The potassium channel Kv1.6 has recently been implicated as a major modulatory channel subunit expressed in primary nociceptors. Furthermore, its expression at juxtaparanodes of myelinated primary afferents is induced following traumatic nerve injury as part of an endogenous mechanism to reduce hyperexcitability and pain-related hypersensitivity. In this study, we compared two mouse models of constitutive Kv1.6 knock-out (KO) achieved by different methods: traditional gene trap via homologous recombination and CRISPR-mediated excision. Both Kv1.6 KO mouse lines exhibited an unexpected reduction in sensitivity to noxious heat stimuli, to differing extents: the Kv1.6 mice produced via gene trap had a far more significant hyposensitivity. These mice (Kcnal6lacZ) expressed the bacterial reporter enzyme LacZ in place of Kv1.6 as a result of the gene trap mechanism, and we found that their central primary afferent pre-synaptic terminals developed a striking neurodegenerative phenotype involving accumulation of lipid species, development of reporter enzyme LacZ in place of Kv1.6 as a result of the gene trap mechanism, and we found that their central primary afferent pre-synaptic terminals developed a striking neurodegenerative phenotype involving accumulation of lipid species, development of “meganeurites,” and impaired transmission to dorsal horn wide dynamic range neurons. The anatomic defects were absent in CRISPR-mediated Kv1.6 KO mice (Kcnal6−/−) but were present in a third mouse model expressing exogenous LacZ in nociceptors under the control of a Nav1.8-promoted Cre recombinase. LacZ reporter enzymes are thus intrinsically neurotoxic to sensory neurons and may induce pathologic defects in transgenic mice, which has confounding implications for the interpretation of gene KOs using lacZ. Nonetheless, in Kcnal6−/− mice not affected by LacZ, we demonstrated a significant role for Kv1.6 regulating acute noxious thermal sensitivity, and both mechanical and thermal pain-related hypersensitivity after nerve injury.

Key words: gangliosides; Kv1.6; LacZ; neuropathic pain; nociception; pain

Significance Statement

In recent decades, the expansion of technologies to experimentally manipulate the rodent genome has contributed significantly to the field of neuroscience. While introduction of enzymatic or fluorescent reporter proteins to label neuronal populations is now commonplace, often potential toxicity effects are not fully considered. We show a role of Kv1.6 in acute and neuropathic pain states through analysis of two mouse models lacking Kv1.6 potassium channels: one with additional expression of LacZ and one without. We show that LacZ reporter enzymes induce unintended defects in sensory neurons, with an impact on behavioral data outcomes. To summarize we highlight the importance of Kv1.6 in recovery of normal sensory function following nerve injury, and careful interpretation of data from LacZ reporter models.
Introduction
The Shaker-like Kv1 potassium channel family comprises 8 α subunits (Kv1.1-Kv1.8), each considered a mammalian homolog of the Shaker channel that produced hyperexcitable sensorimotor phenotypes in Drosophila when mutated. These channels have also been shown to be key regulators of neuronal excitability in mammals (Browne et al., 1994; Smart et al., 1998; Robbins and Tempel, 2012). The most comprehensive recent reports indicate that Kv1.1, Kv1.2, Kv1.4, and Kv1.6 are expressed in mouse sensory neurons (Zeisel et al., 2018; Zheng et al., 2019). Although mouse KO studies have shown that Kv1.1 and Kv1.2 regulate dorsal root ganglion (DRG) neuron excitability, there have been few studies of the role of Kv1.6 (encoded by Kcnab6) in DRG neurons and in sensory coding.

Recent single-cell transcriptomic approaches detected expression of Kcnab6 mRNA throughout the mouse nervous system (Usoskin et al., 2015; Häring et al., 2018; Zeisel et al., 2018) and, within sensory ganglia, Kcnab6 is reported to be the second most abundant potassium channel subunit in nociceptive populations (Zheng et al., 2019). Blockade of Kv1.1/1.2/1.6-containing channels by α-dendrotoxin in Mrgrp-d expressing neurons increased the repetitive firing capability of these nociceptive neurons in vitro (Zheng et al., 2019).

Kv1.6 is upregulated in rodent and human myelinated primary somatosensory neurons after peripheral nerve injury (Calvo et al., 2016). Under these conditions, Kv1.6 subunits emerge at neuronal sites flanking the node of Ranvier and replace native subunits Kv1.1 and Kv1.2, which are known to be downregulated transcriptionally and at the protein level shortly after peripheral nerve injury (Ishikawa et al., 1999; Zhao et al., 2013; Calvo et al., 2016; Hong et al., 2016; González et al., 2017). The timing of Kv1.6 appearance at the juxtaparanode (JXP) and paranode corresponds with both a reduction of ectopic electrical activity and improved withdrawal thresholds for reflexes evoked mechanically by von Frey hairs (Calvo et al., 2016). However, both of these recovery effects were reversed by pharmacological blockade of Kv1.6-containing channels with local application of α-dendrotoxin (Calvo et al., 2016). It is therefore suggested that Kv1.6 subunits are involved in a compensatory response to peripheral nerve injury.

These data suggest that Kv1.6-containing channels provide “brake”-like countercurrents that oppose neural excitation in sensory neurons, as described for other Kv1 subunits (Madrid et al., 2009; Hao et al., 2013; Zhao et al., 2013; González et al., 2017; Dawes et al., 2018). In this study, we have sought to detail the expression of Kcnab6 in the mouse sensory neuraxis, with a predominant focus on primary afferent neurons of the DRGs. In addition to this, through a global gene KO strategy, we have studied sensorimotor behavior in two Kcnab6-null mouse strains (termed Kcnab6lacZlacZ and Kcnab6−/−) to identify a role for Kv1.6-containing channels in pain and/or somatosensation in the contexts of acute and neuropathic pain. Surprisingly, results were not consistent between Kcnab6 KO strains; and we provide anatomic, electrophysiological, and behavioral evidence that the presence or absence of exogenous lacZ reporter cassette accounts for the phenotypic differences between these two mouse strains. Furthermore, we show that expression of lacZ alone on an otherwise phenotypically normal background is sufficient to cause deleterious effects to nociceptive presynaptic terminals in the dorsal horn. Notwithstanding these unexpected consequences of genetic manipulation, the findings of this study support a role for Kcnab6 in acute noxious thermal sensation and in contributing to recovery of normal sensory function in a neuropathic pain model.

Materials and Methods

Animals
Behavioral experiments involving the use of uninjured mice were performed in compliance with the Animals (Scientific Procedures) Act 1986, under UK Home Office-issued project licenses 30/3015 and P1DBEBAB9 held by Prof. David Bennett at the University of Oxford. Behavioral experiments involving the chronic constriction injury (CCI) model of neuropathy were performed at Pontificia Universidad Catolica de Chile and approved by the Institutional Ethics Committee (protocol ID 150714013). In vivo electrophysiological experiments were performed at University College London under UK Home Office project license PEB669065 held by Prof. Anthony Dickenson. Male and female mice were used throughout.

Kcnab6loxP mice: Kcnab6loxP/Mmucd mice were purchased from MMRRC (RRID:MMRRC_011729-UCD) on a mixed C57BL/6J;129S5 background, with heterozygote or WT littermates used as controls in all experiments. The Kcnab6loxP allele was produced by Lexicon Genomics (now Lexicon Pharmaceuticals) by homologous recombination of a gene trap cassette, containing lacZ/neo selection genes, with the coding sequence of Kcnab6 exon 1, depicted in Figure 3A. Kcnab6loxP/loxP; Advrillin–EGFP mice. In order to label primary afferent terminals within the spinal cord, the Kcnab6loxP strain was interbred with a mouse strain expressing a BAC transgene encoding EGFP under the Advrillin promoter, Tg(Avl-EGFP)QD84Gsat/Mmucd (RRID:MMRRC_034769-UCD). Kcnab6−/− mice: Kcnab6−/− (MMRRC_011729-UCD); Mmijak mice were purchased from MMRRC (RRID:MMRRC_024291-JAX) and maintained on a C57BL/6N background, with heterozygote or WT littermates used as controls in all experiments. All of the nature of the mutation is Cas9-mediated excision of the sole coding region, leaving the initial 7 residues intact and resulting in frame shift and premature STOP codon. Navi1.6loxP/loxP; ROSA26loxP/loxP mice: The Gt(Rosa)26Sor+tm1Sor/J mouse is a conditional-ready strain carrying a loxP-neo-polyA-loxP-lacZ cassette in the ubiquitously expressed ROSA26 locus (Soriano, 1999). Thus, there is ubiquitous expression of neomycin phosphotransferase (neo), followed by a poly A tail, which ordinarily prevents read-through transcription of lacZ. The neo-poly A sequence is flanked by loxP sites, so Cre-mediated recombination excises this region and results in expression only of lacZ. Homozygous conditional-ready Gt(Rosa)26Sor+tm1Sor/J mice were gifted kindly by Prof. Shankar Srinivas (DPAG, University of Oxford) to serve as founders of the Navi1.6loxP/loxP; ROSA26loxP/loxP colony by crossing with in-house heterozygous Nav1.8 Cre mice (Nassar et al., 2004; Stirling et al., 2005) gifted by Prof. John Wood, University College London. This produced compound heterozygotes expressing one lacZ allele at the ROSA26 locus in Nav1.8-positive neurons. Nav1.8 Cre-negative littermates did not conditionally express LacZ but constitutively expressed a neomycin phosphotransferase cassette at the ROSA26 locus.

