak bundles were counted (8–13 thin sections per sciatic nerve; $n = 3$ mice/cohort). Thus, the total number of sections imaged per mouse genotype was 25–27 (adult) and 32–37 (P5, P15). Sections that were imaged at 1500 $\times$ were used to quantify myelinated fibers, including A β , A δ fibers, and Remak bundles in adult sciatic nerves and to quantify myelinated and not-myelinated sorted axons in P5 nerves. The density of specific fiber types was determined in randomly selected fields using ImageJ software and is reported based on a 625 μ m² area (mean $\pm$ SEM). High-magnification images (4000 $\times$) were used to determine the mean diameter of A δ fibers and Remak axons, to calculate g-ratios, and to identify and quantify abnormalities in Remak bundle structure. Remak bundle abnormalities that were assessed in adult sciatic nerves included: (1) the presence of SC cytoplasm invaginations; (2) the absence of SC cytoplasm between C-fibers in Remak bundle, defined as poly-axonal pockets; and (3) the lack of SC cytoplasm separating C-fibers from endoneurium at the periphery of the Remak bundle. The area of over 300 randomly selected Remak bundle-associated axons was determined using ImageJ. Each area was converted into a corresponding diameter, assuming the axon was a perfect circle, according to the equation: $A = \pi(\frac{1}{2}D)^2$.

Transcriptome profiling

Total RNA was extracted from three GluN1⁺ and three GluN1[−] DRGs collected at the L3–L4 level as described above and analyzed separately to generate a total of six transcriptome profiles. RNA was assessed for quality using an Agilent TapeStation 4200. RNA sequencing libraries were generated from samples with an RNA integrity number (RIN) >8.0, using the TruSeq Stranded mRNA Sample Prep Kit with TruSeq Unique Dual Indexes (Illumina). Profiling was conducted with an Illumina

HiSeq 4000. Samples were demultiplexed using bcl2fastq v2.20 Conversion Software (Illumina). Quality control of the raw fastq files was performed with the software tool, FastQC v0.11.8 (Wingett and Andrews, 2020). Sequencing reads were trimmed with Trimmomatic v0.38 (Bolger et al., 2014) and aligned to the mouse genome (GRCm38) using the STAR aligner v2.6.0a (Dobin et al., 2013). Read quantification was performed with RSEM v1.3.0 (Li and Dewey, 2011) and the Ensembl release 68 annotation. The R BioConductor packages edgeR (Robinson et al., 2010) and limma (Ritchie et al., 2015) were used to implement the limma-voom method for differential expression analysis (Law et al., 2014). In brief, minimally expressed genes (those without counts per million ≥ 1 in at least three of the samples) were filtered out. Then, trimmed mean of M values (TMM) normalization was applied (Robinson and Oshlack, 2010). The experimental design was modeled on condition ($\sim 0 +$ condition). The voom method was employed to model the mean-variance relationship in the log-cpm values weighted for intersubject correlations in repeated measures of subjects after which lmer was used to fit per-gene linear models and empirical Bayes moderation was applied with the eBayes function. Significance was defined by an adjusted p value cutoff of 0.05 ($n = 3$) after multiple testing correction (Benjamini and Hochberg, 1995) using a moderated t statistic in limma. Functional enrichment of the differentially expressed genes was determined using GSEA (Subramanian et al., 2005), WebGestalt (Zhang et al., 2005), and SPIA (Tarca et al., 2009). The pathway figures were generated using logFC values in Pathview (Luo and Brouwer, 2013).

IENFD analysis

IENFD was assessed in the hind paw footpad. After euthanasia, the plantar glabrous skin was collected, fixed in 4% PFA, and stored at 4°C in sucrose. Floating frozen sections (16 μ m) were immuno-stained with rabbit anti-PGP-9.5 (1:200; Zytomed) as described by Schmid et al. (2014). Primary antibody was detected using Alexa Fluor 488-conjugated donkey anti-rabbit IgG (A21207; Invitrogen). Control sections were treated with secondary antibody only. DAPI (blue) identified nuclei. Sections were mounted and viewed using a confocal laser scanning microscope (LSM 880 with Airyscan; Zeiss). IENFD was quantified in PGP9.5 stained sections by a blinded investigator according to guidelines of Lauria et al. (2010) and expressed as profiles per mm epidermis.

Experimental design and statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc.). All results are expressed as the mean $\pm$ SEM. The normality of data was confirmed using the Kolmogorov-Smirnov test. Comparisons between two groups were performed using two-tailed unpaired, paired or nested t tests. When we compared greater than two groups, one-way ANOVA was performed. Body weight measurements and measurements of inflammatory pain, in which we collected multiple

Figure 1. Specific deletion of *grin1* in SCs. **A–C**, Sciatic nerves were harvested from GluN1+ and GluN1- mice and RT-qPCR experiments were performed to quantify expression level of (**A**) *grin1* mRNA comparing GluN1+ ($n = 3$) and GluN1- ($n = 3$) nerves. Data are expressed as the mean $\pm$ SEM ($t_{(7)} = 4.165$, $**p = 0.004$, unpaired two-tailed t test); (**B**) *grin2b* mRNA comparing GluN1+ ($n = 7$) and GluN1- ($n = 6$) nerves. Data are expressed as mean $\pm$ SEM ($t_{(11)} = 0.9850$, n.s. $p = 0.346$; unpaired two t test); (**C**) *Irf1* mRNA comparing GluN1+ ($n = 3$) and GluN1- ($n = 3$) nerves. Data are expressed as the mean $\pm$ SEM ($t_{(4)} = 1.859$, n.s. $p = 0.1365$; unpaired two-tailed t test). **D**, Primary cultures of mouse SCs were established and immunostained to detect the SCs marker p75^{NTR} (green). Representative IF microscopy images are presented for GluN1+ (left) and GluN1- (right) cells. Nuclei are counterstained with DAPI. Scale bar: 50 μ m. **E**, Total RNA was extracted from primary cultures of SCs, isolated from GluN1+ ($n = 5$ independent cultures) and GluN1- ($n = 3$ independent cultures) mice. *grin1* mRNA expression was measured by RT-qPCR. Data are expressed as the mean $\pm$ SEM ($t_{(6)} = 5.285$, $**p = 0.002$; two-tailed unpaired t test). **F–H**, DRGs were collected from GluN1+ ($n = 3$) and GluN1- ($n = 3$) mice. **F**, RT-qPCR was performed to quantify expression of *grin1* mRNA in DRG extracts. Data are expressed as mean $\pm$ SEM (n.s. $p = 0.7$, Mann–Whitney two-tailed test). **G**, Immunoblot analysis was performed to detect the GluN1 subunit of the NMDA-R. GAPDH serves as a loading control. **H**, Densitometry analysis was performed to determine the relative level of GluN1 protein standardized against the loading control. Data are expressed as a GluN1/GAPDH ratio from GluN1+ ($n = 6$) and GluN1- ($n = 5$) DRGs. Data are mean $\pm$ SEM ($t_{(9)} = 1.069$, n.s. $p = 0.3128$; unpaired two-tailed t test).

observations in individual mice over time, were analyzed by repeated measures ANOVA. Statistical analyses (exact p values, degrees of freedom, effect sizes) for each experiment are reported in the corresponding figure legends; $p < 0.05$ was considered statistically significant.

Results

Targeted disruption of *grin1* in SCs

Mice in which *grin1* is flanked by loxP sites were crossed with mice in which Cre recombinase is expressed under the control of the P0 promoter (Feltri et al., 1999; Orita et al., 2013) to generate

Figure 2. Effect of *grin1* deletion in SCs on mouse physiology and sciatic nerve structure. **A**, Representative images of GluN1+ (top) and GluN1- (bottom) mice. **B**, Histogram showing GluN1+ ($n=6$) and GluN1- ($n=6$) mouse weight at two, three, four, and five months of age. No statistical difference was found at each time point. Data shown are the mean $\pm$ SEM ($F_{(3,40)} = 1.066$, n.s., $p = 0.3747$; repeated measures ANOVA). **C**, Representative images of sciatic nerves from three-month-old GluN1+ and GluN1- mice. **D**, **E**, Representative IHC images of transverse nerve sections for (**D**) P0 and (**E**) Cd11b. Scale bar: 100 μ m. **F**, CD11b+ cells were quantitated in GluN1+ ($n=5$) and GluN1- ($n=4$) nerves. Data shown are the mean $\pm$ SEM ($t_{(6)} = 0.3573$, n.s., $p = 0.7331$; two-tailed unpaired t test). **G**, RT-qPCR was performed to quantify expression of *tnfr* mRNA in GluN1+ ($n=3$) and GluN1- ($n=3$) nerves. Data are mean $\pm$ SEM ($t_{(4)} = 0.2395$, n.s., $p = 0.8225$; two-tailed unpaired t test). **H**, *ccl2* mRNA was determined in GluN1+ ($n=4$) and GluN1- ($n=4$) nerves. Data shown are the mean $\pm$ SEM ($t_{(6)} = 1.236$, n.s., $p = 0.2625$; two-tailed unpaired t test).

