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Local knockdown of the $Na_V1.6$ sodium channel reduces pain behaviors, sensory neuron excitability, and sympathetic sprouting in rat models of neuropathic pain

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Abstract

In the spinal nerve ligation model of neuropathic pain, as in other pain models, abnormal spontaneous activity of myelinated sensory neurons occurs early and is essential for establishing pain behaviors and other pathologies. Sympathetic sprouting into the dorsal root ganglion (DRG) is observed after spinal nerve ligation, and sympathectomy reduces pain behavior. Sprouting and spontaneous activity may be mutually reinforcing: blocking neuronal activity reduces sympathetic sprouting, and sympathetic spouts functionally increase spontaneous activity in vitro. However, most studies in this field have used nonspecific methods to block spontaneous activity, methods that also block evoked and normal activity. In this study, we injected small inhibitory RNA directed against the $Na_V1.6$ sodium channel isoform into the DRG before spinal nerve ligation. This isoform can mediate high frequency repetitive firing, like that seen in spontaneously active neurons. Local knockdown of $Na_V1.6$ markedly reduced mechanical pain behaviors induced by spinal nerve ligation, reduced sympathetic sprouting into the ligated sensory ganglion, and blocked abnormal spontaneous activity and other measures of hyperexcitability in myelinated neurons in the ligated sensory ganglion. Immunohistochemical experiments showed that sympathetic sprouting preferentially targeted $Na_V1.6$-positive neurons. Under these experimental conditions, $Na_V1.6$ knockdown did not prevent or strongly alter single evoked action potentials, unlike previous less specific methods used to block spontaneous activity. $Na_V1.6$ knockdown also reduced pain behaviors in another pain model, chronic constriction of the sciatic nerve, provided the model was modified so that the lesion site was relatively close to the siRNA-injected lumbar DRGs. The results highlight the relative importance of abnormal spontaneous activity in establishing both pain behaviors and sympathetic sprouting, and suggest that the $Na_V1.6$ isoform may have value as a therapeutic target.

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INTRODUCTION

A common feature of many preclinical pain models is hyperexcitability of primary sensory neurons. In particular, abnormal spontaneous activity, primarily in cells with myelinated axons, is an early event in many pain models (Devor, 2009 , Sorkin and Yaksh, 2009, Bedi et al., 2010, Berger et al., 2011, Nieto et al., 2012). Depending on the model used, spontaneous activity may originate at the site of peripheral nerve injury (e.g. sciatic nerve transection and chronic sciatic nerve constriction models) or in the dorsal root ganglia (DRG) (spinal nerve ligation, local DRG inflammation, and chronic DRG compression models). Blocking nerve activity early after the pain model is implemented is highly effective in blocking pain behaviors; a temporary nerve blockade lasting 4 – 7 days can produce an enduring block of pain behaviors (Xie et al., 2005). Blocking nerve activity can also prevent other later occurring pathological changes such as glia activation (Xie et al., 2009).

Another such pathological change is sprouting of sympathetic fibers into the DRG, where both an increased overall fiber density and appearance of “basket” formations of sympathetic fibers wrapping around individual neurons have been observed. This is in contrast to normal DRG, where sympathetic fibers are associated with blood vessels but not neurons. It has been proposed that these abnormal contacts between the sympathetic and sensory neurons contribute to development of pain behaviors and serve as a model for sympathetically maintained pain conditions in humans (Chung et al., 1993, McLachlan et al., 1993, Ramer and Bisby, 1997, Lee et al., 1998, Chien et al., 2005, Xie et al., 2006, Pertin et al., 2007). Many but not all studies demonstrated that reducing sympathetic sprouting reduced some pain behaviors (Neil et al., 1991, Shir and Seltzer, 1991, Kim et al., 1993, Desmeules et al., 1995, Kinnman and Levine, 1995, Pertin et al., 2007, Xie et al., 2010). Direct functional studies of the sympathetic-sensory neuron connection suggest that it is excitatory (particularly at earlier time points), though relatively few such studies have been done (Burchiel, 1984, McLachlan et al., 1993, Devor et al., 1994). There may be a mutually reinforcing relationship between spontaneous activity and sympathetic sprouting. Spontaneous activity precedes sympathetic sprouting, and blocking neuronal activity blocks sprouting (Dong et al., 2002, Zhang et al., 2004, Xie et al., 2007, Xie et al., 2011). Spontaneously active cells preferentially receive sympathetic sprouts in two different neuropathic pain models (Xie et al., 2007, Xie et al., 2011). In one of these studies sprouting was also shown to increase when spontaneous activity was increased by localized application of K channel blockers in vivo. Conversely, there is evidence that sympathetic sprouts once established may contribute to neuronal hyperexcitability and spontaneous activity, perhaps creating a vicious cycle. We showed that, at early time points in the spinal nerve ligation (SNL) model, sprouting sympathetic fibers originate from the dorsal ramus of the spinal nerve, and that stimulating the dorsal ramus in an isolated whole DRG preparation had excitatory effects on medium and large diameter cells that could be attributed to the sympathetic sprouts based on pharmacological and ablation studies (Xie et al., 2010). In a mouse SNL model, blocking sympathetic sprouting by cutting the dorsal ramus eliminated spontaneous activity normally observed three days after the spinal nerve ligation (Xie et al., 2011). Taken together these studies suggest that spontaneous activity may play a key role in

initiating sympathetic sprouting, which may in turn play a key role in maintaining spontaneous activity.

The spontaneous activity observed in myelinated neurons in rodent pain models usually consists of high frequency firing, often in a bursting pattern (Zhang and Strong, 2008, Devor, 2009). Sensory neurons express the $Na_V1.6$ sodium channel isoform (Black et al., 2002, Dib-Hajj et al., 2010), which can give rise to resurgent currents that facilitate high frequency firing (Cruz et al., 2011). We previously showed that small inhibitory (si) RNA-mediated knockdown of the $Na_V1.6$ channel in vivo could prevent pain behaviors and spontaneous activity induced by localized inflammation of the lumbar DRG (Xie et al., 2013). This isoform has also been implicated in chemotherapy induced pain (Sittl et al., 2012). A toxin that increases persistent and resurgent currents in sensory neurons was shown to cause acute pain and itch, mediated by myelinated fibers, when injected into the skin of human volunteers (Klinger et al., 2012). In the present study we examined the effects of local $Na_V1.6$ knockdown in a neuropathic pain model, the spinal nerve ligation model, with particular emphasis on the effects on sympathetic sprouting.

METHODS

Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. Experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Adult Sprague Dawley rats (Harlan, Indianapolis, USA) were used for all experiments. For most experiments, male rats weighing 150 - 200 g at the start of the experiment (for behavior experiments) or females weighing 100 –150 grams at the time of sacrifice (for electrophysiological experiments) were used. Some behavioral experiments were done with female rats where indicated. Rats of both sexes were used for immunohistochemistry experiments.

Procedure for in vivo injection of siRNA into the DRG

siRNAs directed against rat $Na_V1.6$ channel (gene ID 29710) and nontargeting control were designed by and purchased from Dharmacon/ThermoFisher (Lafayette, CO) as previously described (Xie et al., 2013). The $Na_V1.6$-siRNA was siGENOME™ siRNA consisting of a “smartpool” of four different siRNA constructs combined into one reagent. Catalog numbers were M-094591-00 (directed against $Na_V1.6$) and D-001210-02 (nontargeting control directed against firefly luciferase, screened to have minimal off-target effects and least 4 mismatches with all known human, mouse and rat genes according to the manufacturer). 3 μL aliquots containing 80 pmoles of siRNA made up with cationic linear polyethylenimine (PEI)-based transfection reagent (“in vivo JetPEI”, Polyplus Transfection, distributed by WVR Scientific, USA) at a nitrogen/phosphorus ratio of 8 were injected into each L4 and L5 DRG on one side, through a small glass needle (75 μm o.d.) inserted close to the DRG through a small hole cut into the overlying membrane close to the site where the dorsal ramus exits the spinal nerve, as previously described (Xie et al., 2012a). Two injections of

siRNA were done during two separate surgeries, as shown in Fig. 1. During the second surgery the siRNA was injected first and then the pain model was implemented.