Tissue preparation for immunohistochemistry and in situ hybridisation (ISH)
To extract tissue for cryo-sectioning, animals were culled by overdose of pentobarbital delivered via intraperitoneal injection, followed by transcardial perfusion with 15 ml sterile 0.9% w/v saline, then 20 ml 4% PFA in 0.1 M PB. Dissected tissues were postfixed in 4% PFA for varied duration depending on tissue, as determined previously in our laboratory (Dawes et al., 2018): 1.4 DRG, 2 h (room temperature [RT]); spinal cord, 24 h (4°C); glabrous skin, 0.5 h (RT). For analysis of epidermal nerve fibers, skin was dissected from the glabrous region of the hind-paw proximal to the most proximal touch dome, as depicted in Figure 6A. After fixation, tissue was transferred to 30% sucrose in 0.1 M PB at 4°C for at least 24 h. Subsequently, tissue was embedded in OCT medium (TissueTek), rapidly frozen with liquid nitrogen, and stored at −80°C before cryo-sectioning. Sections were slide-mounted on Thermo Scientific SuperFrost Plus slides, with sections cut using a Leica cryostat (Leica Microsystems), at tissue-dependent thickness: DRG, 10 μm; spinal cord, 20 μm; skin, 14 μm.

ISH
Expression of Kcnab6 mRNA was investigated using a chromogenic ISH assay (RNAscope 2.5-RED, Advanced Cell Diagnostics). In brief, slides were removed from −80°C storage and allowed to equilibrate to room temperature before removal of OCT by submersion in DNNase/
RNAse-free PBS. Slides were pretreated with a 10 min application of 
H₂O₂ at room temperature, washed with double-deionized Milli-Q 
water, submerged in 100% EtOH, and allowed to dry (20 min) before a 
10 min application of protease plus in a humidified chamber at 40°C, fol-

donated to the same for all quan-

RNA extraction and cDNA synthesis
Mice were culled by rising CO₂ concentration in a sealed chamber.
Brains were rapidly dissected, immediately frozen in liquid nitrogen, and 
stored at −80°C. Tissue was homogenized in TriPure (Roche) and then 
treated with chloroform before column purification using a High Pure 
RNA isolation kit (Roche). RNA was eluted in RNAase-free water. 
Synthesis of cDNA was achieved using Transcriptor reverse transcriptase 
(Roche) with random hexamers (Invitrogen) and dNTPs (Roche).

qRT-PCR
Quantitative analysis of mRNA expression was conducted on a 
LightCycler 480 II system (Roche), using SYBR Green fluorescence for 
detection. Primers were designed using Primer-BLAST (https://www.
ncbi.nlm.nih.gov/tools/primer-blast/) and were assessed for specificity and 
efficiency by melting curve analysis, and by serial dilution. Primers 
(0.5 μmol) and cDNA (5 ng) were mixed with LightCycler 480 SYBR 
Green master mix (Roche) in a 1:1 ratio in 384-well plates and run on a 
45-cycle protocol. All experimental samples were run in triplicate. 
Quantitation of expression was performed using the 2^ΔΔCt method 
(Livak and Schmittgen, 2001), and Kv1 gene products were first normal-
ized to “housekeeping” genes (Gapdh and Hprt1) and then normalized to 
average WT expression per gene. Primer sequences are detailed in the 
Table 1.

Behavioral assays
Mice were housed in individually ventilated cages with no more than 6 
or no fewer than 2 mice per cage, and fed ad libitum. The light-dark 
cycle period was 12 h, and behavioral experiments were performed dur-
ing the light cycle. Multiple different behavioral assays were performed each day, but the time of day for each behavioral assay was kept constant. 
Before all experiments, animals were habituated to equipment so that 
the experimental environment was not novel during data collection. 
Unless otherwise stated, baseline results were averaged from three base-

The experimenter was blinded to genotype at all times during behavioral 
testing and analysis. Details of individual assays are as follows:

Open field. Mice were placed in the corner of a 50 × 30 cm wooden 
box with a 5 × 3 grid marked on the base and allowed to move freely 
for 3 min. During this time, the number of grid lines crossed and hindlimb 
rearing episodes were recorded.

Rotarod. Mice were required to climb/walk against the direction of the 
platform (Ugo Basile) rotating at 28 rpm beneath them. The 

von Frey. Mice were housed individually in Plexiglas enclosures on a 
metallic grille platform. After an acclimation period of between 30 min 
and 1 h, 50% mechanical withdrawal thresholds were derived using the 
Dixon up-and-down method (Dixon, 1965). Baseline measurements 
were averaged from left and right paw.

Hargreaves. Mice were housed individually in Plexiglas enclosures on a 
glass platform. An infrared heat source (Ugo Basile) was placed 
under the animal, targeted through the glass at the hind-paw. Switching 
the heat source begins a timer, which turns off once the animal 
responds to the stimulus by relocating its paw. Otherwise, the test was 
stopped at a maximum of 20 s.

Pin-prick. Animals were temporally housed individually in Plexiglas 
chambers on a grille used for von Frey testing. A sharp pin was fixed to 
the filament of a 2.0 g von Frey hair and used to provide noxious punc-
tate stimulation to left and right hind-paws. Withdrawal reflexes were 
filmed from below using an iPhone8 in slo-mo mode (720 p, 240 fps) 
such that still frames are ~4 ms apart. The withdrawal latency was 
derived post hoc by scrolling through frames from the time of contact 
with the paw to withdrawal. Two or occasionally three stimuli were 

tinprick, and dry ice results.
temperature of interest (50°C or 53°C). The mouse was then allowed to move freely from plate to plate for 2 min. Movements were recorded by a camera mounted directly above the enclosed space. Data are expressed as the percentage of the time spent on the test plate.

Intraepidermal nerve fiber density
Following immunostaining, three sections each from 4 animals per Kcna6lacZ genotype were analyzed on a Carl Zeiss LSM700 confocal microscope. Using DAPI to mark the dermis/epidermis boundary, the number of Pgp9.5-immunoreactive fibers that could be visualized crossing from dermis to epidermis was counted through the viewfinder with a 63×/C2 oil immersion lens. A post hoc overview image was taken, and the length of epidermis analyzed was measured in ZEN Black software; across all sections, this ranged between 2.1 and 3.3 mm.

DRG subpopulation counts and Atf3 expression
For subpopulation analysis, at least 2 whole DRG sections were imaged and analyzed per animal. DRG “profiles” were defined as being Neurotrace-positive and nucleated. Profiles were designated positive or negative for histochemical markers by eye on images of whole DRG sections taken with identical acquisition settings, and their abundance was calculated as a percentage of Neurotrace-positive profiles (fluorescent Nissl stain). For Atf3 analysis, at least four randomly chosen fields of view were analyzed from DRGs dissected from 4 Kcna6lacZ/lacZ animals and 4 WT littermates.