GluN1- mice. The P0 promoter becomes active specifically in SC precursors at embryonic day 13.5–14.5, including cells that develop into myelinating and non-myelinating SCs. GluN1+ mice carry two copies of floxed *grin1* but are P0-Cre negative. To examine the efficiency of *grin1* gene deletion, first, sciatic nerves were harvested and RNA was isolated. SCs are responsible for >80% of the RNA in uninjured sciatic nerves (Asbury, 1970). *grin1* mRNA was decreased by $69.0 \pm 0.2\%$ in sciatic nerves isolated from GluN1- mice compared with GluN1+ littermate controls ($p < 0.01$; Fig. 1A). Intact NMDA-R cannot assemble in the absence of GluN1 (Lee et al., 2014). *grin2b* mRNA expression was similar in sciatic nerves from GluN1+ and GluN1- mice

(Fig. 1B), as was *lrp1* mRNA (Fig. 1C), which is important because the NMDA-R and LRP1 function as co-receptors in mediating cell signaling in response to protein ligands (Mantuano et al., 2015).

Next, we isolated SCs from sciatic nerves of adult GluN1+ and GluN1- mice and established primary cultures. IF microscopy was performed to examine p75^{NTR}, a biomarker of the non-myelinated and “repair” SC phenotypes. As shown in Figure 1D, SCs isolated from both GluN1+ and GluN1- mice were similar in appearance and immunopositive for p75^{NTR}. RT-qPCR demonstrated a 99% decrease in the mRNA encoding GluN1 in cultured SCs from GluN1- mice compared with GluN1+ mice ($p < 0.01$; Fig. 1E).

The P0 promoter used in our studies is reported to be inactive in CNS glia and in DRG neurons (Feltri et al., 1999, 2002). As a control, L3–L4 DRGs were collected from GluN1+ and GluN1- mice. *grin1* mRNA was quantified by RT-qPCR. Similar levels of *grin1* mRNA were detected in DRGs from GluN1+ and GluN1- mice (Fig. 1F). Similar levels of GluN1 protein also were detected, as shown in the representative immunoblot analysis (Fig. 1G) and by densitometry of five to six separate immunoblots (Fig. 1H). Although these experiments are limited by the diversity of cell types in the DRG, our results support previous studies (Feltri et al., 1999, 2002), indicating that the P0 promoter specifically drives gene deletion in SCs in the PNS.

GluN1- mice do not demonstrate gross abnormalities

GluN1- mice were grossly normal. These mice were fertile and born at the expected Mendelian frequency. Adult GluN1- mice were the same size and weight as GluN1+ mice (Fig. 2A,B). Gait and motor function were qualitatively intact and there was no evidence of neurologic abnormalities.

Sciatic nerves in GluN1- mice appeared normal in size and structure (Fig. 2C). Abnormalities in myelination, which may manifest as a translucent appearance to the sciatic nerve, were not visible using a magnifying glass. No differences were observed when cross-sections of sciatic nerves from GluN1+ and GluN1- mice were compared by IHC for P0 (Fig. 2D). P0 immunostaining confirmed the presence of intact myelinated axons in nerves from both genotypes.

To determine whether the number of resident and blood-derived macrophages changed in uninjured sciatic nerves from GluN1- mice, we performed IHC for CD11b. In addition to macrophages, CD11b-specific antibody immunostains monocytes,

neutrophils, and NK cells (Wong et al., 2011). The density of CD11b-positive cells was unchanged in GluN1[−] mice (Fig. 2E,F). To further confirm that there is no ongoing inflammation in uninjured sciatic nerves in GluN1[−] mice, we quantified expression of the mRNAs encoding the pro-inflammatory cytokine, *tnfa*, and the chemokine, *ccl2*, in sciatic nerve extracts. Expression of *tnfa* and *ccl2* was unchanged (Fig. 2G,H). These results indicate that GluN1 deficiency in SCs is not associated with gross abnormalities in sciatic nerve structure or inflammatory infiltrates.

SC populations are altered in GluN1[−] mice

To determine whether NMDA-R deficiency regulates SC abundance, sciatic nerves from GluN1⁺ and GluN1[−] mice were immunostained to detect Sox10 (Fig. 3A). Sox10 is a transcription factor necessary for SC development, which persists in the nuclei of myelinating and non-myelinating adult SCs (Kuhlbrodt et al., 1998). Morphometric analysis of the Sox10 IHC images demonstrated that the density of Sox10-positive nuclei was increased in GluN1[−] nerves ($p < 0.05$; Fig. 3B), suggesting an increase in the density of SCs. We also quantified *sox10* mRNA expression by RT-qPCR and demonstrated that *sox10* mRNA is increased in GluN1[−] nerves ($p < 0.05$; Fig. 3C). This result would argue that Sox10 expression may be regulated in individual SCs from GluN1[−] mice since most of the RNA harvested from peripheral nerves is SC derived.

Next, we measured GFAP, a biomarker of non-myelinating SCs in uninjured nerves (Jessen and Mirsky, 1984). GFAP immunoreactivity was apparent in nerves from both GluN1[−] and GluN1⁺ mice (Fig. 3D). A trend toward increased GFAP immunoreactivity was observed in GluN1[−] nerves ($p = 0.063$; Fig. 3E). This result may be explained as a trend toward more non-myelinating SCs in GluN1[−] sciatic nerves or a change in the area occupied by each SC.

grin1 deletion regulates proportions of myelinated nerve fibers but does not affect g-ratios

Next, we compared sciatic nerves from adult GluN1[−] and GluN1⁺ mice by TEM. Representative images showing A β fibers, A δ fibers and Remak bundles are shown in Figure 4A. The density of large diameter myelinated A β fibers ($>6\mu\text{m}$) was decreased in sciatic nerves from GluN1[−] mice (6.2 ± 0.4) compared with GluN1⁺ littermate controls (7.9 ± 0.2 ; $p < 0.01$;

Figure 3. Sox10 immunoreactivity and mRNA are increased in GluN1[−] mouse sciatic nerves. **A**, Representative images of Sox10 immunoreactive cells in transverse sections of sciatic nerves from GluN1⁺ and GluN1[−] mice; 400 $\times$. **B**, Quantification of Sox10 immunopositive nuclei per whole transverse nerve section (100 $\times$) from GluN1⁺ ($n = 8$) and GluN1[−] ($n = 8$) mice. Data are expressed as the mean $\pm$ SEM ($t_{(7)} = 2.621$, $*p = 0.0343$; two-tailed paired t test). **C**, RT-qPCR experiments were performed to quantify *sox10* mRNA in sciatic nerves from GluN1⁺ ($n = 5$) and GluN1[−] ($n = 4$) mice. The data shown are the mean $\pm$ SEM ($t_{(7)} = 3.034$, $*p = 0.019$; two-tailed unpaired t test). **D**, Representative images of GFAP immunoreactivity in transverse sections of sciatic nerves from GluN1⁺ and GluN1[−] mice. **E**, Quantification of GFAP in GluN1⁺ ($n = 4$) and GluN1[−] ($n = 4$) sciatic nerve. Data are mean $\pm$ SEM ($t_{(6)} = 2.282$ n.s. $p = 0.063$; two-tailed unpaired t test).

Fig. 4B). By contrast, the density of A δ fibers was increased in GluN1[−] mice (19.7 ± 1.1 vs 11.0 ± 0.6 ; $p < 0.0001$; Fig. 4C). The increase in density of A δ fibers in GluN1[−] mice was not accompanied by a change in the mean diameter of A δ fibers (Fig. 4D).