Surgical procedure for spinal nerve ligation of the DRG

Rats received a unilateral ligation of the ventral ramus of the L5 spinal nerve following the original description by Kim and Chung (Kim and Chung, 1992). Rats were anesthetized by isoflurane. The skin on the back was cut along the spine from S1 to L4 (DRG level). After clearly exposing L5 and L4 transverse processes by separating the overlying back spine paraspinal muscles, the ventral ramus of the L5 spinal nerve could be visualized in between these two transverse processes and freed from surrounding tissue. In some rats the space between L5 transverse process and intervertebral foramen was too narrow to free the L5 spinal nerve from surrounding tissue, in which case a very small round piece of bone (around 0.5 mm diameter) was cut and removed from L5 transverse process to further expose L5 ventral ramus for ligation. Following exposure, the L5 spinal nerve was isolated and tightly ligated with 6-0 silk approximately 2-3 mm distal to the ganglion and then cut distal to the suture. The incision was closed in layers after these surgeries.

Behavior testing

Mechanical sensitivity was tested by applying a series of von Frey filaments to the heel region of the paws, using the up-and-down method (Chaplan et al., 1994). A cutoff value of 15 grams was assigned to animals that did not respond to the highest filament strength used. A wisp of cotton pulled up from, but still attached to a cotton swab was stroked mediolaterally across the plantar surface of the hindpaws to score the presence or absence of a brisk withdrawal response to a normally innocuous mechanical stimulus (light touch-evoked tactile allodynia). This stimulus does not evoke a response in normal animals. Cold sensitivity was scored as withdrawal responses to a drop of acetone applied to the ventral surface of the hind paw. When observed, responses to acetone or light brush strokes consisted of several rapid flicks of the paw and/or licking and shaking of the paw; walking movements were not scored as positive responses.

Electrophysiology

Intracellular recording in current clamp mode was performed at 36 – 37°C using microelectrodes on sensory neurons near the dorsal surface of an acutely isolated whole DRG preparation, as previously described (Xie et al., 2012b). This preparation allows neurons to be recorded without enzymatic dissociation, with the surrounding satellite glia cells and neighboring neurons intact (Song et al., 1999, Zhang et al., 1999). The DRG was continuously perfused with artificial cerebrospinal fluid (in mM: NaCl 130, KCl 3.5, NaH_2PO_4 1.25, $NaHCO_3$ 24, Dextrose 10, $MgCl_2$ 1.2, $CaCl_2$ 1.2, 16 mM HEPES, pH = 7.3, bubbled with 95% O_2/ 5% CO_2). Cells were classified by conduction velocity (stimulation of attached dorsal root) as follows: <1.2 m/s, C fiber; 1.2 – 7.5 m/s, Aδ; >7.5 m/s, Aαβ (Stebbing et al., 1999). Any spontaneous activity observed after impalement of the cell was recorded first, and re-confirmed at the end of the recording period. Action potential (AP) parameters were measured with injected current steps of increasing size. AP threshold, width, and peak voltage were measured during the response to the smallest current that evoked an action potential (rheobase) during a 75 msec depolarization. The AP voltage

threshold was defined as the first point on the rising phase of the spike at which the change in voltage (dV/dt) exceeded $1/10^{th}$ of the maximum value. Duration of the AP was measured at the threshold voltage. An alternative definition of AP duration was also analyzed, by measuring the time between the maximum dV/dt on the rising phase and minimum dV/dt on the falling phase in order to approximate the points of maximum Na^+ influx and K^+ outflux, respectively. After measurement of the threshold action potential parameters, the cell was given longer (270 msec) suprathreshold currents to determine the maximum number of AP that could be evoked and whether subthreshold membrane oscillations could be evoked. Rheobase for spontaneously active cells was defined as zero.

Immunostaining for $Na_V1.6$ and sympathetic fibers with tyrosine hydroxylase in DRG sections

DRG sections were cut at 40 μm on a cryostat after fixation in 4% paraformaldehyde in 0.1M Phosphate Buffer and 4% sucrose. The polyclonal antibody against $Na_V1.6$ was from Alomone (Jerusalem, Israel; catalog ASC-009) used at 1:100 dilution. The antibody specificity was previously demonstrated by a lack of staining in a knockout mouse (Black et al., 2002). The secondary antibody conjugated to Alexa Fluor 594 (Invitrogen, Carlsbad, CA) was used at a dilution of 1:1000. Images from multiple sections of each DRG were captured under an Olympus BX61 fluorescent microscope using Slidebook 4.1 imaging acquisition software (Intelligent Imaging Innovation, Denver, CO). To measure the expression of $Na_V1.6$ in the DRG neurons, the summed intensities of the $Na_V1.6$ signal were measured and normalized by the cellular area in each analyzed section to give an intensity ratio. In all immunohistochemical experiments, data from at least three animals were included to control for interanimal variability. For experiments involving double staining for $Na_V1.6$ and tyrosine hydroxylase (sheep; 1:1000, from Pel- Freeze, Rogers, AR, USA) the procedure was the same except that sequential incubations with the two primary antibodies and two secondary antibodies were conducted, using the same incubation parameters. Secondary antibodies were donkey anti rabbit Alexa Flour 594 and donkey anti Sheep Alexa Flour 488 from Jackson Immuno Research Lab at 1:250 dilution.

Data analysis

Behavioral time course data were analyzed using two-way repeated measures ANOVA with Bonferroni posttest to determine on which days experimental groups differed. For electrophysiological and immunohistochemical data, comparison of values between different experimental groups was done using nonparametric methods for data that did not show a normal distribution based on the D'Agostino and Pearson omnibus normality test. The statistical test used in each case is indicated in the text, or figure legend. Significance was ascribed for $p<0.05$. Levels of significance are indicated by the number of symbols, e.g., *, $p = 0.01$ to <0.05; **, $p = 0.001$ to 0.01; ***, $p < 0.001$. Data are presented as average ± S.E.M.

RESULTS

Local knockdown of $Na_V1.6$ reduces mechanical hypersensitivity induced by spinal nerve ligation

We previously showed that knockdown of $Na_V1.6$ with siRNA injected locally into the dorsal root ganglion almost completely blocked development of mechanical pain behaviors induced by local inflammation of the lumbar DRG. In the present study we used a similar approach to examine the effect of $Na_V1.6$ knockdown in neuropathic pain models. A pool of 4 siRNAs directed against $Na_V1.6$, or control non-targeting siRNA (directed against firefly luciferase) was injected into the L4 and L5 DRG. The siRNA construct used was the same as described in our previous study (Xie et al., 2013), in which we showed marked knockdown of the channel in DRG cell bodies as measured by immunohistochemistry and presented detailed evidence that off-target effects did not account for the observed effects. As shown in Figure 2 and in agreement with our previous findings, in normal animals, prior to implementing a pain model, these siRNA injections had no effect on mechanical or cold sensitivity measured 4 - 7 days later (compare POD 0 time point to prior baseline in Figure 2). On POD 0, a second siRNA injection was made and during the same surgery the L5 spinal nerve was ligated (SNL model). Compared with animals receiving control siRNA, animals receiving the $Na_V1.6$ siRNA showed a marked reduction of SNL-induced mechanical hypersensitivity. In male animals, the von Frey response (Fig. 2A) lasted for the duration of the experiment (up to 8 weeks), while the response to stroking with a light cotton wisp (Fig. 2C) resolved after 45 days; consistent with previous reports that these two types of mechanical pain have distinct mechanisms and pharmacological sensitivity (Field et al., 1999). Cold hypersensitivity was not significantly reduced by the siRNA treatment except on POD1 (Fig. 2 E).