Transmission electron microscopy (TEM)
Adult mice received an intraperitoneal overdose of pentobarbital and were then transcardially perfused with 0.9% saline followed by a 1% PFA, 1% glutaraldehyde fixative in 0.1M PB. Spinal cords were then removed and postfixed in the same solution for 24 h and transferred to 0.1M PB before sectioning; 60 μm sections were prepared using a vibratome, postfixed with osmium tetroxide OsO4, dehydrated, and flat-embedded in epoxy resin. At this stage, some images were acquired using light microscopy. Subsequently, ultrathin sections were cut using a Diatome diamond knife and Leica Ultracut S.
mate mice were used; the mean ± SD ages of animals were as follows: 17.3 ± 1.1 (Kcnab6lacZ/lacZ), 14.7 ± 1.1 (Kcnab6lacZ/lacZ), and 17.1 ± 2.5 (Kcnab6lacZ/lacZ) weeks. Mice were initially anesthetized with 3.5% v/v isoflurane for the remainder of the experiment (1.5% isoflurane delivered in a 3:2 ratio of nitrous oxide and oxygen. Once areflexic, mice were transferred to a thermostatically controlled water-perfused warming blanket, and respiratory rate was visually monitored throughout. A laminectomy was performed to expose the L3-L5 segments of the spinal cord; mineral oil was then applied to prevent dehydration. Extracellular recordings were made from deep dorsal horn wide dynamic range (WDR) neurons with receptive fields on the glabrous skin of the toes using 0.127 mm 2 mΩ Parylene-coated tungsten electrodes (A-M Systems). Searching involved light tapping of the hind-paw while manually moving the electrode. All recordings were made at depths delineating the deep dorsal horn laminae (Watson et al., 2009), and were classified as WDR on the basis of neuronal sensitivity to dynamic brushing (i.e., gentle stroking with a squirrel-hair brush), and noxious punctate mechanical (15 g) and heat (48°C) stimulation of the receptive field.

The receptive field was then stimulated using a wider range of natural stimuli (brush, von Frey filaments, heat, cold, and 15 g, and heat 32°C, 42°C, 45°C, and 48°C) applied over a period of 10 s per stimulus and the evoked response quantified. The heat stimulus was applied with a constant water jet onto the center of the receptive field. Ethyl chloride (50 μl) was applied to the receptive field, described previously as an evaporative noxious cooling stimulus (Leith et al., 2010). Evoked responses to room temperature water (25°C) were subtracted from ethyl chloride-evoked responses to control for any concomitant mechanical stimulation during application. Natural stimuli were applied starting at the lowest intensity stimulus with ~40 s between stimuli in the following order: brush, von Frey, cold, heat, electrical. Receptive fields were determined using a 15 g von Frey. An area was considered part of the receptive field if a response of >30 action potentials over 5 s was obtained. A rest period of 30 s between applications was used to avoid sensitization. Receptive field sizes are expressed as a percentage area of a standardized paw measured using ImageJ (National Institutes of Health). Electrical stimulation of WDR neurons was delivered transcutaneously via needles inserted into the receptive field. A train of 16 electrical stimuli (2 ms pulses, 0.5 Hz) was applied at 3 times the threshold current for C-fiber activation. Responses evoked by A-/C-fibers (50-250 ms) were separated and quantified on the basis of latency. Neuronal responses occurring after the C-fiber latency band were classified as postdischarge. The input and the corresponding function were measured by calculating the input-output function. Responses evoked by A- (0-50 ms) and C-fibers (50-250 ms) were recorded and digitized at a rate of 20 kHz. Data were captured and analyzed by a Cambridge Electronic Design 1401 interface coupled to a computer with Spike2 software with poststimulus time histogram and rate functions. In some cases where a single-unit recording could not be obtained, spike sorting was performed post hoc with Spike2 using fast Fourier transform followed by three-dimensional principal component analysis of waveform features for multunit discrimination (electrical stimulation was not performed for these neurons). One to three neurons were characterized per mouse. In total, 19 mice were characterized from 14 WT mice (7 male, 7 female), 12 neurons from 6 Kcnab6lacZ/lacZ mice (10 male, 6 female), and 19 neurons from 15 Kcnab6lacZ/lacZ mice (6 male, 9 female). Of these neurons, 15 per genotype were successfully stimulated transcutaneously so that electrical properties could be interrogated. In vivo electrophysiological procedures were nonrecovery; at the end of experiments, mice were terminally anesthetized with isoflurane.

In vivo dorsal horn recordings
Both male and female Kcnab6lacZ/lacZ carriers, but not WT littermates, express β-galactosidase under the Kcnab6 promoter, detectable in DRG cells by immunostaining. In addition to prominent β-galactosidase immunoreactivity in small neurons, staining revealed LacZ-expressing satellite glia ensheathing DRG neurons, confirmed with an antibody raised against satellite glial marker, glutamine synthetase (GluN1, GluN2A, and GluN2B). The appearance of identifiable glomerular structures was interpreted qualitatively. The signal was amplified (∼6000), bandpass filtered (low-/high-frequency cutoff 0.5/2 kH), and digitized at a rate of 20 kHz. Data were captured and analyzed by a Cambridge Electronic Design 1401 interface coupled to a computer with Spike2 software with poststimulus time histogram and rate functions. In some cases where a single-unit recording could not be obtained, spike sorting was performed post hoc with Spike2 using fast Fourier transform followed by three-dimensional principal component analysis of waveform features for multunit discrimination (electrical stimulation was not performed for these neurons). One to three neurons were characterized per mouse. In total, 19 neurons were characterized from 14 WT mice (7 male, 7 female), 21 neurons from 16 Kcnab6lacZ/lacZ mice (10 male, 6 female), and 19 neurons from 15 Kcnab6lacZ/lacZ mice (6 male, 9 female). Of these neurons, 15 per genotype were successfully stimulated transcutaneously so that electrical properties could be interrogated. In vivo electrophysiological procedures were nonrecovery; at the end of experiments, mice were terminally anesthetized with isoflurane.

Figure 2. LacZ (β-galactosidase) expression in Kcnab6lacZ/lacZ DRG. A, Kcnab6lacZ carriers, but not WT littermates, express β-galactosidase under the Kcnab6 promoter, detectable in DRG cells by immunostaining. B, In addition to prominent β-galactosidase immunoreactivity in small neurons, staining revealed LacZ-expressing satellite glia ensheathing DRG neurons, confirmed with an antibody raised against satellite glial marker, glutamine synthetase (GluN1, GluN2A, and GluN2B). The appearance of identifiable glomerular structures was interpreted qualitatively.
Experimental design and statistical analyses

GraphPad Prism software was used for all statistical calculations. In general, unless otherwise stated, the unit of statistical analysis was the individual animal and significance is reported as multiplicity-adjusted p values. N values and details of mouse sex are included in figure legends as appropriate.

ISH. Expression of Kcna6 mRNA was quantified by calculating the mean red channel pixel intensity (converted from 0 to 255 8-bit color value to a percentage scale) in ROIs drawn in ImageJ software around cells with identifiable nuclei; thus, expression is normalized to cell size (sum of pixel intensities/number of pixels). “Background” was from ROIs containing nerve root. Mean red channel pixel intensity (Wilk test and means compared with a two-tailed, unpaired Student’s t test) was from Shapiro–Wilk test. If they passed, a Kruskal–Wallis test was performed instead with Dunn’s test to make multiple comparisons versus WTs or between all genotypes, respectively. For characteristics that failed the normality test, a nonparametric Kruskal–Wallis test was performed instead with Dunn’s test to make multiple comparisons versus WTs or between all genotypes.

In vivo electrophysiology. In all experiments, the neuron was considered to be the experimental unit of replication. Minimum group sizes were determined by a priori calculations using the following assumptions (n = 0.05, 1-β 0.8, e 1, effect size range d = 0.5-0.8). Effect sizes were based on historical datasets. Mechanical and heat responses were analyzed by two-way repeated-measures ANOVA with Dunnett’s post hoc test to compare to WT results. Brush, cold, fiber threshold, electrical results, and receptive field area were assessed for normality using the Shapiro–Wilk test. If they passed, each characteristic was assessed by one-way ANOVA with Dunnett’s or Tukey’s post hoc test to make multiple comparisons versus WTs or between all genotypes, respectively. For characteristics that failed the normality test, a Kruskal–Wallis test was performed instead with Dunnett’s post hoc multiple comparisons test. Multiplicity-adjusted p values are reported in each case.