Representative images of small myelinated A δ fibers are shown in Figure 4E. To confirm that *grin1* deletion in SCs did not affect myelination, myelinated axons (90–95 fibers) with diameters ranging from 3 to 10 μm were selected randomly from images of sciatic nerves from three separate GluN1[−] and GluN1⁺ mice; g-ratios, which report the mean axonal diameter divided by the mean fiber diameter, were calculated. As shown

Figure 4. Ultrastructural analysis of sciatic nerves in GluN1[−] mice. **A**, Representative TEM images showing myelinated A β , A δ , and non-myelinated C-fibers in sciatic nerves from GluN1⁺ (left) and GluN1[−] (right) mice (1500 $\times$; scale bar: 5 μ m). **B**, Quantification of large diameter, A β axons per field (625 μ m²) in GluN1⁺ (n = 27) and GluN1[−] (n = 25) sciatic nerves. The data shown are the mean $\pm$ SEM ($t_{(50)} = 3.564$ $^{**}p$ = 0.0008, two-tailed unpaired t test). **C**, Quantification of A δ fibers per field (625 μ m²) in sciatic nerves from GluN1⁺ (n = 27) and GluN1[−] (n = 25) mice. Data are the mean $\pm$ SEM ($t_{(50)} = 6.833$ $^{***}p$ < 0.0001, two-tailed unpaired t test). **D**, Measure of the diameter of A δ fibers in sciatic nerves from GluN1⁺ (n = 86) and GluN1[−] (n = 147) mice. A δ fibers are <5 μ m in diameter. The data shown are the mean $\pm$ SEM ($t_{(230)} = 1.395$ n.s. p = 0.8892, two-tailed unpaired t test). **E**, Representative TEM images of A δ axons (2000 $\times$; scale bar: 1 μ m). **F**, Scatter plots showing g -ratios versus axon diameter for myelinated axons from GluN1⁺ (n = 90) and GluN1[−] (n = 95) mice. Simple linear regression revealed $F_{(1,181)} = 0.011$ n.s. p = 0.9176. No difference in the slopes for the g -ratios was observed. All images are of four-month-old mice.

in Figure 4F, the g -ratios distributed similarly when nerves from GluN1[−] and GluN1⁺ mice were compared.

***grin1* deletion in SCs alters Remak bundle abundance and ultrastructure**

Given the limited sensitivity of GFAP immunoreactivity in determining the density of non-myelinating SCs, we analyzed TEMs

at high magnification (4000 $\times$) to more accurately study the abundance and structure of Remak bundles in sciatic nerves from GluN1[−] and GluN1⁺ mice. Image analysis of electron micrographs confirmed that the density of Remak bundles was significantly increased in GluN1[−] sciatic nerves (10.6 ± 0.9 vs 4.2 ± 0.4 ; p < 0.0001; Fig. 5A). This corresponded with an increased number of Remak bundle-associated axons in GluN1[−] mice (p < 0.0001; Fig. 5B). The number of axons per Remak bundle in GluN1[−] mouse nerves distributed into a pattern that was not obviously changed compared with GluN1⁺ mice (Fig. 5C) and consistent with the distribution anticipated in wild-type mice (Murinson and Griffin, 2004).

High-magnification ultrastructural analysis of Remak bundles showed that in GluN1⁺ mice, the axons were uniformly ensheathed by extensions of SC cytoplasm so that the outer axonal membranes of each axon were approximately equidistant from each other throughout the Remak bundle (Fig. 5D, top panels). By contrast, the axons in Remak bundles in GluN1[−] mice demonstrated highly irregular ensheathment by SC cytoplasm (Fig. 5D, bottom panels). The abnormalities observed included invagination of SC cytoplasm (Fig. 5D, bottom left panel), poly-axonal pockets (Fig. 5D, asterisks), and direct exposure of the axon to the endoneurium because of locally absent or thinned SC ensheathment (Fig. 5D, arrowheads).

Quantitative image analysis revealed a greater than twofold increase in the percentage of Remak bundle-associated axons impacted by apparent abnormalities in ultrastructure in GluN1[−] mice, compared with GluN1⁺ mice (p < 0.001; Fig. 5E). Poly-axonal pockets are rare in Remak bundles in wild-type peripheral nerves (Murinson and Griffin, 2004); however, in sciatic nerves from GluN1[−] mice, the number of Remak bundles containing one or more poly-axonal pocket doubled (p < 0.05; Fig. 5F). The frequency of exposure of C-fibers to the endoneurium increased more than fourfold in GluN1[−] mice (p < 0.0001; Fig. 5G). These results demonstrate that *grin1* deletion in SCs affects Remak bundle ultrastructure.

When abnormalities in the PNS involve principally Remak bundles, this may be attributed to a late axonal sorting defect or a postsorting defect in differentiation of non-myelinating SCs (Feltri et al., 2016). Late axonal sorting defects are characterized by an increase in large axons (>1.0 μ m) within Remak bundles. We therefore measured

Figure 5. *grin1* deletion in SCs alters Remak bundles. **A**, Quantification of Remak bundle density (per 625 μm^2) in transverse nerve sections from GluN1+ ($n=27$) and GluN1- ($n=25$) mice (four-month-old). Data are mean $\pm$ SEM ($t_{(50)} = 6.34$, $p < 0.0001$; two-tailed unpaired t test). **B**, Total number of Remak axons in transverse nerve sections (625 μm^2) from GluN1+ and GluN1- mice, $t_{(31)} = 5.931$, $***p < 0.001$. **C**, Analysis of Remak bundles showing the number of C-fibers ensheathed within each Remak bundle in GluN1+ ($n=26$) and GluN1- ($n=26$) nerve sections (one axon, $t_{(50)} = 2.732$, $**p = 0.0087$; 2–10 axons, $t_{(50)} = 2.602$, $*p = 0.0121$; 11–20 axons, $t_{(50)} = 3.423$, $**p = 0.0012$; and >20 axons, $t_{(50)} = 1.785$, $p = 0.0803$). The data shown are the mean $\pm$ SEM for each group analyzed by a two-tailed unpaired t test. **D**, Representative images of Remak bundles in GluN1+ (top panel) and GluN1- (bottom panel) sciatic nerves (4000 $\times$; scale bar: 500 nm). **E–H**, Quantification of abnormalities in Remak bundles. Abnormalities are defined as (1) SCs cytoplasm invagination (bottom left image); (2) poly-axonal pockets (asterisk); and (3) axons in direct contact with the endoneurium (arrowhead). **E**, Abnormal Remak bundles were counted and expressed as a percentage of total number of Remak bundles in GluN1+ ($n=30$) and GluN1- ($n=30$) nerves. The data shown are the mean $\pm$ SEM ($t_{(66)} = 4.134$, $***p < 0.0001$, two-tailed unpaired t test). **F**, The number of Remak bundles containing one or more poly-axonal pockets in GluN1+ ($n=13$) and GluN1- ($n=17$) nerves was determined and expressed as a percentage of the total number of Remak bundles. The data shown are the mean $\pm$ SEM ($t_{(28)} = 4.134$, $*p = 0.0326$, two-tailed unpaired t test). **G**, Axons in direct contact with the endoneurium that were not ensheathed correctly by SCs cytoplasm were counted and expressed as a percentage of total axons per Remak bundle in GluN1+ ($n=28$) and GluN1- ($n=39$) nerves. The data shown are the mean $\pm$ SEM ($t_{(65)} = 4.899$, $***p < 0.0001$, two-tailed unpaired t test). **H**, Remak axon diameter sizes in GluN1+ and GluN1- mice did not reveal large axons $>1 \mu\text{m}$; ($n=6$). The data shown are the mean $\pm$ SEM; $t_{(4)} = 0.8472$, n.s., two-tailed nested t test.

Figure 6. Absence of GluN1 in SCs manifests defects in development during radial sorting. **A**, Electron micrographs of P5 and P15 sciatic nerves from GluN1+ and GluN1- mice. In P5, bundles of unsorted axons are indicated by white asterisks. Blue arrows point to examples of myelinated axons and yellow stars identify sorted but not myelinated axons. In P15, bundles of not-myelinated axons are indicated by purple asterisks. Blue arrows point to examples of myelinated axons. Scale bar: 5 μm . **B–D**, Quantification of morphologic features of P5 sciatic nerves. The number of myelinated axons, $t_{(66)} = 0.4387$, n.s. (**B**), the number of not-myelinated sorted axons, $t_{(66)} = 3.599$, $***p < 0.001$ (**C**), and the number of total sorted axons, $t_{(66)} = 2.247$, $*p < 0.05$ (**D**) were determined per cross section (625 μm^2). All results are the mean $\pm$ SEM and analyzed by two-tailed unpaired t test. **E, F**, Quantification of TEM in P15 nerves. **E**, The number of myelinated axons, $t_{(52)} = 0.4633$, n.s. were determined per cross section (625 μm^2). **F**, The number of not-myelinated bundles, $t_{(54)} = 3.182$, $**p < 0.01$. All results are the mean $\pm$ SEM and analyzed by two-tailed unpaired t test.

over 300 randomly selected Remak bundle-associated axons in electron micrographs of nerves from GluN1- and GluN1+ mice. The mean axonal diameter was slightly decreased in nerves from GluN1- mice. However, most importantly, the fraction of large axons was not increased in GluN1- Remak bundles (Fig. 5H).