In male animals, in the control siRNA group, SNL led to contralateral von Frey responses which were significantly different from baseline but much smaller than ipsilateral responses (smallest average threshold was 11.9 grams on POD 7). These contralateral responses were significantly reduced in the $Na_V1.6$ siRNA group on most days, to values not significantly different from baseline. Contralateral withdrawal responses to stroking with a cotton wisp were never observed in the $Na_V1.6$ siRNA group; on most POD the control siRNA group had average responses significantly higher than baseline though these were much smaller than those observed ipsilaterally. In the control group the contralateral responses showed noticeable interanimal variability; 4 of 11 animals showed large responses on multiple days while the rest responded to ipsilateral but never to contralateral stimuli. Contralateral responses to acetone were also small, and were not significantly affected by $Na_V1.6$ siRNA.

In light of important sex differences in pain, and because electrophysiological experiments were conducted using female animals, we confirmed the basic effect of $Na_V1.6$ knockdown in female animals. As shown in Fig. 2 (B, D, F), $Na_V1.6$ knockdown was also effective in reducing mechanical behaviors in females. There were no significant differences between males and females in von Frey responses or cotton wisp responses compared within either the $Na_V1.6$ or nontargeting siRNA injected groups at comparable times. As in males,

contralateral von Frey responses in females were small (never less than 11 grams); there was no effect of siRNA type on any contralateral responses at most time points.

Using the same protocol (injecting siRNA on 2 days; Fig. 1), knockdown of $Na_V1.6$ by the siRNA treatment was confirmed by immunohistochemical staining for the $Na_V1.6$ channel in DRG sections on POD4 (i.e., 4 days after the second siRNA injection; Figure 3). Comparison of the control siRNA injected DRGs with normal animals showed no significant differences in $Na_V1.6$ expression due to the SNL procedure.

Local knockdown of $Na_V1.6$ reduces hyperexcitability of myelinated A***β sensory neurons induced by spinal nerve ligation

Abnormal spontaneous activity, subthreshold membrane potential oscillations, and the ability to fire multiple action potentials are characteristic and likely interrelated (Ratte et al., 2014) properties of hyperexcitable sensory neurons in several neuropathic pain models including SNL. Microelectrode recordings from cells with myelinated axons conducting in the Aβ range (based on dorsal root conduction velocities) in isolated whole DRG preparations showed that spinal nerve ligation increased spontaneous activity in the (ligated) L5 DRG on POD4. The fractions of cells that could fire multiple action potentials or show subthreshold oscillations in response to suprathreshold current injections also increased after SNL. All three of these properties were restored to values not significantly different from normal by $Na_V1.6$-siRNA injection (Figure 4). The rheobase was also markedly reduced by SNL and slightly but significantly increased in the SNL plus $Na_V1.6$-siRNA group. Similar significant effects of SNL and of $Na_V1.6$-siRNA on rheobase were still observed when only non-spontaneously active cells were considered (spontaneously active cells were defined to have a rheobase of zero). The action potential broadening induced by SNL was unaffected by $Na_V1.6$-siRNA (Fig. 4H) when the action potential width was measured at threshold. The same result was obtained when an alternative method of defining action potential width was used, measuring the time between the maximum rate of change, dV/dt max on the rising phase and minimum dV/dt on the falling phase. Using this method the AP duration was 0.52± 0.03 msec after SNL in the control siRNA group and 0.50 ± 0.03 msec in the SNL + $Na_V1.6$-siRNA group, both significantly longer than the normal n.t. group value of 0.31 ± 0.02 msec ($p<0.001$, Kruskal-Wallis test). The average resting potential of myelinated Aβ cells was significantly more positive after SNL (−65.9 ± 0.48 mV vs. −59.4 ± 0.45 mV), a value which was only partially normalized in cells treated with $Na_V1.6$-siRNA (−61.95 ± 0.38 mV; each group was significantly different from the other two groups, $p<0.001$, Kruskal-Wallis test). We have previously shown that $Na_V1.6$ siRNA injection has relatively minor effects on most action potential parameters of myelinated neurons in the absence of a pain model, with the primary effect being a reduction in the ability of cells to fire repetitively (Xie et al., 2013).

In C cells, in contrast, there were no significant effects of SNL or of Nav1.6-siRNA on spontaneous activity (which was zero in all groups), or on subthreshold membrane oscillations or multiple action potential firing in response to suprathreshold currents. SNL decreased rheobase and increased action potential width but these effects were not mitigated by $Na_V1.6$-siRNA (Figure 5). The same result regarding action potential width was obtained

using the alternative method of defining action potential width (see above): the AP duration so defined was 2.5 ± 0.20 msec after SNL in the control siRNA group and 2.6 ± 0.14 msec in the SNL + $Na_V1.6$-siRNA group, both significantly longer than the normal n.t. group value of 1.45 ± 0.10 msec ($p<0.001$, one-way ANOVA with Bonferroni posttest). The average resting potential of C cells was significantly more positive after SNL (−57.9 ± 1.5 mV vs. −52.4 ± 1.2 mV). In the SNL + $Na_V1.6$-siRNA group, the value was −55.7 ± 0.92 mV; a value that was not significantly different from either of the other two groups (Kruskal-Wallis test).

In the relatively small number of Aδ cells recorded, 4.3% of the cells had spontaneous activity in the SNL + nontargeting siRNA group; however this was not significantly different from the values of zero observed in the other two groups (Fig. 6A). As in the Aβ cells, $Na_V1.6$ knockdown reduced the percentages of cells capable of firing multiple action potentials (Fig. 6C) or generating subthreshold membrane potential oscillations (Fig. 6B) after SNL. SNL reduced the rheobase but there was no further effect of NaV1.6 knockdown (Fig. 6D). The membrane potential was depolarized by SNL (from −64.5± 1.5 to −59.8 ± 1.7 mV) but was not significantly affected further by $Na_V1.6$ knockdown (−58.8 ± 1.0 mV; Kruskal Wallace test). Action potential broadening after SNL did not quite reach significance ($p = 0.07$) although the $Na_V1.6$ knockdown group action potential duration was significantly broader than the normal group (Fig. 6F). Using the alternative definition of action potential duration (see above) no significant differences were observed between the 3 groups (0.79 ± 0.09, 0.95 ± 0.18, and 1.43 ± 0.17 msec for the normal + n.t., SNL + n.t., and SNL + NaV1.6 siRNA groups, respectively; ANOVA with Dunn's posttest).