Figure 3. Kcna6ΔlacZ mice are hyposensitive to noxious heat. A, The <tm1Lex> vector, containing a lacZ cassette, is introduced to the coding sequence (CDS) of Kcna6 exon 1 by homologous recombination, resulting in Kcna6 KO as confirmed by qRT-PCR (a.u., arbitrary units relative to WT expression) on brain RNA extract using 2 sets of primers. Expression of two other Kv1 subunits (Kv1.1 and Kv1.2) is unaffected by Kcna6 KO. n = 4 WT, 3 HET, 4 KO. ***p < 0.0001 (ordinary one-way ANOVA with Tukey’s post hoc multiple comparisons test). B–E, Kcna6ΔlacZ–/– and Kcna6ΔlacZ+/– mice are hyposensitive to 50°C and 53°C hot plate assays, while both heterozygous and homozygous mutants are hyposensitive on the Hargreaves radiant heat assay. I, Neither groups differ from WTs in their response to mechanical stimulation by von Frey hairs. Data points represent individual animal values, plotted with group mean ± SEM. n = 20 Kcna6+/+, 15 Kcna6ΔlacZ+/− (13 male, 2 female), 22 Kcna6ΔlacZ−/− (11 male, 11 female), apart from the beam test (E): n = 12 Kcna6+/+ (6 male, 6 female), 12 Kcna6ΔlacZ−/− (10 male, 2 female), 10 Kcna6ΔlacZ−/− (6 male, 4 female). ***p < 0.001, ****p < 0.0001 versus Kcna6+/+ and Kcna6ΔlacZ−/− (F, G) or versus Kcna6ΔlacZ+/− (E) (one-way ANOVA with Tukey’s post hoc multiple comparisons test).
Expression of Kcna6 in the mouse DRG

Using a chromogenic ISH protocol (ACD), we visualized Kcna6 expression in WT mouse L4 DRG neurons (Fig. 1A). Overall Kcna6 expression was higher in smaller versus larger DRG neuronal populations. Across all cells examined from 4 animals, there is a significant negative correlation between mean Kcna6 pixel intensity and cell area (Fig. 1Bii); and across biological replicates, small-diameter neurons had significantly greater expression than large-diameter neurons (Fig. 1Biii). We quantified Kcna6 expression in five immunohistochemically defined subpopulations of sensory neurons: namely, myelinated neurons expressing neurofilament 200 (Nf200); peptidergic nociceptors expressing calcitonin gene-related peptide (Cgrp); C-low

Figure 4. Abnormal anatomy in the Kcna6lacZ dorsal horn. A, LacZ (β-gal) staining in Kcna6lacZ homozygote spinal cord sections revealed striking abnormal, large profiles within superficial dorsal laminae. LacZ immunoreactivity also colocalized with smaller NeuN-negative, DAPI-positive profiles throughout gray and white matter. Inset, High-power (63 × objective) single z-slice image of abnormal swollen, vacuolated LacZ+ profiles in laminae I-II. Scale bars: 100 μm; inset, 10 μm. B, Abnormal LacZ+ profiles do not colocalize with Pax2 (a marker of inhibitory interneurons), nor do smaller LacZ+ profiles in deeper gray and white matter. Scale bar, 100 μm. C, In superficial laminae, LacZ does not colocalize with markers of astrocytes (GFAP) or microglia (IBA1), but glial activation is often observed in close apposition to the abnormal LacZ+ profiles. Scale bar, 10 μm. D, Markers of nonpeptidergic (IB4) and peptidergic (Cgrp) nociceptive populations known to terminate in superficial laminae show strong colocalization with enlarged profiles in Kcna6lacZ homozygote spinal cord sections. Scale bars: 100 μm; inset, 25 μm.
threshold mechanoreceptors expressing tyrosine hydroxylase (Th1C-LTMRs); and nonpeptidergic nociceptors binding to isolectin B4 (IB4). Overlap between the Nf2001 and Cgrp1 population demarcates the fifth subpopulation of myelinated peptidergic nociceptors. Signal from the ISH was detected significantly above background fluorescence in nociceptive populations but not in the Th1 or Nf2001/Cgrp− populations (Fig. 1C).

The strongest expression of Kcna6 was observed in myelinated peptidergic neurons (Nf2001/Cgrp1). We also noted Kcna6 expression in satellite glial cells ensheathing DRG neurons, as identified by staining for glutamine synthetase (Figs. 1A and 2).

Kcna6lacZ KO mice exhibit altered nociceptive behavior

In order to investigate the contribution of Kv1.6 subunits to pain-like behavior in mice, we procured a Kcna6 KO mouse produced by homologous recombination with a <tm1Lex> cassette containing a lacZ reporter allele and neomycin phosphotransferase, henceforth termed Kcna6lacZ (MMRRC stock #011729-UCD). We confirmed expression of LacZ protein (β-galactosidase) by immunohistochemical detection in appropriate DRG populations and satellite glia (Fig. 1C).

Behavioral phenotyping of this KO strain included an array of sensorimotor assays. Heterozygous or homozygous Kcna6lacZ mice performed comparably to WT littermates on open field assays and motor coordination tasks (Fig. 3B–E). Homozygous Kcna6lacZ/lacZ KOs had a striking deficit in noxious thermal sensitivity on both hot plate (50°C and 53°C) and Hargreaves assays, whereas heterozygous littermates were hyposensitive only to stimulation via the Hargreaves test (Fig. 3F–H). Responsiveness to light punctate mechanical stimuli via von Frey hair application was unaltered in either genotype compared with WT littermates (Fig. 3I).

Kcna6lacZ mice develop abnormal synapse-forming primary afferent terminals in the superficial dorsal horn

The most striking finding was a gross anatomic abnormality in the spinal dorsal horn of Kcna6lacZ mice. These abnormalities

Figure 5. Degeneration of Kcna6lacZ primary afferents is progressive and gene dose-dependent. A, An Advillin-driven EGFP reporter mouse was crossed to the Kcna6lacZ homozygotes to label DRG primary afferents marked by Nissl staining. Scale bar, 50 μm. B, EGFP colocalized with the abnormal spinal profiles, confirming that they are primary afferent in origin. The pathology was present by 6 weeks postnatally but also appeared to worsen with age. Scale bar, 100 μm. C, Kcna6lacZ heterozygotes had a late-onset, slowly progressing pathology over a period of 11 months, evidenced by LacZ (C, ii) or IB4 (C, iii) staining, whereas WTs were unaffected at 11 months (C, i). Scale bar, 100 μm. D, At 11 months, despite developing primary afferent terminal pathology, Kcna6lacZ−/− heterozygote mice did not display any behavioral hyposensitivity to noxious heat compared with WT age-matched littermate controls. n = 4 Kcna61/−, 4 Kcna6lacZ−/− (2 male, 2 female per genotype). p = 0.845 (50°C) and p = 0.7273 (53°C) by unpaired, two-tailed t test.
Figure 6. Intraepidermal nerve fiber morphology and density are unaltered in KcnalacZ/lacZ mice. A, Representative images of epidermal innervation from the indicated hind-paw region of KcnalacZ/lacZ mice and WT littermates. In KO mice, β-galactosidase immunoreactivity colocalizes with the pan neuronal marker Pgp9.5 in the dermal nerve plexus. Scale bar, 15 μm. B, Nerve fiber density in the epidermis did not differ significantly between KO and WT mice. Data are mean ± SEM; mean values from individual animals. n = 4 KcnalacZ/lacZ(2 male, 2 female per genotype). p = 0.4427 (unpaired, two-tailed t test).