Loss of GluN1 in SCs alters early radial sorting of axons

To identify abnormalities in development of GluN1- mice that may explain the ultrastructural changes observed in adult sciatic nerves, we analyzed P5 and P15 sciatic nerves by TEM. In both GluN1- and GluN1+ mice, P5 sciatic nerve included unsorted bundles of axons, myelinated axons, and not-myelinated sorted axons (Fig. 6A). Image analysis revealed that the density of myelinated axons was unchanged in GluN1- mice, compared with GluN1+ mice (Fig. 6B); however, the density of not-myelinated sorted axons was significantly increased in GluN1- mice ($p < 0.05$; Fig. 6C), and contributed to an increase in the overall density of sorted axons (Fig. 6D). At P15, once again that the density of myelinated fibers was not altered in GluN1- mice (Fig. 6E); however, the density of axon bundles, partially or fully ensheathed by a single non-myelinating SC (early Remak bundles) almost doubled in the GluN1- mouse ($p < 0.01$; Fig. 6F).

GluN1- mice demonstrate mechanical and thermal hypersensitivity

GluN1+ and GluN1- mice were compared in experiments examining mechanical and thermal sensitivity in the absence of sciatic nerve injury. First, mechanical sensitivity was measured by non-noxious probing of the hindpaw using von Frey filaments. In GluN1- mice, 50% PWTs were significantly lower compared with GluN1+ mice ($p < 0.05$; Fig. 7A), a result that indicates tactile hypersensitivity. Next, the response of GluN1+ and GluN1- mice to sharp stimuli was compared using the pinprick test, which consists of gently applying an insect pin to the lateral toe and the most lateral part of the hind paw. Again, GluN1- mice demonstrated a statistically significant increase in response ($p < 0.05$; Fig. 7B), suggesting increased mechanical sensitivity. In thermal hyperalgesia experiments, using the Hargreaves apparatus, the latency for paw withdrawal from a focal radiant heat source was significantly decreased in GluN1- mice ($p < 0.05$; Fig. 7C), indicating thermal hyperalgesia in these animals. By contrast, no change in motor function was evident when GluN1+ and GluN1- mice were compared using the Rotarod test (Fig. 7D).

To test response to inflammatory pain, mice were subjected to formalin injections in the hind paw. As anticipated, formalin

Figure 7. GluN1[−] mice demonstrate altered thermal and mechanical nociception. **A**, **B**, Mechanical sensitivity was measured by innocuous and noxious probing of the hind paw using (**A**) von Frey filaments in gram weight (g) and (**B**) pin prick, respectively, in GluN1⁺ ($n = 12$) and GluN1[−] ($n = 12$) mice. The data shown are the mean $\pm$ SEM ($t_{(22)} = 2.195$, $*p = 0.0390$, two-tailed unpaired t test (von Frey) and $t_{(22)} = 2.577$, $*p = 0.0219$, two-tailed unpaired t test (pinprick)). **C**, Thermal sensitivity was measured using an Hargreaves apparatus. The paw withdrawal latency was measured in seconds using an infrared intensity of 30 in GluN1⁺ ($n = 8$) and GluN1[−] ($n = 8$) mice. The data shown are the mean $\pm$ SEM ($t_{(14)} = 2.324$, $*p = 0.0357$, two-tailed unpaired t test). **D**, Motor function was assessed using an accelerated Rotarod (4–40 rpm over a 500-s period) in GluN1⁺ ($n = 12$) and GluN1[−] ($n = 12$) mice. The data shown are the mean $\pm$ SEM ($t_{(22)} = 0.6120$, n.s., $p = 0.5468$, two-tailed unpaired t test). **E**, Formalin testing in GluN1⁺ ($n = 7$) and GluN1[−] ($n = 8$) mice was performed to assess the response to inflammatory pain. The time course of paw-licking following a subcutaneous injection of 20 μ l of formalin (5% in saline) in the hind paw is shown (left diagram). Each point represents the average amount of time a mouse spent licking the injected paw during a 5-min period. The data shown are the mean $\pm$ SEM ($F_{(10,154)} = 0.011$, n.s., $p = 0.9755$, repeated measures ANOVA). Age matched mice, three to five months, in both genotypes were compared.

induced vigorous licking of the paw in two distinct periods (Hunskar et al., 1985); an “early” phase 1 response within the first 5 min followed by a “later stage” second phase lasting from 20 to 50 min (Fig. 7E). Analysis of the individual phases, by cumulative time spent licking during the early and the late phase, revealed no statistically significant differences between GluN1⁺ and GluN1[−] mice (Fig. 7E), demonstrating that *grin1* deletion in SCs does not regulate inflammatory pain.

Transcriptome profiling of DRGs

We compared the DRG transcriptomes of uninjured adult GluN1[−] and GluN1⁺ mice. A total of 138 differentially regulated genes were identified, as shown in the heat map and volcano plot (Fig. 8A,B). The data have been submitted to GEO, a public database (accession number is GSE154182). The largest share of regulated genes was in the category, “biological regulation” (Fig. 8C). A large fraction of the regulated genes was localized to the plasma membrane (Fig. 8D). KEGG pathway analysis (entry mmu04080) revealed key regulated genes that control neuronal function, including GABA and opioid receptors, which were downregulated, and the cholecystinin 2 receptor, *cckbr*, the G-coupled receptor, *Gpr151*, and the serotonin transporter, *SLC6A4*, which were upregulated (Fig. 8B,E).

Overall, greater than one-third of all differentially regulated genes were associated with pain pathways and are activated in persistent or chronic pain. Heat map analysis of the 50 most significant pain-related genes are shown in Figure 9A. *ATF3*, *NPY*, and *sprr1a* were validated by RT-qPCR and were increased by 14-fold, 45-fold, and 160-fold, respectively, in DRGs from GluN1[−] mice ($*p < 0.05$, $**p \leq 0.01$; Fig. 9B–D). *ATF3* and *NPY* are upregulated in previously described injury-induced neuropathic pain models. In GluN1[−] mice, regulation of expression of *ATF3* and *NPY* occurs in the absence of nerve injury. The 15 most significantly regulated pain genes, together with the fold-change in expression ($2^{\Delta\log FC}$), adjusted p values, and references to pain processing are listed in Table 1.

Deletion of *grin1* in SCs alters peripheral nerve terminals

Because decreased IENFD is characteristic of SFN and observed in other painful syndromes such as fibromyalgia (Clauw, 2015), we performed IF to detect PGP9.5 (green) in skin biopsies of GluN1[−] and GluN1⁺ mice. PGP9.5 is a pan-axonal marker and identifies small nerve fibers in cutaneous skin (Schmid et al., 2014). The density of PGP9.5-positive nerve profiles in skin was significantly decreased in GluN1[−] mice, compared with GluN1⁺ mice ($p < 0.05$; Fig. 10).

Discussion

In the United States, the prevalence of chronic pain ranges from 11% to 40% in adults (Dahlhamer et al., 2018). Identifying molecular mechanisms that underlie chronic pain remains an important scientific objective. Recent studies in mouse model systems have identified dysfunctional SCs as a cause of neuropathic pain (Orita et al., 2013; Faroni et al., 2014). Herein, we demonstrated that conditional *grin1* deletion in SCs causes hypersensitivity in pain processing in the absence of nerve injury. This abnormality was associated with changes in the density of small fibers and ultrastructural changes in Remak bundles. A decrease in IENFD was observed, consistent with heightened sensitivity to pain. Furthermore, deletion of *grin1* in SCs regulated the “pain-associated” DRG transcriptome, suggesting regulation of sensory neuron gene expression. GluN1[−] mice may represent a novel model system for studying SFN, fibromyalgia, and other poorly understood conditions in which chronic unremitting pain is experienced by patients.