SNL-induced sympathetic sprouting occurs preferentially around $Na_V1.6$-positive neurons and is reduced by $Na_V1.6$-siRNA treatment

We have previously shown that spontaneously active cells are much more likely to be the targets of abnormal sympathetic fiber sprouting into the DRG in the SNL model in mice (Xie et al., 2011) as well as in rats using a different pain model that also induces sympathetic sprouting (sciatic nerve transection) (Xie et al., 2007). As shown in Fig. 4, $Na_V1.6$-siRNA strongly reduced spontaneous activity induced by SNL, as we have shown in another rat pain model (local inflammation of the DRG) (Xie et al., 2013). In that study we also showed that spontaneously active cells were more likely to be immuno-positive for the $Na_V1.6$ channel. Based on these data we hypothesized that sympathetic sprouting induced by SNL would occur preferentially around neurons expressing $Na_V1.6$, and that $Na_V1.6$-siRNA treatment would reduce sympathetic sprouting. As shown in Fig. 7, co-labeling experiments for $Na_V1.6$ and the sympathetic marker tyrosine hydroxylase in fixed DRG sections showed that 90 ± 1.3 % of neurons surrounded by sympathetic fiber "basket" formations were $Na_V1.6$ positive, compared to 58 ± 0.6 % of neurons lacking sympathetic baskets ($p < 0.05$).

The degree of sympathetic sprouting 4 days after SNL was also reduced by $Na_V1.6$-siRNA, as shown in Fig. 8. Sympathetic sprouting was quantified by determining the number of neurons surrounded by tyrosine hydroxylase – positive "basket" formations, normalized to the cellular area in each section.

$Na_V1.6$ knockdown in the L5 DRG alone also reduces pain behaviors

The SNL model was originally developed as a neuropathic pain model in which cell bodies of the injured, axotomized neurons (L5) and of the uninjured neurons (L4) are separate from each other. In this model the electrophysiological and gene expression changes are much more marked in the L5 DRG than in the L4 DRG. It was therefore of interest to determine whether knocking down $Na_V1.6$ in only the axotomized L5 DRG, instead of in both L4 and L5 DRGs as done in the preceding experiments, was also effective in reducing pain behaviors. The experimental protocol was the same as used for Figure 2 and as schematized in Figure 1, except that the two siRNA injections were done into only the L5 DRG. As shown in Figure 9, a marked reduction in pain behaviors was still observed when comparing $Na_V1.6$ injected animals to control siRNA injected animals. The effect size was similar to that seen when both L5 and L4 DRGs were injected.

In this experiment, mechanical hypersensitivity (von Frey test) on the contralateral side was minimally affected by SNL in the control siRNA injected group, differing from baseline only on POD 3, 7, and 14. There were no significant effects of $Na_V1.6$ siRNA injection on the contralateral von Frey responses. No animals in either group showed contralateral responses to stroking with a cotton wisp on any day. The differences in degree of contralateral mechanical responding even in the control siRNA injected group in this experiment compared with the experiment shown in Fig. 2 most likely reflect the greater interanimal variability in contralateral responses as discussed above; responses still differed when only 6 animals per group were analyzed for the Fig. 2 data to equalize animal numbers between the two experiments. Contralateral acetone responses were also small and not significantly affected by $Na_V1.6$ siRNA.

Local $Na_V1.6$ knockdown improves pain behaviors in a second neuropathic pain model

In order to test the generality of the our findings, we examined the behavioral effects of $Na_V1.6$ knockdown in a second neuropathic pain model, the chronic constriction of the sciatic nerve (CCI) model (Bennett and Xie, 1988). We used the same protocol as used for the SNL model, of injecting the L4 and L5 DRG with siRNA, waiting 4 days, then re-injecting the siRNA at the time the model was implemented. Preliminary experiments using the conventional CCI model, in which the sciatic nerve is partially constricted at the mid-thigh level, showed that $Na_V1.6$-siRNA injection had little effect on pain behaviors ($n = 4$ rats per group, data not shown). In the CCI model the injury site is the initial origin site of spontaneous activity. We reasoned that effects of siRNA on sodium channel density at this distance from the cell body where the siRNA was injected might occur only very slowly. We next tested the effect of $Na_V1.6$-siRNA injection in a modified version of the CCI model, in which the constriction was made at a site closer to the DRG, at the most proximal portion of the sciatic nerve just distal to the juncture of its component spinal nerves. The modified CCI model caused behavioral sensitivity similar to that observed with the conventional CCI model, but in the modified model $Na_V1.6$-siRNA was effective in reducing mechanical hypersensitivity, as shown in Fig. 10.

The modified CCI model showed almost no contralateral effects that differed significantly from baseline in any of the three behavior tests, and there were no significant effects of $Na_V1.6$ siRNA on contralateral responses.

DISCUSSION

We found that local knockdown of the $Na_V1.6$ channel greatly reduced pain behaviors, sympathetic sprouting, and sensory neuron spontaneous activity in the SNL model. Some previous studies using nerve blockade to investigate the relationship between spontaneous activity and sympathetic sprouting used local application of TTX or lidocaine (Zhang et al., 2004, Xie et al., 2005). These agents might be expected to give a general block of all neural activity. In the present study, we used siRNA directed against $Na_V1.6$. We have previously shown that acute knockdown of $Na_V1.6$ does not affect behavior in normal animals and that it blocks spontaneous activity induced by DRG inflammation while having relatively minor effects on most other electrophysiological parameters (Xie et al., 2013). Similarly, in the same DRG inflammation model, local application to the inflamed DRG of riluzole concentrations that did not affect behavior in normal animals were effective in reducing pain behaviors; the sodium channel blocker riluzole preferentially suppresses spontaneous activity at lower concentrations than are required to block evoked action potentials (Xie et al., 2012b). In conjunction with the present study these findings highlight the importance of abnormal spontaneous activity, not just neuronal activity in general, in initiating both pain behaviors and sympathetic sprouting. In this regard it is also of interest that another measure of increased neuronal excitability induced by SNL, action potential broadening, was not affected at all by $Na_V1.6$ knockdown; nor was the reduction in rheobase by SNL completely restored by $Na_V1.6$ knockdown. Spontaneous activity in Aβ cells is the electrophysiological parameter that seems to be the best correlated to mechanical pain behaviors.

We initially chose to investigate the $Na_V1.6$ isoform because its properties allow it to mediate high frequency repetitive firing, such as occurs in spontaneously active cells in many pain models including the SNL and DRG inflammation models. In both of these models $Na_V1.6$ knockdown was very effective in reducing both spontaneous activity and mechanical pain behaviors. The gene expression changes in these two models are quite different. Functionally, both potassium and sodium currents are upregulated in the local DRG inflammation model (Wang et al., 2007), in which there is no axon transection. In contrast, most studies of Na channel expression (in the axotomized L5 DRG) in the SNL model show that most Na channel isoforms, including $Na_V1.6$, are downregulated. The exception is the $Na_V1.3$ isoform, a form usually present in DRG only during development that is markedly upregulated in SNL and other models involving axon transection (Kim et al., 2001, 2002, Berta et al., 2008, Fukuoka et al., 2008, Wang et al., 2011b). It may seem counterintuitive that knockdown of $Na_V1.6$ reduces pain behaviors and spontaneous activity, given that $Na_V1.6$ is downregulated in the SNL model. However, most studies on Na channel expression in the SNL model were done at the mRNA level, not the protein level; their interpretation is confounded by other studies reporting changes in channel localization, protein expression, and regulation by $Na_V\beta$ subunits in this model (Pertin et al., 2005, Berta et al., 2008, Dib-Hajj et al., 2010, Wang et al., 2011a) . In addition, one study suggested that the absolute expression level of $Na_V1.3$ remains quite low (Berta et al., 2008), and knockout

mice lacking this channel still develop pain behaviors in response to SNL (Nassar et al., 2006). Most functional studies on Na currents have been done in small diameter neurons which were probably predominantly unmyelinated in vivo (e.g., (Flake et al., 2004)), but changes in excitability of larger, myelinated Aβ neurons play important roles in this and other models, and it is these cells that develop spontaneous activity after ligation in the L5 DRG. Our observation that knocking down $Na_V1.6$ reduces spontaneous activity and repetitive firing does not mean that increased $Na_V1.6$ is responsible for the increased excitability and spontaneous firing in the SNL model. Our results simply suggest that reducing activity of this channel is a very effective way to reduce spontaneous or repetitive firing. There can be multiple ways of manipulating ion channels to arrive at the same firing pattern, depending on the balance of opposing currents (Rho and Prescott, 2012, Ratte et al., 2014). For example, reduction of pain behaviors and repetitive firing in the SNL model was also achieved by countering the SNL-induced downregulation of the Kcna2 potassium channel (Zhao et al., 2013).