Figure 7. KcnalacZ/lacZ mice do not exhibit signs of cellular injury to or loss of DRG subpopulations. A, Despite pathology of primary afferent terminals, a lack of Atf3 upregulation suggests that injury-related gene-expression changes are not initiated in KcnalacZ/lacZ DRGs. A positive control image is provided from a WT mouse that underwent spared nerve injury. Scale bar, 50 μm. B, Representative images of whole DRG sections KcnalacZ/lacZ mice and WT littermates. Inset, Magnification of dashed ROI. Sections were stained for Nf200 and Cgrp, or IB4 and Th, with a fluorescent Nissl counterstain (Neurotrace). Scale bar, 100 μm. C, Mean ± SEM percentages for each DRG subpopulation marker relative to Neurotrace (Nissl)-positive neurons. There is no significant difference between KcnalacZ/lacZ mice and WT littermates. Adjusted p values comparing genotypes are indicated from multiple unpaired t tests, performed with 5% false discovery rate using the Benjamini, Krieger, and Yekutieli two-stage setup method. n = 4 KcnalacZ/lacZ(2 male, 2 female per genotype).
relate to the manipulation of the Kcna6 locus, since WT littermates were anatomically normal and the abnormal structures in Kcna6lacZ/lacZ dorsal horn were intensely immunoreactive for the lacZ gene product β-galactosidase/LacZ (Fig. 4A–D). Specifically, we identified that central axonal projections of primary afferent neurons had swollen features in superficial as well as deep dorsal horn laminae. These abnormal swellings did not colocalize with neuronal (NeuN, Pax-2) or glial (GFAP, astrocytes; Iba1, microglia) markers (Fig. 4A–C). There was, however, immunohistochemical colocalization with IB4 and Cgrp (Fig. 4D), and interbreeding Kcna6lacZ/lacZ mice with Advillin-EGFP reporter mice (MMRRC stock #034769-UCD) demonstrated colocalization of EGFP (Fig. 5A,B), confirming their primary afferent origin. The abnormal profiles were detected in both male and female Kcna6lacZ/lacZ mice. Some β-galactosidase staining distinct from the primary afferent terminals was detected throughout the gray and white matter, occasionally colocalizing with Pax-2 (Fig. 4A,B). Since this LacZ reporter is driven by the endogenous Kcna6 promoter, this likely reflects

**Figure 8.** Ultrastructural analysis of degenerative Kcna6lacZ primary afferents. A, The 60-μm-thick osmicated sections showing normal anatomy in WT tissue (AI), and lipid-rich profiles in the superficial Kcna6lacZ/lacZ dorsal horn (Aii). B, C, Swollen terminals (red dashed areas) vary in size and electron density and contain many small vesicular components. D, Some of the abnormal vesicle-containing profiles originate from myelinated afferents in Lissauer’s tract. E, Numerous profiles were observed adjacent to postsynaptic dendrites (asterisk) and sprouting intervaricose axons (arrow) but lacked presynaptic neurotransmitter vesicles. F, Some normal synapses were observed. G, Presumed Type I glomerular central boutons (C1) originating from IB4+ neurons appeared abnormal, as did (H) presumed Type II glomerular central boutons (C2) and (I) peptidergic boutons containing dense-cored vesicles. Red dashed areas indicate the abnormal afferent terminals. J, Axons in the dorsal root were normal.
known low-level expression of Kcna6 in some excitatory and inhibitory interneuron populations (Häring et al., 2018; Zeisel et al., 2018), or reported Kcna6 expression in mouse and rat mature and progenitor oligodendrocytes (Attali et al., 1997; Chittajallu et al., 2002) and astrocytes (Smart et al., 1997; Zhu et al., 2014).

Six-week-old Kcna6lacZ/lacZ;Advillin-EGFP mice presented with clear swollen terminals, although the profiles were much reduced in size compared with at 24 weeks (Fig. 5B). Meanwhile, heterozygous Kcna6lacZ+/ mice appeared to have a degree of protection against the development of this central terminal pathology but the phenotype progressed with increasing age (Fig. 5Ci–Ciii). No evidence of degeneration was found in WT littermates at 11 months old (Fig. 5Civ). Compared with age-matched WTs, there appeared to be no accompanying thermal hyposensitivity in a small cohort of these aged Kcna6lacZ+/ heterozygotes (Fig. 5D), although these animals developed a structural abnormality, albeit to a lesser degree than the homozygotes. This pathology was only detected in the central terminals of Kcna6lacZ/lacZ primary afferents; morphology and counts of intra-epidermal nerve fiber density in the hind-paw skin were normal (Fig. 6).

Pathology to sensory neuron terminals did not initiate upregulation of neural injury marker, Atf3 (Tsujino et al., 2000), in Kcna6lacZ/lacZ DRG neurons relative to WT littermates and proportion of Atf3+ DRG neurons in both genotypes was far lower.
than positive control DRG tissue sampled from WT mice after spared nerve injury (Decosterd and Woolf, 2000) (Fig. 7A). There was also no obvious loss of neurons in any DRG subpopulation (Fig. 7B).

TEM was used to analyze these dorsal horn structures at nanoscale. The abnormal axon terminals identified previously were stained intensely by osmium when observed using light microscopy in Kcna6lacZ/lacZ tissue, suggesting the presence of a high concentration of lipids (Fig. 8Ai, Aii). TEM revealed densely packed, lipid-rich vesicular bodies contained within membrane-delimited profiles (Fig. 8B). Both the abnormal profiles and the vesicles within were highly variable in size and electron density (Fig. 8C). Some profiles were ensheathed by myelin, within or in close proximity to Lissauer’s tract (Fig. 8Di, Dii). Others made clear synaptic contacts with adjacent dendrites containing postsynaptic densities, and often appeared to send out multiple cytoplasmic projections from their swollen region, tentatively designated as intervaricose axons (Fig. 8E).

While some normal presynaptic boutons (of unknown origin) could be found in the superficial laminae (Fig. 8F), there was a notable reduction of normal Type I (C1) and Type II (C2) glomerular central boutons, originating from IB4+ neurons and Aδ- or C-LTMRs, respectively (West et al., 2015; Gutierrez-Mecinas et al., 2016; Larsson and Broman, 2019). The few examples of central glomerular boutons appeared highly irregular in form and content (Fig. 8G–I). Type I central boutons (C1) are described as having “a dark small central terminal of indented contour with closely packed spheroidal vesicles of variable diameter and few mitochondria” (Ribeiro-Da-Silva and Coimbra, 1982), but the example in Figure 8G lacks vesicles and is very abnormal. Some Type II central boutons (C2) and peptidergic axonal boutons (containing dense-cored vesicles) were observed, although similarly these did not look normal and potentially included vesicular pathology (Fig. 8H, I). The dorsal root was unaffected (Fig. 8F), so the accumulation of membranous, vesicular bodies appeared to occur once afferents had penetrated the cord.

Dorsal horn WDR neurons from Kcna6lacZ mice have reduced electrophysiological responses to peripheral stimuli
We reasoned that the abnormality in primary afferent terminals was highly likely to underlie behavioral deficits in Kcna6lacZ mice via impaired neurotransmission from first- to second-order afferent

Figure 10. Anatomical and behavioral consequences of CRISPR-mediated Kcna6 deletion independent of lacZ expression. A, Comparison of IB4+ and Cgrp+ central terminal staining in CRISPR-mediated Kcna6+/− homozygote KOs and WT littermates >15 weeks old reveals normal morphology. Scale bar, 100 μm. B, Measurement of exploratory behavior (gridline crosses and rearing episodes) averaged across 3 baselines. C, Motor performance on rotarod was unaffected in Kcna6+/− and Kcna6−/− mice. D-I, Withdrawal threshold or latency to plantar hind-paw stimulation by von Frey (D), pin-prick (E), dry ice (F), Hargreaves (G), and hot plate (H, I). *p < 0.05 versus Kcna6+/*+ by one-way ANOVA (B) with Dunnett’s multiple comparisons test. n = 8 Kcna6+/*+/C0 (4 male, 4 female), 8 Kcna6+/*− (4 male, 4 female), 8 Kcna6−/*− (5 male, 3 female) (B–G); n = 12 Kcna6+/*+ (4 male, 8 female), 14 Kcna6+/*− (6 male, 8 female), 13 Kcna6−/*− (6 male, 7 female) (H, I). Data are mean ± SEM.
neurons. We therefore undertook electrophysiological analysis of the response of deep dorsal horn WDR neurons to hind-paw stimulation. WT, Kcnal6lacZ+/+ and Kcnal6lacZ/lacZ WDR firing rates were characterized after "natural" stimulation by calibrated von Frey filaments, graded temperatures of water, brush, and evaporative cooling. In all genotypes, the graded intensity of mechanical and thermal stimuli was coded in the WDR firing rate, as is expected of these neurons (Fig. 9A,B). However, at the noxious end of the mechanical and thermal repertoire, there were significant deficits.