Although a number of laboratories have reported that SCs express NMDA-Rs (Evans et al., 1991; Fink et al., 1999; Campana et al., 2017), the function of the NMDA-R in SCs remains unclear. We previously showed that cultured SCs respond to glutamate, which activates cell signaling and promotes SC migration (Campana et al., 2017). A second important

Figure 8. Differentially regulated genes in DRGs of GluN1+ and GluN1- mice. **A**, Hierarchical clustering and heat map analysis of differentially expressed genes (138 genes; adjusted $p < 0.05$). The scaled expression value (row Z score) is shown in a blue-red color scheme with red indicating higher expression and blue indicating lower expression. **B**, Volcano plot showing large fold changes that are also statistically significant. These may be the most biologically significant genes. The dashed line shows where $p = 0.05$, with points above having $p < 0.05$ and points below having $p > 0.05$. The gray points have a fold change < 2 ($\log_2 = 1$). **C**, **D**, Gene ontology pathways that are enriched in differentially expressed genes. The number of genes enriched in (C) biological processes or (D) molecular functions are indicated. **E**, KEGG pathway analysis demonstrating molecular interactions in the neuroactive ligand receptor network. The pathway was significantly regulated ($p < 0.05$). Yellow boxes are upregulated genes and blue boxes are down regulated genes. All data compare mice that are three months old.

function of the NMDA-R is to function as an essential component of a receptor system which, together with low-density lipoprotein receptor-related protein 1 (LRP1), activates cell signaling and cell migration in response to protein ligands in SCs (Mantuan et al., 2015). When the gene encoding LRP1 was conditionally deleted in SCs in mice (scLRP1- mice), using the same P0 promoter applied in the current study, neuropathic pain was exacerbated following partial nerve ligation (Orita et al., 2013). Abnormalities were

identified in Remak bundles in scLRP1- mice and, although we did not complete comprehensive DRG transcriptome profiling in scLRP1- mice, a number of genes were regulated, which also were regulated in GluN1- mice, including ATF3 and *spr1a* (Poplawski et al., 2018). Thus, there is overlap in the phenotypes observed when the genes encoding either the NMDA-R GluN1 subunit or LRP1 are deleted in SCs. The GluN1- phenotype appears more severe in its effects on small fibers and pain processing.

In adult GluN1[−] mice, changes in the density of A δ and A β myelinated axons were detected by TEM without evidence for structural impairment of myelin. In addition, the density of Remak bundles was increased by greater than twofold. Overall, this change in proportion of fiber types was consistent with our observation that the density of SCs was increased in GluN1[−] mice, as determined by both TEM and IHC for Sox10-positive nuclei. To understand these changes, we studied P5 and P15 nerves from GluN1[−] mice by TEM. P5 nerves were most remarkable for an increase in the density of not-myelinated sorted axons, whereas, at P15, the main feature was an increase in the abundance of clusters of non-myelinated axons apparently in association with a single SC. We viewed these latter structures as immature Remak bundles, as they contained both unsorted axons and axons individualized by SC basal lamina. Collectively, our TEM developmental studies are consistent with results in adult nerves. Although not a commonly observed developmental feature, an increase in the abundance of not-myelinated sorted axons at P5 has been observed before (Ommer et al., 2019), yet this abnormality evolved into a defect in myelinated axon populations. Our results suggest that not-myelinated sorted axons also may coalesce, with the extra SCs undergoing apoptosis, to form immature Remak bundles. Alternatively, a greater number of not-myelinated sorted axons observed in the GluN1[−] nerves contributed to the greater number of A δ fibers observed. This model is consistent with SC elongation, apoptosis and proliferation being intimately connected to radial sorting (Webster et al., 1973).

We considered the possibility that changes in PNS ultrastructure in GluN1[−] mice may be related to PI3K and ERK1/2 signaling. Activation of PI3K and ERK1/2 is essential in early SC differentiation (Maurel and Salzer, 2000; Newbern et al., 2011) and our studies performed with cultured SCs and using *in vivo* model systems indicate that the NMDA-R/LRP1 system may be a major determinant of the basal level of activation of these cell-signaling factors in SCs (Campana et al., 2006, 2017). Axon segregation and SC elongation are PI3K dependent (Maurel and Salzer, 2000). Thus, SCs with a lower level of PI3K activation may ensheath decreased lengths of axons, allowing for an increase in the number of SCs that associate with each axon, independently of one another, throughout the length of the sciatic nerve. In the absence of the NMDA-R, other receptor tyrosine kinases still activate PI3K and ERK1/2 pathways in SCs and are important for survival and myelination (Stewart et al., 1996; Campana et al., 1999; Taveggia et al., 2005).

Our results suggested that late radial sorting defects that impact Remak bundle structure did not occur in GluN1[−] nerves

Figure 9. Expression of pain-related genes in GluN1[−] DRGs. **A**, Hierarchical clustering and heat map of 50 differentially regulated genes associated with pain in individual mouse DRGs. The scaled expression value (row Z score) is shown in a blue-red color scheme with red indicating higher expression and blue indicating lower expression. **B–D**, RT-qPCR was performed to validate the level of mRNA expression in DRGs harvested from GluN1⁺ ($n = 3$) and GluN1[−] ($n = 3$) mice for (**B**) *atf3* (mean $\pm$ SEM, $t_{(4)} = 4.604$, $*p = 0.01$, two-tailed unpaired t test); (**C**) *npv* (mean $\pm$ SEM, $t_{(4)} = 5.506$, $*p = 0.0053$, two-tailed unpaired t test); and (**D**) *sprr1a* (mean $\pm$ SEM, $t_{(4)} = 3.275$, $*p = 0.0306$, two-tailed unpaired t test).

because an increase in the frequency of large axons in bundles was not observed. The ensheathment abnormalities identified in Remak bundle SCs in adult GluN1[−] mice are likely because of postradial sorting changes in SC differentiation as defined by Feltri et al. (2016). These aberrant axon ensheathments were prevalent, and resulted in highly variable spacing of neighboring axons. This is significant because when C-fibers come into contact, they provide a mechanism for impulse conduction through the nerve membrane in either direction and spontaneous ephaptic transmission (Sadjadpour, 1975; Ueda, 2008). When the distance between axons is abnormally large, nerve firing also may become aberrant. Changes in Remak bundle ultrastructure and pain were observed previously in mice with genetic deletion of LRP1 (Orita et al., 2013) or the GABA receptor (Faroni et al., 2014) in SCs.

Functional NMDA-Rs are expressed by peripheral glia in addition to SCs, including DRG satellite cells (Castillo et al., 2013). Our comprehensive analysis of DRG mRNA and protein showed that GluN1 levels were unchanged in GluN1[−] mice. However, DRGs contain multiple cell types including satellite

Table 1. Top 15 most significantly regulated pain genes in GluN1 DRGs

Symbol	ENSEMBL (ENSEMUSG000000-)	ENTREZ ID	LogFC	GluN1 + average	GluN1– average	Average expression	Adjusted <i>p</i> value	Association with pain	References
Gal	24907	14419	1.9250	3.6055	5.5292	4.5673	0.00039	Functions as a predictive and prognostic gene of neuropathic pain	
ATF3	26628	11910	2.86	2.9835	5.8508	4.4172	0.00039	High protein-protein interaction with NPY; prognostic and therapeutic target for neuropathic pain	Tang et al. (2020)
Sprr1a	50359	20753	6.9990	−1.2523	5.7724	2.2600	0.00243	Upregulated in DRGs 5-d spared nerve injury; a neuropathic pain model	Tang et al. (2020)
Fgf3	1074	14174	3.8194	−2.5147	1.3031	−0.6058	0.00243	Serum biomarker for sensory neuropathy	Antoine et al. (2015)
Cckbr	30898	12426	4.1617	−1.5512	2.6217	0.5352	0.00527	Activation of Cckbr inhibits opioid analgesia	Yang et al. (2018)
Gpr151	42816	240239	2.6794	0.4850	3.1804	1.8327	0.00567	Facilitates pathogenesis of neuropathic pain via ERK1/2 signaling	Jiang et al. (2018)
Fxyd2	59412	11936	−0.9510	8.6866	7.7362	8.2114	0.00921	Underlies persistent hypersensitivity to pain induced by inflammation	Wang et al. (2015)
P2y1	27765	18441	−0.8313	6.5726	5.7413	6.1570	0.00974	Loss of inhibition of N-type voltage-activated calcium channels	Gerevich et al. (2004)
Slc6a4	20838	15567	3.6839	−1.4063	2.2752	0.4344	0.01034	Migraine	Kowalska et al. (2016)
NPY	29819	109648	5.8372	−2.6726	3.1761	0.2518	0.01034	High protein-protein interaction with ATF3; prognostic and therapeutic target for neuropathic pain	Tang et al. (2020)
Slc17a8 (VGLUT3)	19935	216227	−0.9671	4.5242	3.5543	4.0392	0.01034	Injury induced mechanical hypersensitivity by low threshold mechanoreceptors	Seal et al. (2009)
Sox11	63632	20666	1.0725	1.5369	2.6027	2.0698	0.02142	Injury induced transcription factor that upregulates BDNF	Salerno et al. (2012)
Scn3b	49281	235281	−0.5600	6.9464	6.3862	6.6663	0.02307	Control neuronal excitability	Namadurai et al. (2015)
Ttyh2	34714	117160	0.6934	3.5562	4.2489	3.9026	0.02729	Chloride anion channel transport/BDNF; sensitive to calcium	Boudes et al. (2009)
Csf1	14599	12977	1.0658	3.9589	5.0226	4.4907	0.03166	Drives arthritis pain	Collison (2018)