We found that $Na_V1.6$ knockdown in only the ligated L5 DRG was effective in reducing pain behaviors; simultaneous injection into the unligated L4 DRG (that presumably provides the main transmission pathway for the pain behaviors in this model) was not required. This is consistent with earlier studies showing that the electrophysiological changes are much more marked in the L5 DRG, and with studies suggesting a key role for inputs to the spinal cord from the L5 DRG (Sheen and Chung, 1993, Yoon et al., 1996); however, see also (Eschenfelder et al., 2000, Li et al., 2000).

We only observed effects of siRNA in the CCI model when the lesion site was moved closer to the DRG. This modified model was qualitatively similar to the conventional CCI model in behavioral effects. In the CCI model the initial site of spontaneous activity is at the lesion site (Tal and Eliav, 1996). We speculate that it may be difficult to manipulate Na channel levels far from the cell body with one or two siRNA injections over a few days. However, we cannot rule out the possibility that the ionic basis of the modified model differs, for example because the closer lesion site exposes the DRG cell bodies to higher levels of inflammatory mediators. Our previous study showed that recovery from $Na_V1.6$ knockdown (based on immunohistochemical staining for the channel in DRG sections) was significant by 7 days (Xie et al., 2013). We presume that the long-lasting (30 to 60 days) behavioral effects of $Na_V1.6$ knockdown observed in both the SNL and the modified CCI models in this study are not based on prolonged knockdown of $Na_V1.6$, but rather on the fact that blocking spontaneous activity at early stages of the pain model is an effective way to block establishment of the pain state (Xie et al., 2005).

The electrophysiological effects of $Na_V1.6$ knockdown were consistent with previous reports showing that this isoform, which can give rise to resurgent and persistent currents, facilitates repetitive firing (Raman and Bean, 1997, Khaliq et al., 2003, Cruz et al., 2011). Interpretation of effects on some electrophysiological parameters such as the threshold, maximum rate of voltage change or peak voltage is confounded by the depolarization observed after SNL, which was also partially mitigated by $Na_V1.6$ knockdown. However, depolarization per se cannot induce in normal Aβ cells the high incidence of spontaneous activity observed after SNL.

This study joins several previous studies indicating that $Na_V1.6$ may be a useful target in pain. Much of the focus in pain research has been on the $Na_V1.7$ isoform because its genetic deletion causes congenital indifference to pain in humans. However, a recent study of several pain models using mice lacking specific Na channel isoforms showed that $Na_V1.7$ was not required for certain types of neuropathic pain including oxaliplatin-induced pain (Minett et al., 2014), which has previously been shown to depend on $Na_V1.6$ (Sittl et al., 2012, Deuis et al., 2013). This study also showed that SNL-induced pain could be observed after $Na_V1.7$ was deleted from sensory neurons. A recent study (Kalezic et al., 2013) showed that several sodium channel blockers' ability to inhibit $Na_V1.7$ in vitro correlated well with their ability to inhibit spontaneous activity in vivo in the SNL model. Several factors may contribute to the apparent discrepancy between this study and the data presented here. We used $Na_V1.6$ knockdown early in the SNL model, whereas Kalezic et al. studied later time points (e.g. up to POD 14); our data may be more relevant to initiation of the pain state rather than maintenance. Although most of the drugs Kalezic et al. tested were from an $Na_V1.7$ discovery program, for those for which we could find other published data, the isoform specificity was usually based on comparison to potency for blocking $Na_V1.5$ but not explicitly compared to that for $Na_V1.6$. Finally, their study used fiber recording whereas our electrophysiological data were based on recordings from the cell body. We propose that $Na_V1.6$, particularly its conformations that allow repetitive firing, should still be considered a possible therapeutic target for neuropathic pain conditions.

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Highlights

- $Na_V1.6$ channel was knocked down with small inhibitory RNA in DRG before spinal nerve ligation.
- Mechanical pain behaviors were reduced at all time points.
- Spontaneous activity of myelinated neurons was reduced.
- Sympathetic sprouting occurred around $Na_V1.6$-expressing neurons and was reduced by knockdown.
- Knockdown also reduced pain behavior in a sciatic nerve constriction model.

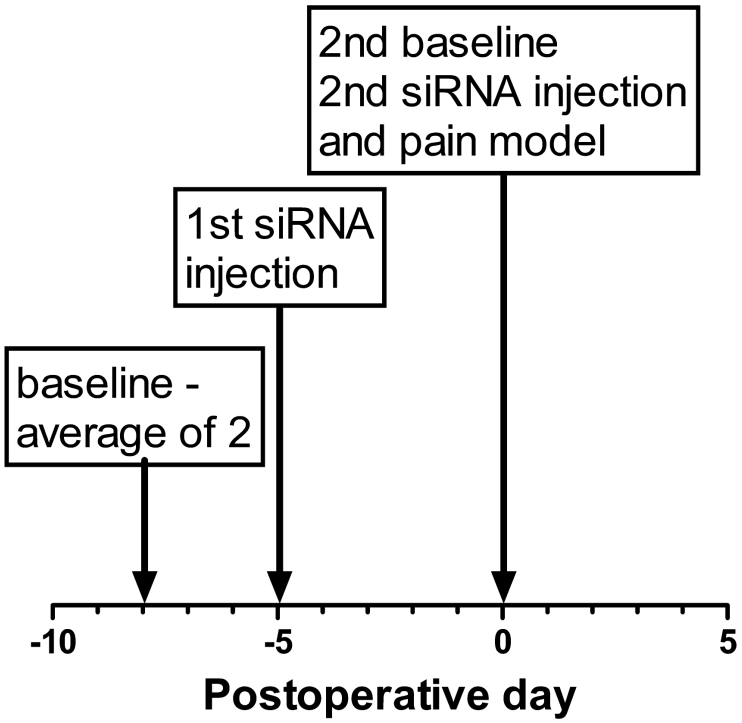

Figure 1.

Protocol for behavior experiments. At least two baseline measurements were performed prior to any surgeries; their average value is plotted as POD -8. A first surgery was performed to inject siRNA (either control nontargeting, or directed against $Na_V1.6$) into the L4 and L5 DRGs. Four to seven days later, on POD 0, a second behavioral baseline was obtained to determine the effects of the siRNA alone. This is plotted as POD 0. Immediately after the behavior testing, a second surgery was performed to inject a second aliquot of the siRNA and to implement the pain model (spinal nerve ligation or chronic constriction of the sciatic nerve). Behavioral measurements then continued for up to two months, as indicated in the figures. In one set of experiments only the L5 DRG was injected.