Figure 11. Anatomical consequences of nociceptor-specific LacZ expression independent of Kcnal6 deletion. A, No Cre-mediated LacZ expression in Nav1.8 Cre-negative mice >17 weeks old expressing the ROSA26lacZ reporter allele. IB4+ terminals in the dorsal horn appear normal in these mice despite expressing neo globally. B, In >17-week-old mice carrying one ROSA26lacZ allele and one Nav1.8 Cre allele, lacZ is expressed in small-diameter, IB4+ and CGRP+ DRG neurons. Swollen nociceptor terminals containing β-galactosidase are clearly visible in the superficial laminae. A, B, Images are representative paired DRG and dorsal horn sections from the same animals. C, One-year-old Cre-negative mice remain phenotypically normal. In 1-year-old Nav1.8Cre/+;ROSA26lacZ/+ mice, dorsal horn pathology was much more extensive, including in CGRP+ afferents projecting to deeper laminae. Scale bars: DRG, 50 μm; dorsal horn, 100 μm.
in spike generation in both Kcnab\textsuperscript{lacZ} heterozygote and homozygote mice following 15 g von Frey or 48°C heat stimulation (Fig. 9A,B). Responses to non-noxious mechanical brush stimulation did not differ between genotypes (Fig. 9C). Evaporative cooling with ethyl chloride did not elicit significantly different responses across genotypes (Fig. 9D).

Electrical activation thresholds for A- and C-fiber-evoked spikes were unchanged in heterozygous or homozygous Kcnab\textsuperscript{lacZ} animals (Fig. 9E) versus WT mice, nor were there statistical differences in the total evoked spikes by either fiber class after a train of 16 electrical stimuli to the receptive field on the paw (Fig. 9F). The number of spikes generated after the C-fiber latency window (termed postdischarge) was also not affected (Fig. 9F). While the total number of spikes attributed to “wind-up” of WDRs (i.e., the progressive increase in responsivity with each stimulus) was not different between genotypes (Fig. 9F), there were some considerable differences in the kinetics of wind-up between WT mice and Kcnab\textsuperscript{lacZ} mutants. Specifically, WDR responses in WTs followed a typical hyperbolic pattern, gradually reaching a maximal discharge rate toward the end of the 16-stimulus paradigm (Fig. 9G). KO mice had a similar initial rate of wind-up but reached a lower plateau, while wind-up in heterozygotes formed a much more linear relationship with increasing stimuli. Epidermal receptive fields providing input to WDRs were similar in size across all genotypes, as was the range of sampled neuron depths and the age of mice at the time of experiment (Fig. 9H–I).

**LacZ-negative Kcnab\textsuperscript{−/−} mice have milder behavioral phenotypes with no pathology in presynaptic primary afferent terminals**

The degenerative phenotype affecting the central terminals of primary afferents in Kcnab\textsuperscript{lacZ/lacZ} mice was unexpected and had not previously been reported in other Shaker-like potassium channel mutants. For comparison with the very unusual phenotype of the Kcnab\textsuperscript{lacZ} strain, we characterized a different commercially available CRISPR-mediated constitutive Kcnab KO mouse (MMRRC stock #042391-JAX), containing no exogenous lacZ cassette. Spinal cord tissue was collected from these heterozygous Kcnab\textsuperscript{lacZ} mice aged >15 weeks, by which time point dorsal horn pathology had fully developed in Kcnab\textsuperscript{lacZ/lacZ} mice. However, there was no sign of a similar effect on central primary afferent terminals; the superficial dorsal horn neuropil appeared normal, with dense peptidergic and nonpeptidergic fiber reactivity in lamina II (Fig. 10A).

Responses to a wide range of sensory (noxious and non-noxious) stimuli across multiple modalities were assessed, and mice were also challenged with multiple motor tasks. Performance across tasks, such as the open field and rotarod challenges, was comparable between Kcnab\textsuperscript{−/−}, Kcnab\textsuperscript{+/−}, and Kcnab\textsuperscript{+/−} littermate mice (Fig. 10B,C). Responses to punctate mechanical stimulation by von Frey hairs and more noxious pin-prick were also similar (Fig. 10D,E), as well as responses to noxious cooling of the hind-paw with the dry ice test (Fig. 10F) (Brenner et al., 2012). Compared with the phenotype of Kcnab\textsuperscript{lacZ} KOs, the thermal hyposensitivity of this Kcnab\textsuperscript{lacZ} cohort was much milder, with no discernible difference in withdrawal latency to the Hargreaves test or a 50°C hot plate (Fig. 10G,H). However, with more intense noxious hot plate stimulation at 53°C, the Kcnab\textsuperscript{lacZ} cohort had a mildly but significantly delayed response time (Fig. 10I).

**Nav1.8\textsuperscript{Cre+/−} dependent expression of LacZ is sufficient to induce presynaptic pathology in primary afferent terminals**

The absence of primary afferent degeneration in Kcnab\textsuperscript{−/−} mice suggested that the expression of exogenous LacZ was responsible for the pathology identified in the dorsal horn of Kcnab\textsuperscript{lacZ} mice.
However, it remained possible that this phenomenon arose because of: (1) the neomycin resistance cassette also present in the <tm1Lex> alleles of Kcna6lacZ mice (Fig. 3A); or (2) an unanticipated interaction between the loss of Kv1.6 subunits and expression of LacZ. To seek definitive evidence that LacZ expression alone is intrinsically harmful to the central terminals of nociceptive primary afferent neurons, we used a Nav1.8 promoter-driven Cre recombinase (Nassar et al., 2004; Stirling et al., 2005) to induce expression of exogenous LacZ (Soriano, 1999) on a Kcna6+/+ background in primary afferent nociceptors. The breeding of Nav1.8Cre+/+ and ROSA26lacZ/lacZ produced Nav1.8Cre+/+; ROSA26lacZ/lacZ compound heterozygotes expressing LacZ protein in Nav1.8+ neurons, which overlap considerably with Kcna6+ neurons in the DRG (Usoskin et al., 2015; Zeisel et al., 2018; Zheng et al., 2019).

Neither LacZ protein nor dorsal horn abnormalities were detected by immunohistochemistry in 17-week-old Cre-negative Nav1.8Cre+/+;Rosa26lacZ/lacZ mice which, without the presence of Cre recombinase, express a neomycin resistance cassette globally (Fig. 11A). This suggests that exogenous neomycin phosphotransferase was not responsible for primary afferent terminal degeneration in Kcna6lacZ mice. In Nav1.8 Cre-positive mice also carrying the ROSA26lacZ allele, exogenous LacZ expression was detected and abnormal LacZ+ presynaptic terminals were once again visible within the dorsal horn, colocalizing with IB4 and Cgrp staining (Fig. 11B). These were, however, less numerous than in the Kcna6lacZ animals at the equivalent age. Nav1.8Cre+/+; ROSA26lacZ/lacZ mice had far more extensive evidence of primary afferent degeneration in the dorsal horn at ~1 year of age, including lesions of axons within the attached dorsal root and those projecting into deeper laminae (Fig. 11C). Nav1.8 Cre-negative littermates demonstrated normal dorsal horn morphology at this time point.

Viewed with TEM, the ultrastructure of pathologic Nav1.8Cre+/+; ROSA26lacZ/lacZ afferents (Fig. 12) was similar to those of the Kcna6lacZ/lacZ mice (Fig. 8), while normal morphology of primary afferent terminals was observed in Nav1.8-Cre-negative littermate controls (Fig. 12) and in Kcna6−/− KO mice not expressing lacZ (Fig. 13). Clearly, the presence of exogenous LacZ protein in DRG neurons is sufficient to cause age-dependent deterioration of primary afferent presynaptic boutons.

**Ganglioside metabolism is implicated in LacZ-mediated pathophysiology**

LacZ is a bacterial enzyme with β-galactosidase activity (Juers et al., 2012), and is therefore well placed to interfere with endolysosomal metabolism of complex gangliosides (sialic acid-containing glycosphingolipids), the major constituent of mammalian cell membranes. Neurons chiefly synthesize a- and b-series gangliosides, including GM1, cleavage of which to GM2 is achieved through the endo-lysosomal pathway by GM1-β-galactosidase removing a terminal β-galactose residue. We hypothesized that overexpression of exogenous LacZ may result in accumulation of GM1, GM2, or other neuronal ganglioside species, and tested this using immunohistochemical techniques. GM1 is bound by cholera toxin-B (CTxB) (Merritt et al., 1994), and we identified positive staining for GM1 with fluorophore-conjugated CTxB in the dorsal root and dorsal column white matter tracts, but also colocalizing with markers of LacZ-affected nociceptor terminals from Kcna6lacZ/lacZ and Nav1.8Cre+/+;Rosa26lacZ/lacZ mice in the superficial dorsal horn, including Cgrp and β-galactosidase (Fig. 14). Other colocalizing ganglioside species were identified via monoclonal antibody staining against GD2 itself or GD3-based...
derivatives GT1b/GQ1b (Boffey et al., 2005; Clarke et al., 2017) (Fig. 15).