Table includes gene symbols, ENSEMBL numbers (last five digits), entrez ID, LogFC, average expression levels for GluN1+ and GluN1– mice, adjusted *p* values, the association of the gene with pain processing, and references. All data were collected from DRGs (*n* = 3/genotype) comparing differential expression in GluN1+ and GluN1– mice.

Figure 10. IENFD is decreased in GluN1– mice. **A**, Images of hind paw skin biopsies immunostained for the pan-axonal marker, PGP9.5 (green) in uninjured GluN1+ (upper panel) and GluN1– (lower panel) mice. Nuclei are stained with DAPI (scale bar: 20 μ m). **B**, Quantification of the number of IENFD profiles (100 μ m in diameter) in GluN1+ (*n* = 6) and GluN1– (*n* = 4) skin biopsies (mean $\pm$ SEM, $t_{(8)} = 3.285$, $p = 0.0111$, two-tailed unpaired *t* test). Age matched, four- to seven-month-old, mice from both genotypes were compared.

cells and our analysis of whole tissue may have missed modest changes in a single cell type. Satellite cells do not express P0 (Jessen and Mirsky, 2005); however, because they are derived from the neural crest, we cannot completely rule out some level of P0-Cre activity in these cells. Importantly, activated satellite

cells can induce nociceptor sensitization (Ferrari et al., 2014). Determining whether satellite cells contribute to the observed changes in pain processing in GluN1– mice is an important future goal.

grin1 deletion in SCs was associated with a substantial change in the transcriptome of DRGs in adult mice in the absence of injury. The GluN1– DRG transcriptome demonstrated many similarities with the transcriptome of mice experiencing neuropathic pain (Yang et al., 2018; Sun et al., 2020; Tang et al., 2020). Amongst the pain-related genes upregulated in the DRGs of GluN1– mice, *npv* and *atf3* have been reported to be biomarkers of neuropathic pain when collectively upregulated. *npv* contributes to the pathogenesis of pain (Son et al., 2007) and *ATF3* is a biomarker for neuronal activation and regeneration

(Seijffers et al., 2007; Inoue et al., 2018). *Cckbr* encodes a G-protein coupled receptor, which is a pain therapeutic target (Bernstein et al., 1998) since antagonizing *Cckbr* minimizes opioid use in patients with severe burn injury (Yin et al., 2016).

fgf3 is upregulated in a subgroup of patients with paraneoplastic sensory neuron neuropathy (Antoine et al., 2015). *SLC6A4* encodes the serotonin transporter and is expressed at increased levels in neuropathic pain, idiopathic trigeminal neuralgia and migraine (Cui et al., 2014; Kowalska et al., 2016; Calvo et al., 2019). *SLC6A4* has been linked to the NMDA-R in the context of addiction (Karel et al., 2018) and fibromyalgia (Tour et al., 2017). Collectively, GluN1[−] mice have a molecular expression signature that models SFN, fibromyalgia, and other chronic pain syndromes in which peripheral nerve injury is not obvious.

To further assess the abnormalities in GluN1[−] mice, we analyzed the IENFD in hind paws (Devigili et al., 2019). The IENFD was decreased in GluN1[−] mice. Decreases in IENFD are observed in painful HIV neuropathy, SFN caused by diabetes, and fibromyalgia (Periquet et al., 1999; Polydefkis et al., 2002; Pittenger et al., 2004; Evdokimov et al., 2019). It is assumed that decreased IENFD results from selective degeneration of small fibers near or in the skin. Because the NMDA-R is expressed by non-myelinating SCs (Campana et al., 2017) and terminal SCs (Woo et al., 2012) and both types of SCs are located in peripheral terminals, loss of terminal fibers may be related to SC abnormalities in GluN1[−] mice. Glutamate signaling is essential for proper development, maintenance, and differentiation of glial cells in the skin (Woo et al., 2012). These SCs have been shown to participate in sensation of mechanical stimuli (Abdo et al., 2019).

In summary, the NMDA-R is a major determinant of normal SC ultrastructure and function, particularly in non-myelinating SCs. *grin1* deletion in SCs alters the DRG transcriptome is associated with pain-related behaviors. Overall, these studies demonstrate an important relationship between SC physiology and neuropathic pain.