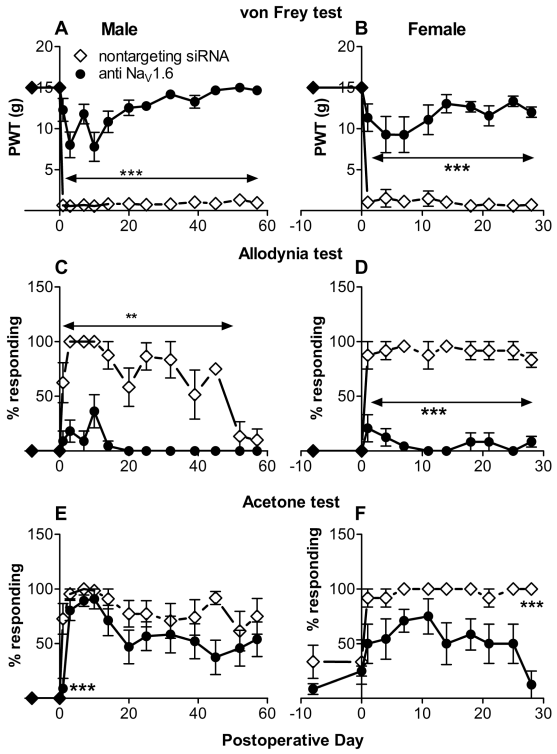

Figure 2.

Effect of $Na_V1.6$ knockdown in spinal nerve ligation model. Data from male animals, as used for most other behavioral experiments in this study, are on the left. Data from female animals such as those used for electrophysiological studies are on the right; experimenter was blinded as to type of siRNA injected. Baseline values for the von Frey test (A, B), cotton wisp test (C, D) and acetone cold sensitivity test (E, F) were measured twice and the average is plotted at POD -8. The first surgery was done to inject $Na_V1.6$-siRNA or nontargeting control siRNA into the L4 and L5 DRG on one side. Four to seven days later, on POD 0, behaviors were measured again (presurgical value is plotted on day 0), at which time there was no significant effect of the siRNA treatment on any of the measured behaviors in either sex, and then the second surgery was done immediately after the behavior testing to ligate the L5 spinal nerve and inject a second dose of the siRNA. $Na_V1.6$-siRNA significantly reduced von Frey sensitivity on all days after the spinal nerve ligation (***, $p<0.001$ all time-points), and reduced responding to stroking with a cotton wisp (**, $p<0.01$, ***, $p<0.001$, at all time-points from POD1 to POD 45). Cold hypersensitivity was significantly affected by the $Na_V1.6$-siRNA in males only on POD1 and in females only on POD 28. Values compared using two-way repeated measures ANOVA with Bonferroni posttest. N = 11 male animals or 4 female animals per group. PWT, paw withdrawal threshold. Data shown are from the ipsilateral side; see text for discussion of contralateral results.

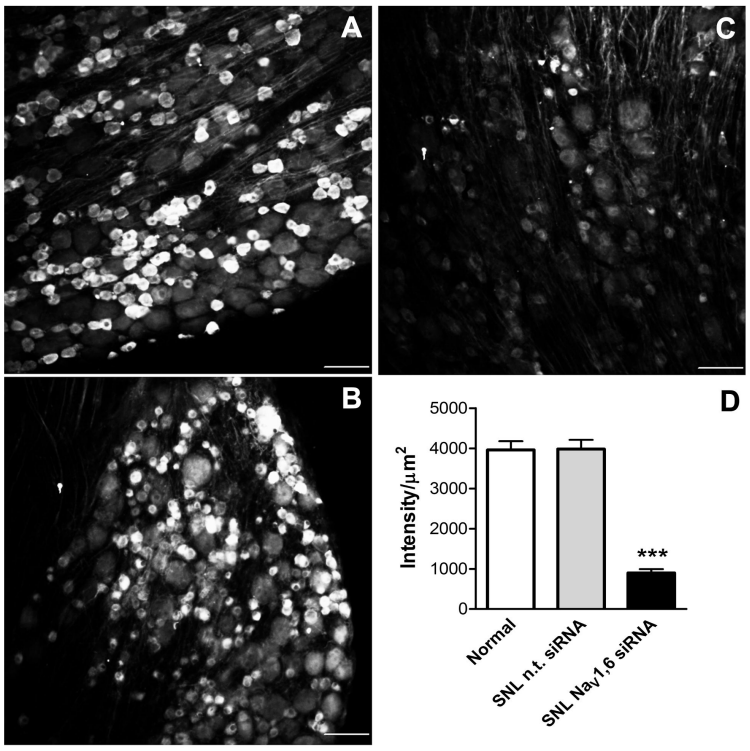

Figure 3.

Knockdown of $Na_V1.6$ was confirmed by immunohistochemical staining for the channel. Sections of L5 DRG from normal animals (A), and from animals 4 days after L5 spinal nerve ligation with 2 preceding siRNA injections for control nontargeting siRNA (B) or $Na_V1.6$ siRNA (C) were stained for $Na_V1.6$. The intensity of $Na_V1.6$ staining in each section was normalized to the cellular area (D). N = 79 to 87 sections from 9 different animals (3 per group). ***, significant difference between nontargeting (n.t.) and $Na_V1.6$-siRNA injected groups, Kruskal-Wallis test with Dunn's Multiple Comparison posttest. Scale bars, 100 μm.

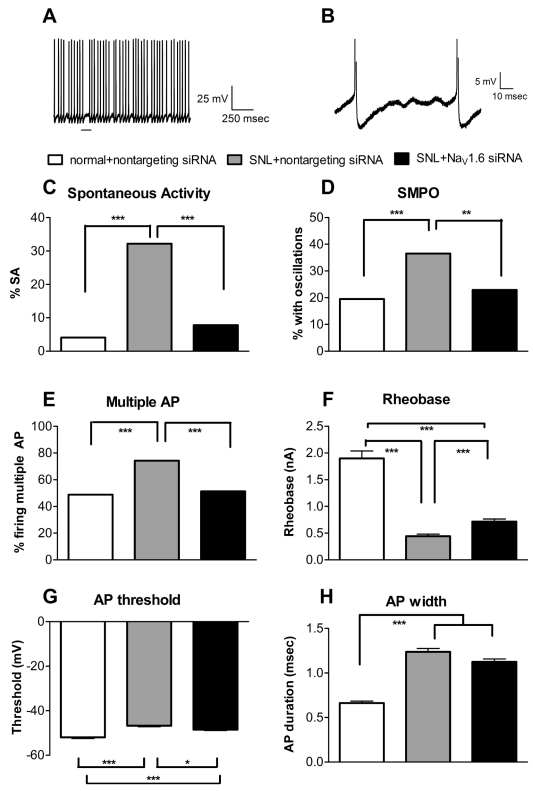

Figure 4.

Effect of spinal nerve ligation and $Na_V1.6$-siRNA on electrophysiological parameters of myelinated Aβ neurons. Sharp microelectrode recordings in isolated whole L5 DRG were performed in normal DRG 4 days after injection with non-targeting control siRNA, or in DRG 4 days after ligation of the L5 spinal nerve (SNL) and second injection of non-targeting or $Na_V1.6$-siRNA. (A) Example of spontaneous activity in a neuron after SNL + nontargeting siRNA injection. (B) Expansion of the portion of the trace in (A) indicated by the underline to show subthreshold oscillations between groups of action potentials. (C) Spontaneous activity was increased by SNL and normalized by $Na_V1.6$-siRNA (chi-square test). Suprathreshold current injections could induce subthreshold membrane potential oscillations (SMPO; D) and multiple (>2) action potentials (AP; E) in a larger percentage of cells after SNL and these values were normalized by $Na_V1.6$-siRNA (chi-square test). Other measures of hyperexcitability: rheobase (F); action potential threshold (G), and action potential duration (H) were increased by SNL but not completely normalized by $Na_V1.6$-siRNA. Values were compared using Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. ***, $p<0.001$, significant difference between the indicated groups. N = 123 normal + nontargeting siRNA cells from 4 rats, 183 SNL + nontargeting siRNA cells from 6 rats, and 230 SNL + $Na_V1.6$-siRNA cells from 7 rats.