Mice lacking *Kcna6* exhibit prolonged mechanical and thermal hypersensitivity after peripheral nerve injury

Uptregulation of *Kcna6* in painful rat and human neuromas has implicated this subunit in a compensatory response to nerve injury (Calvo et al., 2016). Furthermore, local α-dendrotoxin-mediated blockade of *Kcna6*-containing channels in the context of neuroma produces a persistent mechanical hypersensitivity (Calvo et al., 2016), revealing their role in setting neuropathic mechanosensory thresholds. We investigated whether this subunit contributes to pain sensation in a similar fashion in mice, using a CCI model in *Kcna6*−/− mice lacking *Kcna6* but not expressing *lacZ*. At baseline, as per Figure 10, *Kcna6* KOs were again hyposensitive to 53°C but not 50°C noxious heat in a TPP assay versus 25°C (Fig. 16A,B), and displayed no difference in sensitivity to Hargreaves or von Frey assays compared with littermate controls retaining a functional *Kcna6* allele (Fig. 16A,B). Behavioral outcomes were followed for 28 d after injury. Across 50°C TPP, Hargreaves, and von Frey assays, mice completely lacking *Kcna6* remained persistently hypersensitive compared with littermate controls, which displayed a degree of recovery from the initial day 7 hypersensitivity (Fig. 16A,C,D). With follow-up across all of these assays, mice lacking *Kcna6* exhibited an observable injury-mediated hypersensitivity on 53°C versus 25°C TPP because of their baseline hyposensitivity; however, in the post-injury period, there were discernible differences between *Kcna6*−/− and wild-type mice.

![Figure 14](image-url)
and \textit{Kcna6}^{−/−} mice at day 28 whereby \textit{Kcna6}-null mice were hypersensitive compared with littermate controls (Fig. 16).

**Discussion**

**Neurotoxicity of exogenous \textit{LacZ} expression**

Through investigation of several transgenic mouse strains, a major finding was that exogenous \textit{lacZ} cassettes expressed in primary afferent nociceptors cause pathologic accumulation of lipid species within central presynaptic arborizations. These lipid-rich “organelles” resemble endo-/lyso-/autophagosomal structures involved in turnover of plasma membrane gangliosides (Figs. 8 and 12). Loss-of-function mutations affecting GM1-\(\beta\)-galactosidase or the group of enzymes that catabolize GM2 (\(\beta\)-hexosaminidases) result in fatal neurodegenerative ganglioside storage disorders (Breiden and Sandhoff, 2019), with accumulation of the substrates GM1 and GM2, respectively, in neuronal lysosomes.

GM1 and GM2 gangliosidoses feature “ballooned” axons (Breiden and Sandhoff, 2019), visible by Golgi staining in feline models (Purpura and Baker, 1978; Walkley et al., 1981, 1990; Sango et al., 1995) remarkably similar to swollen terminals of \textit{Kcna6}^{lacZ/lacZ} and Nav1.8\textit{Cre}^{+/+}; ROSA26\textit{lacZ}/+ afferents (Figs. 8C–I and 12E,F). Similar vacuolated “inclusion bodies” were identified via TEM in DRG neurons from \(\alpha\)-galactosidase KO mice in a Fabry disease model (Miller et al., 2018).

Endogenous \(\beta\)-galactosidase is undetectable in WT primary afferents by immunohistochemistry (Fig. 2), or RNA sequencing (Zeisel et al., 2018). We argue that exogenous \textit{LacZ}/\(\beta\)-galactosidase overrides endogenous ganglioside metabolism, resulting in aberrant accumulation of several ganglioside species identified via immunostaining (Figs. 14 and 15). Within the DRG, GM1 gangliosides are mostly associated with large myelinated primary afferent neurons (Robertson and Grant, 1989), and GM1 staining is almost absent from the superficial dorsal horn in mice, although GM1 upstream precursors GD1a, GD1b, and GT1b are abundant (Vajn et al., 2013). We clearly labeled GM1-, GD2-, and GD3-derived gangliosides in LacZ-affected terminals, suggesting that endogenous ganglioside metabolism was being diverted through abnormal biochemical pathways (Breiden and Sandhoff, 2019).

Endogenous GM1-\(\beta\)-galactosidase expression accumulates over time and is a marker of cellular senescence (Dimri et al., 1995; Lee et al., 2006; Geng et al., 2010). GM2 gangliosidoses, including Tay-Sachs disease and Sandhoff disease, have an early
onset, although variants with delayed onset and reduced severity correlate with improved catabolic GM2 clearance (Leinekugel et al., 1992; Sandhoff and Harzer, 2013; Breiden and Sandhoff, 2019). LacZ-induced ganglioside accumulation may therefore accelerate cellular aging processes, consistent with slower pathologic progression in Kcnab1lacZ/+ heterozygotes (Fig. 5) and Nav1.8Cre+/ROSA26lacZ/+ mice (Fig. 11B, C), which only expressed one lacZ allele. Another feature of GM2 gangliosidosid, demyelination, involves microglia (Ogawa et al., 2018), which were also detected adjacent to swollen Kcnab1lacZ profiles (Fig. 4C).

Although LacZ was detectable in Kcnab1lacZ distal nerve terminals innervating glabrous skin (Fig. 6), structural abnormalities were only observed in central afferent axons, mostly once they had penetrated the spinal cord but occasionally in the dorsal root of severely affected, aged animals. This is curious and accentuates the polarized structure and function of sensory neurons. It remains unclear why presynaptic axons were affected specifically, but neurotransmitter release is dependent on effective lysosomal and ganglioside function (Hirai et al., 2005), impaired in acquired autoimmune diseases, such Guillain-Barré syndrome (Buchwald et al., 2007; Plomp and Willison, 2009). Gangliosides, such as GM1, can colocalize with presynaptic proteins in lipid rafts (Taverna et al., 2004), known to regulate exocytosis (Salaün et al., 2004). Age-related presynaptic accumulation of GM1 also initiates degenerative change via amyloid deposition (Yamamoto et al., 2008).

We noted a predominant effect on nociceptive versus nonnociceptive afferent terminals in Kcnab1lacZ mice, which likely reflects the higher expression of Kv1.6 (and hence LacZ) in nociceptors (Figs. 1 and 2). Peptidergic and nonpeptidergic nociceptors form morphologically distinct specialized central boutons (Ribeiro-Da-Silva and Coimbra, 1982; West et al., 2015; Gutierrez-Mecinas et al., 2016; Larsson and Broman, 2019). The propensity for degeneration of IB4+ more than Cgrp+ terminals is interesting; it suggests that these subpopulations may express distinct species of gangliosides or rely on differential ganglioside metabolism to maintain presynaptic bouton integrity. IB4-binding terminals retract following peripheral nerve injury (Tajit et al., 1988; Molander et al., 1998; Shehab et al., 2004), whereas both IB4+ and Cgrp+ terminals retract following systemic administration of HIV therapeutic stavudine (Huang et al., 2013). These findings might probe further investigation into the role of ganglioside metabolism in maintaining primary afferent synaptic structures, especially in the context of peripheral neuropathy.

Our focus has been on lacZ expression in DRG neurons; one previous study has investigated expression in cortical neurons and raised concerns regarding the use of LacZ reporters (Reichel et al., 2016). The authors found that lacZ expression in cortical glutamatergic neurons caused severe morphologic and behavioral impairments, such as decreased hippocampal volume, reduced dendritic branching in hippocampal neurons, and deficits in hippocampus-dependent memory. They did not report abnormal lipid accumulations in axon terminals, although it is possible that these were not specifically sought. Our combined findings from lacZ expression in central and peripheral neurons mean that anatomic, electrophysiological, and behavioral data from transgenic mice expressing lacZ require interpretation with extreme caution. We advocate using alternative reporters, such as GFP or YFP, and note we and others have not observed any abnormalities in primary afferent terminals of Advillin-EGFP mice (Sikandar et al., 2017; Hunter et al., 2018). This is particularly relevant as a significant proportion of the lines produced in high throughput phenotypic screens, such as the International Mouse Phenotyping Consortium, use LacZ as a reporter (Skarnes et al., 2011; Meehan et al., 2017).