References

- Abdo H, Calvo-Enrique L, Martinez Lopez J, Song J, Zhang MD, Usoskin D, El Manira E, Adameyko I, Hjerling-Leffler J, Ernfors P (2019) Specialized cutaneous Schwann cells initiate pain sensation. *Science* 365:695–699.
- Antoine J, Boutahar N, Lassabliere F, Reynaud E, Ferraud K, Rogemond V, Paul S, Honnorat J, Camdessanche J (2015) Anti-fibroblast growth factor receptor 3 antibodies identify a subgroup of patients with sensory neuropathy. *J Neurol Neurosurg Psychiatry* 86:1347–1355.
- Asbury AK (1970) The histogenesis of phagocytes during Wallerian degeneration: Radio-autographic observations. *Proceedings of the VIth International Congress of Neuropathology*, pp 666–82. Paris: Masson & Cie.
- Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B Stat Methodol* 57:289–300.
- Bernstein Z, Yucht S, Battista E, Lema M, Spaulding M (1998) Proglumide as a morphine adjunct in cancer pain management. *J Pain Symptom Manage* 15:314–320.
- Bolger A, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114–2120.
- Boudes M, Sar C, Menigoz A, Hilaire C, Péquignot M, Kozlenkov A, Marmorstein A, Carroll P, Valmier J, Scamps F (2009) Best1 is a gene regulated by nerve injury and required for Ca²⁺ activated CL[−] current expression in axotomized sensory neurons. *J Neurosci* 29:10063–10071.
- Calvo M, Davies A, Hébert H, Weir G, Chesler E, Finnerup N, Levitt R, Smith B, Neely G, Costigan M, Bennett D (2019) The genetics of neuropathic pain from model organisms to clinical application. *Neuron* 104:637–653.
- Campana W (2007) Schwann cells: activated peripheral glia and their role in neuropathic pain. *Brain Behav Immun* 21:522–527.
- Campana WM, Darin SJ, O'Brien JS (1999) Phosphatidylinositol 3-kinase and Akt protein kinase mediate IGF-1- and prosaptide-induced survival in Schwann cells. *J Neurosci Res* 57:332–341.
- Campana WM, Li XQ, Dragojlovic N, Janes J, Gaultier A, Gonias SL (2006) The low density lipoprotein receptor related protein is a pro-survival receptor in Schwann cells: possible implications in peripheral nerve injury. *J Neurosci* 26:11197–11207.
- Campana WM, Mantuano E, Azmoon P, Henry K, Banki M, Kim J, Pizzo D, Gonias S (2017) Ionotropic glutamate receptors activate cell signaling in response to glutamate in Schwann cells. *FASEB J* 31:1744–1755.
- Castillo C, Norcini M, Martin Hernandez LA, Correa G, Blanck TJJ, Recio-Pinto E (2013) Satellite glia cells in dorsal root ganglia express functional NMDA receptors. *Neuroscience* 240:135–146.
- Chaplan S, Bach F, Pogrel J, Chung J, Yaksh T (1994) Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 53:55–63.
- Chen T, Kukley M (2020) Glutamate receptors and glutamatergic signalling in the peripheral nerves. *Neural Regen Res* 15:438–447.
- Christensen P, Welch N, Brideau C, Stys P (2016) Functional ionotropic glutamate receptors on peripheral axons and myelin. *Muscle Nerve* 54:451–459.
- Clauw D (2015) What is the meaning of “small fiber neuropathy” in fibromyalgia? *Pain* 156:2115–2116.
- Collison J (2018) CSF1 drives pain in arthritis. *Nat Rev Rheumatol* 14:562.
- Cui W, Yu X, Zhang H (2014) The serotonin transporter gene polymorphism is associated with the susceptibility and the pain severity in idiopathic trigeminal neuralgia patients. *J Headache Pain* 15:42–47.
- Dahlhamer J, Lucas J, Zelaya C, Nahin R, Mackey S, DeBar L, Kerns R, Von Korff M, Porter L, Helmick C (2018) Prevalence of chronic pain and high-impact chronic pain among adults — United States, 2016. *MMWR Morb Mortal Wkly Rep* 67:1001–1006.
- Devigili G, Tugnoli V, Penza P, Camozzi F, Lombardi R, Melli G, Broglio L, Granieri E, Lauria G (2008) The diagnostic criteria for small fibre neuropathy: from symptoms to neuropathology. *Brain* 131:1912–1925.
- Devigili G, Rinaldo S, Lombardi R, Cazzato D, Marchi M, Salvi E, Eleopra R, Lauria G (2019) Diagnostic criteria for small fibre neuropathy in clinical practice and research. *Brain* 142:3728–3736.
- Dixon W (1980) Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol* 20:441–462.
- Dobin A, Davis C, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras T (2013) STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29:15–21.
- Evans P, Reale V, Merzon R, Villegas J (1991) N-methyl-D-aspartate (NMDAR) and non-NMDAR (metabotropic) receptors modulate the membrane potential of the Schwann cell of the squid giant nerve fibre. *J Exp Biol* 173:229–279.
- Evdokimov D, Frank J, Klitsch A, Unterecker S, Warrings B, Serra J, Papagianni A, Saffer N, Meyer zu Altschildesche C, Kampik D, Malik R, Sommer C, Üçeyler N (2019) Reduction of skin innervation is associated with a severe fibromyalgia phenotype. *Ann Neurol* 86:504–516.
- Faroni A, Castelnovo L, Procacci P, Caffino L, Fumagalli F, Melfi S, Gambarotta G, Bettler B, Wrabetz L, Magnaghi V (2014) Deletion of GABA-B receptor in Schwann cells regulates Remak bundles and small nociceptive C-fibers. *Glia* 62:548–565.
- Feltri ML, D'Antonio M, Previtali S, Fasolini M, Messing A, Wrabetz L (1999) P0-Cre-transgenic mice for inactivation of adhesion molecules in Schwann cells. *Ann NY Acad Sci* 883:116–123.
- Feltri ML, Porta DG, Previtali SC, Nodari A, Migliavacca B, Casseti A, Littlewood-Evans A, Reichardt LF, Messing A, Quattrini A, Mueller U, Wrabetz L (2002) Conditional disruption of beta 1 integrin in Schwann cells impedes interactions with axons. *J Cell Biol* 156:199–209.
- Feltri ML, Poitelon Y, Previtali SC (2016) How Schwann cells sort axons: new concepts. *Neuroscientist* 22:252–265.
- Ferrari LF, Lufu CM, Araldi D, Rodrigues MA, Macedo LP, Ferreira SH, Parada CA (2014) Inflammatory sensitization of nociceptors depends on activation of NMDA receptors in DRG satellite cells. *Proc Natl Acad Sci USA* 111:18363–18368.
- Fink T, Davey D, Ansellin A (1999) Glutamatergic and adrenergic receptors expressed on adult guinea pig Schwann cells in vitro. *Can J Physiol Pharmacol* 77:204–210.
- Gasparotti R, Padua L, Briani C, Lauria G (2017) New technologies for the assessment of neuropathies. *Nat Rev Neurol* 13:203–216.
- Gerevich Z, Borvendeg SJ, Schröder W, Frank H, Wirkner K, Nörenberg W, Fürst S, Gillen C, Illes P (2004) Inhibition of N-type voltage activated calcium channels in rat dorsal root ganglion neurons by P2Y receptors is a possible mechanism of ADP-induced analgesia. *J Neurosci* 24:797–807.
- Griffin J, Thompson W (2008) Biology and pathology of non-myelinating Schwann cells. *Glia* 56:1518–1531.

- Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32:77–88.
- Harty B, Monk K (2017) Unwrapping the unappreciated: recent progress in Remak Schwann cell biology. *Curr Opin Neurobiol* 47:131–137.
- Hunskaar S, Fasmer O, Hole K (1985) Formalin test in mice, a useful technique for evaluating mild analgesics. *J Neurosci Methods* 14:69–76.
- Inoue M, Uchida Y, Edagawa M, Hirata M, Mitamura J, Miyamoto D, Taketani K, Sekine S, Kawauchi J, Kitajima S (2018) The stress response gene ATF3 is a direct target of the Wnt/ β -catenin pathway and inhibits the invasion and migration of HCT116 human colorectal cancer cells. *PLoS One* 13:e0194160.
- Jessen KR, Mirsky R (1984) Nonmyelin-forming Schwann cells coexpress surface protein and intermediate filaments not found in myelin-forming cells: a study of Ran-2, A5E3 antigen and glial fibrillary acidic protein. *J Neurocytol* 13:923–934.
- Jessen KR, Mirsky R (2005) The origin and development of glia cells in peripheral nerves. *Nat Rev Neurosci* 6:671–682.
- Jiang BC, Zhang WW, Yang T, Guo CY, Cao DL, Zhang ZJ, Gao YJ (2018) Demethylation of G-protein-coupled receptor 151 promoter facilitates the binding of Krüppel-like factor 5 and enhances neuropathic pain after nerve injury in mice. *J Neurosci* 38:10535–10551.
- Karel P, Calabrese F, Riva M, Brivio P, Van der Veen B, Reneman L, Verheij M, Homberg J (2018) d-Cycloserine enhanced extinction of cocaine-induced conditioned place preference is attenuated in serotonin transporter knockout rats. *Addict Biol* 23:120–129.
- Kinkelin I, Bröcker E, Koltzenburg M, Carlton S (2000) Localization of ionotropic glutamate receptors in peripheral axons of human skin. *Neurosci Lett* 283:149–152.
- Kowalska M, Prendecki M, Kozubski W, Lianeri M, Dorszewska J (2016) Molecular factors in migraine. *Oncotarget* 7:50708–50718.
- Kuhlbrodt K, Herbarth B, Sock E, Hermans-Borgmeyer I, Wegner M (1998) Sox10, a novel transcriptional modulator in glial cells. *J Neurosci* 18:237–250.
- Lauria G, Bakkers M, Schmitz C, Lombardi R, Penza P, Devigili G, Smith A, Hsieh S, Mellgren S, Umapathi T, Ziegler D, Faber C, Merkies I (2010) Intraepidermal nerve fiber density at the distal leg: a worldwide normative reference study. *J Periphere Nerv Syst* 15:202–207.
- Law C, Chen Y, Shi W, Smyth G (2014) voom: precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol* 15:R29.
- Lee C, Lü W, Michel J, Goehring A, Du J, Song X, Gouaux E (2014) NMDA receptor structures reveal subunit arrangement and pore architecture. *Nature* 511:191–197.
- Li B, Dewey C (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12:323.
- Luo W, Brouwer C (2013) Pathview: an R/bioconductor package for pathway-based data integration and visualization. *Bioinformatics* 29:1830–1831.
- Mantuano E, Lam M, Shibayama M, Campana W, Gonias S (2015) The NMDA receptor functions independently and as an LRP1 co-receptor to promote Schwann cell survival and migration. *J Cell Sci* 128:3478–3488.
- Maurel P, Salzer JL (2000) Axonal regulation of Schwann cell proliferation and survival and the initial events of myelination requires PI 3-kinase activity. *J Neurosci* 20:4635–4645.
- Murinson B, Griffin J (2004) C-fiber structure varies with location in peripheral nerve. *J Neuropathol Exp Neurol* 63:246–254.
- Namadurai S, Yereddi NR, Cusdin FS, Huang CLH, Chirgadze DY, Jackson AP (2015) A new look at sodium channel B subunits. *Open Biol* 5:140192.
- Newbern JM, Li X, Shoemaker SE, Zhou J, Zhong J, Wu Y, Bonder D, Hollenback S, Coppola G, Geschwind DH, Landreth GE, Snider WD (2011) Specific functions for ERK/MAPK signaling during PNS development. *Neuron* 69:91–105.
- Oaklander A (2016) What is the meaning of “small-fiber polyneuropathy” in fibromyalgia? An alternate answer. *Pain* 157:1366–1367.
- Ommer A, Figlia G, Pereira JA, Datwyler AL, Gerber J, DeGeer J, Lalli G, Suter U (2019) Ral GTPases in Schwann cells promote radial axonal sorting in the peripheral nervous system. *J Cell Biol* 218:2350–2369.
- Orita S, Henry K, Mantuano E, Yamauchi K, De Corato A, Ishikawa T, Feltri M, Wrabetz L, Gaultier A, Pollack M, Ellisman M, Takahashi K, Gonias S, Campana W (2013) Schwann cell LRP1 regulates Remak bundle ultrastructure and axonal interactions to prevent neuropathic pain. *J Neurosci* 33:5590–5602.
- Periquet M, Novak V, Collins M, Nagaraja H, Erdem S, Nash S, Freimer M, Sahenk Z, Kissel J, Mendell J (1999) Painful sensory neuropathy: prospective evaluation using skin biopsy. *Neurology* 53:1641–1641.
- Pittenger G, Ray M, Burcus N, McNulty P, Basta B, Vinik A (2004) Intraepidermal nerve fibers are indicators of small-fiber neuropathy in both diabetic and non-diabetic patients. *Diabetes Care* 27:1974–1979.
- Polydefkis M, Yiannoutsos C, Cohen B, Hollander H, Schifitto G, Clifford D, Simpson D, Katzenstein D, Shriver S, Hauer P, Brown A, Haidich A, Moo L, McArthur J (2002) Reduced intraepidermal nerve fiber density in HIV-associated sensory neuropathy. *Neurology* 58:115–119.
- Poplawski G, Ishikawa T, Brifault C, Lee-Kubli C, Regestam R, Henry K, Shiga Y, Kwon H, Ohtori S, Gonias S, Campana W (2018) Schwann cells regulate sensory neuron gene expression before and after peripheral nerve injury. *Glia* 66:1577–1590.
- Ritchie M, Phipson B, Wu D, Hu Y, Law C, Shi W, Smyth G (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43:e47.
- Robinson M, Oshlack A (2010) A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol* 11:R25.
- Robinson M, McCarthy D, Smyth G (2010) edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26:139–140.
- Sadjadpour K (1975) Postfacial palsy phenomena: faulty nerve regeneration or ephaptic transmission? *Brain Res* 95:403–406.
- Salerno KM, Jing X, Diges CM, Cornuet PK, Glorioso JC, Albers KM (2012) Sox11 modulates brain derived neurotrophic factor expression in an exon promoter specific manner. *J Neurosci Res* 90:1011–1019.
- Schmid A, Bland J, Bhat M, Bennett D (2014) The relationship of nerve fibre pathology to sensory function in entrapment neuropathy. *Brain* 137:3186–3199.
- Seal RP, Wang X, Guan Y, Raja SN, Woodbury CJ, Basbaum AI, Edwards RH (2009) Injury induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. *Nature* 462:651–655.
- Seijffers R, Mills C, Woolf C (2007) ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. *J Neurosci* 27:7911–7920.
- Shillo P, Sloan G, Greig M, Hunt L, Selvarajah D, Elliott J, Gandhi R, Wilkinson I, Tesfaye S (2019) Painful and painless diabetic neuropathies: what is the difference? *Curr Diab Rep* 19:32.
- Shy ME, Garbern JY, Kamholz J (2002) Hereditary motor and sensory neuropathies: a biological perspective. *Lancet Neurol* 1:110–118.
- Son S, Lee K, Jeon S, Park E, Park K, Cho H (2007) Activation of transcription factor c-jun in dorsal root ganglia induces VIP and NPY upregulation and contributes to the pathogenesis of neuropathic pain. *Exp Neurol* 204:467–472.
- Sopacua M, Hoeijmakers J, Merkies I, Lauria G, Waxman S, Faber C (2019) Small-fiber neuropathy: expanding the clinical pain universe. *J Peripher Nerv Syst* 24:19–33.
- Stewart HJ, Bradke F, Taberner A, Morrell D, Jessen KR, Mirsky R (1996) Regulation of rat Schwann cell Po expression and DNA synthesis by insulin like growth factors in vitro. *Eur J Neurosci* 8:553–564.
- Subramanian A, Tamayo P, Mootha V, Mukherjee S, Ebert B, Gillette M, Paulovich A, Pomeroy S, Golub T, Lander E, Mesirov J (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102:15545–15550.
- Sun W, Kou D, Yu Z, Yang S, Jiang C, Xiong D, Xiao L, Deng Q, Xie H, Hao Y (2020) A transcriptomic analysis of neuropathic pain in rat dorsal root ganglia following peripheral nerve injury. *Neuromolecular Med* 22:250–263.
- Tang S, Jing H, Huang Z, Huang T, Lin S, Liao M, Zhou J (2020) Identification of key candidate genes in neuropathic pain by integrated bioinformatic analysis. *J Cell Biochem* 121:1635–1648.
- Tarca A, Draghici S, Khatri P, Hassan S, Mittal P, Kim J, Kim C, Kusanovic J, Romero R (2009) A novel signaling pathway impact analysis. *Bioinformatics* 25:75–82.
- Taveggia C, Zanazzi G, Petrylak A, Yano H, Rosenbluth J, Einheber S, Xu X, Esper ML, Loeb JA, Shrager P, Chao MV, Falls DL, Role L, Salzer JL (2005) Neurotrophin-1 tyrosine II determines the ensheathment fate of axons. *Neuron* 47:681–694.
- Tour J, Löfgren M, Mannerkorpi K, Gerde B, Larsson A, Palstam A, Bileviciute-Ljungar I, Bjersing J, Martin I, Ernberg M, Schalling M, Kosek

- E (2017) Gene-to-gene interactions regulate endogenous pain modulation in fibromyalgia patients and healthy controls—antagonistic effects between opioid and serotonin-related genes. *Pain* 158:1194–1203.
- Ueda H (2008) Peripheral mechanisms of neuropathic pain — involvement of lysophosphatidic acid receptor-mediated demyelination. *Mol Pain* 4:11.
- Wang F, Cai B, Li KC, Hu XY, Lu YJ, Wang Q, Bao L, Zhang X (2015) FXRD2, a γ subunit of Na^+ , K^+ -ATPase, maintains persistent mechanical allodynia induced by inflammation. *Cell Res* 25:318–334.
- Wang H, Wang X, Zhong S, Shen Z (2013) Novel method for culturing Schwann cells from adult mouse sciatic nerve in vitro. *Mol Med Rep* 7:449–453.
- Webster HD, Martin JR, O'Connell MF (1973) The relationship between interphase Schwann cells and axons before myelination: a quantitative electron microscopic study. *Dev Biol* 32:401–416.
- Wingett SW, Andrews S (2020) FastQ Screen: A tool for multi-genome mapping and quality control. *F1000Res* 7:1338.
- Wong C, Gilkes D, Zhang H, Chen J, Wei H, Chaturvedi P, Fraley S, Wong C, Khoo U, Ng I, Wirtz D, Semenza G (2011) Hypoxia-inducible factor 1 is a master regulator of breast cancer metastatic niche formation. *Proc Natl Acad Sci USA* 108:16369–16374.
- Woo S, Baba Y, Franco A, Lumpkin E, Owens D (2012) Excitatory glutamate is essential for development and maintenance of the piloneural mechanoreceptor. *Development* 139:740–748.
- Yang J, He J, Lu J, Jie L (2018) Jun, Gal, Cd74, and C1qb as potential indicator for neuropathic pain. *J Cell Biochem* 119:4792–4798.
- Yin K, Deuis J, Lewis R, Vetter I (2016) Transcriptomic and behavioural characterisation of a mouse model of burn pain identify the cholecystokinin 2 receptor as an analgesic target. *Mol Pain* 12:174480691666536.
- Zhang B, Kirov S, Snoddy J (2005) WebGestalt: an integrated system for exploring gene sets in various biological contexts. *Nucleic Acids Res* 33:W741–W748.