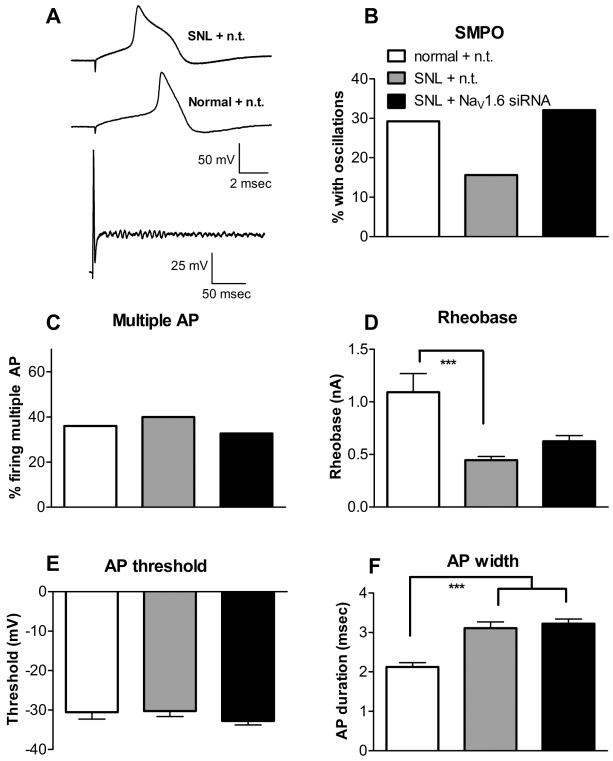

Figure 5.

Effect of spinal nerve ligation and $Na_V1.6$-siRNA on electrophysiological parameters of unmyelinated C neurons. Sharp-microelectrode intracellular recordings in isolated whole L5 DRG were performed in normal DRG 4 days after injection with non-targeting control siRNA, or in DRG 4 days after ligation of the L5 spinal nerve (SNL) and second injection of non-targeting or $Na_V1.6$-siRNA. (A) Examples of evoked action potentials (top) and subthreshold membrane potential oscillations evoked by larger, longer current pulses (bottom). Spontaneous activity was not observed in any C cells. Suprathreshold current injections could induce subthreshold membrane potential oscillations (SMPO; B) and multiple (>2) action potentials (AP; C) in a percentage of cells but these percentages were not affected by SNL or by the siRNA treatment (chi-square test). Some other measures of hyperexcitability: rheobase (D), and AP duration (F) but not AP threshold (E), were increased by SNL but not affected by $Na_V1.6$-siRNA. Values were compared using Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. ***, $p<0.001$, significant difference between the indicated groups. N = 25 normal + nontargeting siRNA cells from 4 rats, 35 SNL + nontargeting siRNA cells from 6 rats, and 56 SNL + $Na_V1.6$-siRNA cells from 7 rats.

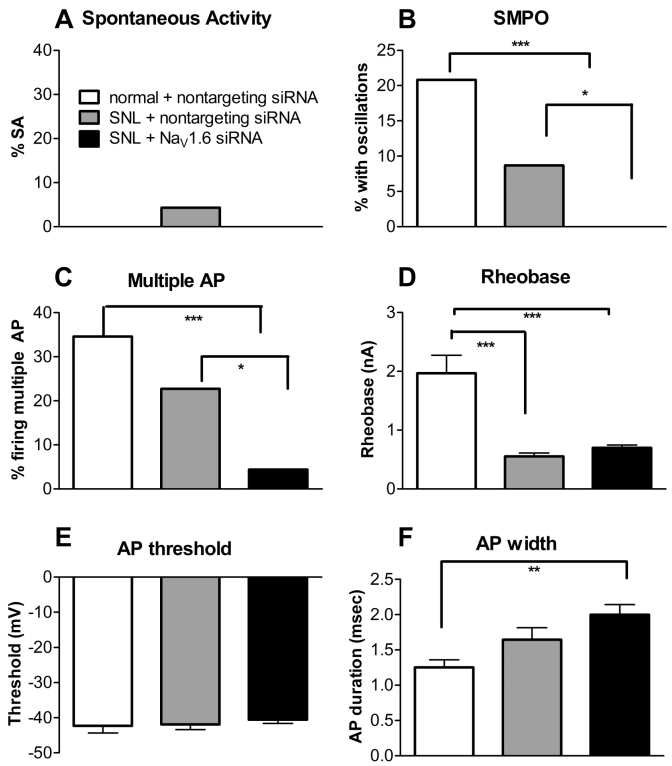

Figure 6.

Effect of spinal nerve ligation and $Na_V1.6$-siRNA on electrophysiological parameters of Aδ neurons. Sharp microelectrode recordings in isolated whole L5 DRG were performed in normal DRG 4 days after injection with non-targeting control siRNA, or in DRG 4 days after ligation of the L5 spinal nerve (SNL) and second injection of non-targeting or $Na_V1.6$-siRNA. (A) Spontaneous activity was zero except in the SNL + non-targeting siRNA group and there were no differences between the groups (chi-square test). Suprathreshold current injections could induce subthreshold membrane potential oscillations (SMPO; B) and multiple (>2) action potentials (AP; C) in a smaller percentage of cells after SNL and these values were even smaller after $Na_V1.6$-siRNA (chi-square test). Rheobase was decreased by SNL but there was no further effect of $Na_V1.6$-siRNA (D). Action potential threshold did not differ between the three groups (E). Action potential duration (F) increase by SNL did not reach significance ($p = 0.07$) but was significantly higher in the SNL + $Na_V1.6$-siRNA group. Values were compared using Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. ***, $p<0.001$, significant difference between the indicated groups. N = 26 normal + nontargeting siRNA cells from 4 rats, 22 SNL + nontargeting siRNA cells from 6 rats, and 45 SNL + $Na_V1.6$-siRNA cells from 7 rats.

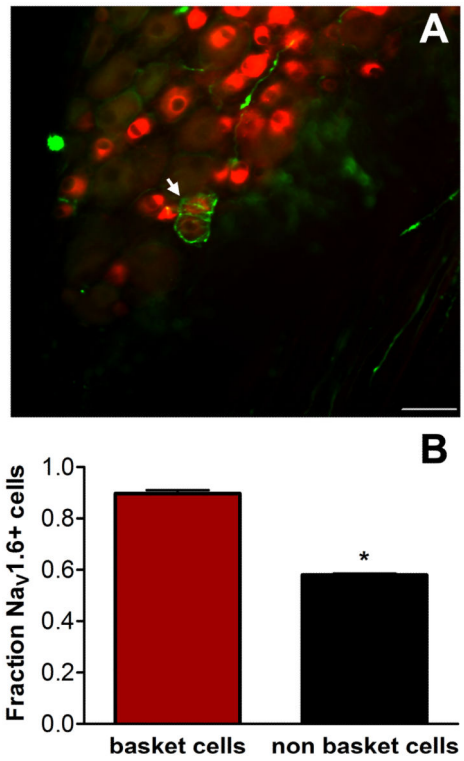

Figure 7.