The role of Kv1.6 in thermosensation
This study also revealed a role for Kv1.6 in regulating thermal sensitivity. The unexpected neurodegenerative phenotype of LacZ-expressing animals was considered likely to underlie the differences in severity of thermal behavioral phenotypes between Kcnab1lacZ and Kcnab1 mice, especially since Gb3 ganglioside accumulation in Fabry disease models was shown to interfere with Trpv1 signaling and heat sensation (Hofmann et al., 2018). The intricate mechanisms through which gangliosides modulate pain sensation specifically, including regulation of ion channels and receptors, have been reviewed recently.
### Table 1. Appendix

| Antigen | Host/conjugate | Source/RRID | Dilution |
|---------|----------------|-------------|----------|
| **Antibodies** | | | |
| Primary antibodies | | | |
| **Atf3** | Rabbit | Santa Cruz Biotechnology (sc188) | 1:500 |
| **β -III tubulin** | Mouse | Sigma-Aldrich (T8578) | 1:500 |
| **β -gal (E. coli)** | Chicken | Abcam (ab9361) | 1:500 |
| **Cgrp** | Sheep | Enzo Life Sciences (BML-CA1137) | 1:500 |
| **Cgrp** | Rabbit | Peninsula Laboratories (T-4032) | 1:500 |
| **IB4** | (biotinylated) | Sigma-Aldrich (LT-2140) | 1:200 |
| **GD2** | Mouse | Kindly gifted by Dr. Juliet Gray, University of Southampton | 2 μg/ml |
| **GD3 (EG4)** | Mouse | As per Boffey et al. (2005) and Clark et al. (2017) | 2 μg/ml |
| **Gfap** | Mouse | Sigma-Aldrich | 1:500 |
| **Glutamine synthetase** | Rabbit | Sigma-Aldrich (G2781) | 1:1000 |
| **Homer1** | Goat | Frontier Institute | 1:500 |
| **Iba1** | Rabbit | Abcam (ab178847) | 1:500 |
| **NeuN** | Chicken | Millipore (ABN91) | 1:500 |
| **NeuN** | Rabbit | Abcam (ab177487) | 1:500 |
| **Neurotrace (red)** | NA | Invitrogen (N21482) | 1:100 |
| **Neurotrace (blue)** | NA | Invitrogen (N21479) | 1:100 |
| **NF200** | Mouse | Sigma-Aldrich (N-0142) | 1:500 |
| **Pax2** | Rabbit | Thermo Fisher Scientific (71-6000) | 1:200 |
| **Pgp9.5** | Rabbit | Thermo Fisher Scientific (P-31582) | 1:500 |
| **Synaptophysin** | Rabbit | Thermo Fisher Scientific (P-21206) | 1:500 |
| **Th** | Sheep | Thermo Fisher Scientific (P-10994) | 1:500 |
| **Secondary antibodies** | | | |
| **Biotin** | Streptavidin/Pacific Blue | Thermo Fisher Scientific (S11222) | 1:500 |
| **Chicken IgG** | Goat/Alexa-488 | Thermo Fisher Scientific (A-11039) | 1:500 |
| **Chicken IgG** | Goat/Alexa546 | Thermo Fisher Scientific (A-11040) | 1:500 |
| **GM1** | Cholera toxin B/Alexa-488 | Invitrogen (C34775) | 2 μg/ml |
| **Goat IgG** | (biotinylated) | Vector Laboratories (BA-9500) | 1:200 |
| **Mouse IgG** | Goat/Pacific Blue | Thermo Fisher Scientific (P-31582) | 1:500 |
| **Mouse IgG** | Donkey/Alexa-488 | Thermo Fisher Scientific (A-21202) | 1:500 |
| **Rabbit IgG** | Donkey/Cy3 | Jackson Immunoresearch Laboratories (711-166-152) | 1:500 |
| **Rabbit IgG** | Goat/Pacific Blue | Thermo Fisher Scientific (P-10994) | 1:500 |
| **Sheep IgG** | Donkey/Alexa-488 | Thermo Fisher Scientific (A-11015) | 1:500 |

### qRT-PCR primers

| Gene | Description | Forward (5'-3') | Reverse (5'-3') |
|------|-------------|----------------|-----------------|
| Kcn01 | Coding sequence | TGGCCAG6GATGAGAACCCAC | TACTGAGAGCTGAGATGAC |
| Kcn02 | Coding sequence | CCGCGCAG6GACTGCTT | TGGTTCGATCACTGAGAC |
| Kcn1 | Exon-spanning | TTTTGCCAG6GATGAGAACCCAC | ACACCTTTACCAAGAGGAGT |
| Kcn2 | Exon-spanning | CACTGCGAG6GACAGCTCAG | AGTTAGAAAGAGGACCA |
| Hprt1 | Exon-spanning | GTGCTGAG6GCTGCTGCTG | TGCGAGAGGACAGACTGAC |
| Gipd1 | Exon-spanning | TGGTGCGAG6GCTGCTGCTG | TGCTGAGAGGACAGACTGAC |
(Sántha et al., 2020). The common feature between the two strains was hyposensitivity to noxious heat. At present, it is unclear through what mechanism this hyposensitivity manifests.

Most known functions of Kv1 channels would instead predict a hypersensitive phenotype on removal of Kv1.6. Its upregulation following peripheral nerve injury in WT animals reduces spontaneous electrical discharge in injured nerve fibers and behavioral hypersensitivity, while pharmacological blockade of Kv1.6 in this context maintains spontaneous discharge and persistent mechanical hypersensitivity (Calvo et al., 2016). Blockade of Kv1 currents in nonpeptidergic nociceptors isolated from Mrgprd-GFP mice (where Kv1.6 is the major Kv1 subunit) also increased their maximal action potential firing rate substantially (Zheng et al., 2019). The channel’s acute effect on membrane excitability thus limits excitability, in line with canonical Kv1 channel properties in neurons (Browne et al., 1994; Smart et al., 1998; Glazebrook et al., 2002; Chi and Nicol, 2007; Robbins and Tempel, 2012; Hao et al., 2013; Dawes et al., 2018). We had therefore been expecting, if anything, thermal hypersensitivity.

It is possible that these paradoxical effects relate to subunit stoichiometry of heteromeric Kv1 channels, which can significantly influence activation thresholds. Kv1.2 subunits promote a more depolarized activation potential than Kv1.1 (Akhtar et al., 2002), and Kv1.6 a still more depolarized activation potential (Grupe et al., 1990) ($V_{\text{0.5max}}$ for homomeric channels: Kv1.1, −33 mV; Kv1.2, −26 mV; Kv1.6, −17 mV). KO of Kv1.6 in sensory neurons could result in overrepresentation of Kv1.1/1.2 subunits and reduced neuronal excitability. It is noted that Kcnal6 is expressed throughout the mouse nervous system (Zeisel et al., 2018); and in two global Kv1.6 KO strategies, there are myriad explanations for hyposensitive behavioral traits, requiring further interrogation.

### Kcnal6 promotes recovery from nerve injury

Intriguingly, in the context of neuropathic CCI, Kcnal6-null animals behave in a more canonical fashion, displaying more severe and prolonged hypersensitivity to thermal and mechanical stimuli, while littermate controls with functioning Kv1.6 gradually recover. We were able to observe the hyposensitive phenotype to acute noxious thermal stimuli reported in both Kcnal6 KO strains (Figs. 3 and 10) and the persistent post-neuropathic hypersensitive phenotypes predicted by Calvo et al. (2016), in the same cohort of animals (Fig. 16). We believe this reconciles the two contrasting datasets and provides robust evidence that both phenomena are genuine consequences of Kv1.6 loss of function.

In conclusion, we have identified a role for Kv1.6 subunits in acute thermal nociception and the recovery response to nerve injury. We also present evidence that transgenic insertion of exogenous lacZ-containing cassettes can have pathologic downstream consequences for neuronal structure and function. LacZ induced accumulation of gangliosides in presynaptic primary nociceptor terminals in the dorsal horn. This anatomic defect exacerbated the underlying behavioral phenotype of Kv1.6 KO mice, demonstrated by comparison between Kcnal6lacZ and CRISPR-mediated Kcnal6−/− mice. This confirms that exogenous lacZ cassettes can be markedly neurotoxic. We recommend caution when interpreting past and future results from transgenic mice expressing exogenous LacZ. We also illustrate a role for Kv1.6 in the recovery of normal sensory function following nerve injury, thus enhancing that Kv1.6 function could be a therapeutic target in neuropathic pain.

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