Co-localization of $Na_V1.6$ and sprouting sympathetic fibers. DRG sections were labeled for $Na_V1.6$ (red) and tyrosine hydroxylase (green). As shown in the sample section (A), 4 days after spinal nerve ligation sympathetic fibers formed basket formations around some neurons (white arrow), structures rarely observed in normal DRGs. Scale bar, 50 μm. Cells with basket formations were scored as $Na_V1.6$ positive or negative. In a subset of the same sections, all the neurons lacking sympathetic baskets were also scored as $Na_V1.6$ positive or negative. The overall fraction of $Na_V1.6$-positive neurons was calculated for each rat from analysis of multiple sections (B). *, significant difference between groups (Mann-Whitney test). Data presented are average fractions from 4 rats, derived from scoring of 1043 basket cells observed in 291 sections, and 4936 non-basket cells observed in 57 sections.

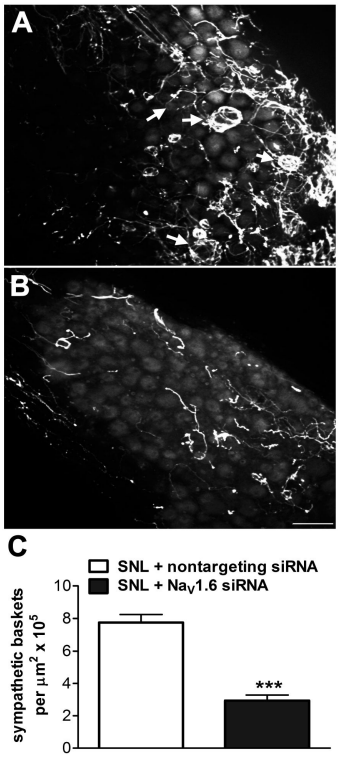

Figure 8.

Effect of $Na_V1.6$-siRNA on sympathetic sprouting in the L5 DRG. DRG sections were labeled for tyrosine hydroxylase. As shown in the sample sections, 4 days after spinal nerve ligation sympathetic fibers formed basket around some neurons in the ligated L5 DRG (as indicated by white arrows) (A), structures rarely observed in normal DRGs, and these were reduced in animals receiving siRNA directed against $Na_V1.6$ (B). Scale bar=100 μm. To quantify this observation, the number of neurons with tyrosine hydroxylase-positive basket formations was counted in each section, and normalized to the cellular area (C). Data are from 105 sections from 3 rats treated with $Na_V1.6$-siRNA and 101 sections from 3 rats treated with nontargeting control siRNA. ***, significant difference between the 2 groups, $p<0.001$, Mann-Whitney test.

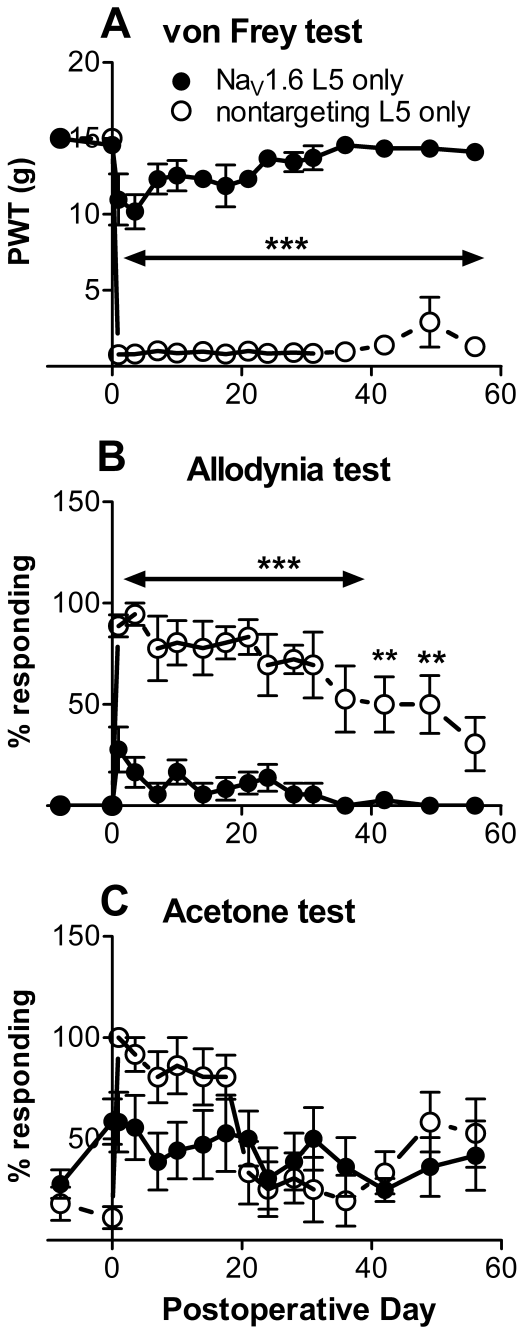

Figure 9.

Effect of Na$_V$1.6 knockdown in L5 DRG alone in spinal nerve ligation model. Baseline values for the von Frey test (A), cotton wisp test (B), and acetone cold sensitivity test (C) were measured twice and the average is plotted at postoperative day -8. The first surgery was done to inject Na$_V$1.6-siRNA or nontargeting control siRNA into only the L5 DRG on one side. Three days later, on postoperative day 0, behaviors were measured again (presurgical value is plotted on day 0), at which time there was no significant effect on any of the measured behaviors, and then the second surgery was done immediately after the

behavior testing to ligate the L5 spinal nerve and inject a second dose of the siRNA. $Na_V1.6$-siRNA significantly reduced von Frey sensitivity on all days after the spinal nerve ligation. (***, $p<0.001$ all time-points), and reduced responding to stroking with a cotton wisp (***, $p<0.001$ at all time- points except POD 42 and 49, ** $p<0.01$; POD56 not significant). Cold hypersensitivity was not significantly affected by the $Na_V1.6$-siRNA (C). Values compared using two-way repeated measures ANOVA with Bonferroni post-test. N = 6 animals per group. The experimenters were blinded as to the siRNA treatment. Data are from the ipsilateral side; see text for discussion of contralateral effects.

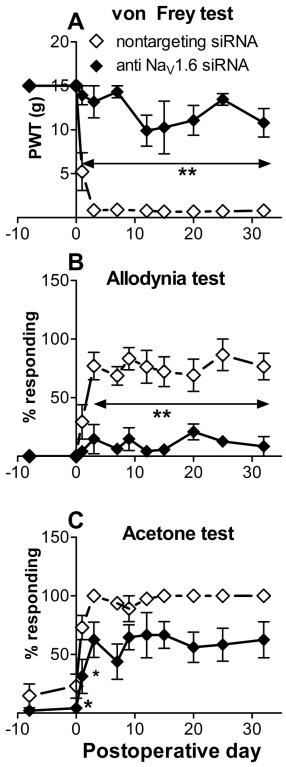

Figure 10.

Effect of Na$_V$1.6-siRNA on pain behaviors in a modified chronic constriction injury model. After baseline pain measurements (average plotted on POD -8), siRNA was injected into L4 and L5 DRGs. Pain behaviors were measured again 4 days later (plotted on POD 0), and then the chronic constriction injury was implemented on the most proximal part of the sciatic nerve, just distal to the junction of the component spinal nerves. Mechanical hypersensitivity (A) and allodynia (B) were significantly reduced on all post CCI days tested; cold allodynia (C) was reduced on the two indicated days. *, $p<0.05$, **, $p<0.01$, significant difference between the two groups (two-way repeated measures ANOVA with Bonferroni posttest). N = 8 animals per group; the control group consisted of 4 males and 4 females; the NaV1.6 group consisted of 6 males and 2 females; no obvious differences between the sexes were observed.