RELATIONSHIP OF AXONAL VOLTAGE-GATED SODIUM CHANNEL 1.8 (NaV1.8) mRNA ACCUMULATION TO SCIATIC NERVE INJURY-INDUCED PAINFUL NEUROPATHY IN RATS

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Running Title: Axonal NaV1.8 mRNA and painful neuropathy

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Background: Painful neuropathy is an unsolved disease with increased voltage-gated sodium channel (NaV) activities.

Results: NaV1.8 shRNA treatment attenuated injury-induced pain behavior and normalized the NaV1.8 mRNA levels in the affected axons but not in somata of sensory neurons.

Conclusion: Painful neuropathy may causally involve axonal NaV1.8 mRNA.

Significance: Nerve injury may induce axonal NaV1.8 mRNA accumulation, which may offer a novel therapeutic target.

SUMMARY

Painful peripheral neuropathy is a significant clinical problem; however, its pathological mechanism and effective treatments remain elusive. Increased peripheral expression of tetrodotoxin-resistant voltage-gated sodium channel 1.8 (NaV1.8) has been shown to associate with chronic pain symptoms in humans and experimental animals. Sciatic nerve entrapment (SNE) injury was used to develop neuropathic pain symptoms in rats, resulting in increased NaV1.8 mRNA in the injured nerve, but not in dorsal root ganglia (DRG). To study the role of NaV1.8 mRNA in the pathogenesis of SNE-induced painful neuropathy, NaV1.8 short hairpin RNA (shRNA) vector was delivered by subcutaneous injection of cationized gelatin/plasmid DNA polyplex into the rat hindpaw and its subsequent retrograde transport via sciatic nerve to DRG. This in vivo NaV1.8 shRNA treatment reversibly and repeatedly attenuated the SNE-induced pain symptoms, an effect became apparent following a distinct lag period of 3-4 days and lasted for 4-6 days before returning to pretreatment levels. Surprisingly, apparent knockdown of NaV1.8 mRNA occurred only in the injured nerve, not in the DRG, during the pain alleviation period. Levels of heteronuclear NaV1.8 RNA were unaffected by SNE or shRNA treatments suggesting that transcription of the Scn10a gene encoding NaV1.8 was unchanged. Based on these data we postulate that increased axonal mRNA transport results in accumulation of functional NaV1.8 protein in the injured nerve and the development of painful neuropathy symptoms. Thus, targeted delivery of agents that interfere with axonal NaV1.8 mRNA may represent effective neuropathic pain treatments.

Injuries to peripheral nerves often cause chronic pain manifesting as post-herpetic neuralgia, painful diabetic neuropathy and painful post-traumatic neuroma. Chronic peripheral neuropathy affects ~1.5 % of the general population (1) and greatly impairs quality of life, as current treatments remain unsatisfactory for
most patients. Painful neuropathies commonly share clinical features such as light touch-evoked pain (alldynia) (2-5), burning sensation, exaggerated responses to noxious stimuli (hyperalgesia), and either spontaneous or evoked unpleasant abnormal sensations (dysesthesia) (6). Although several pathological mechanisms have been proposed (7-9), the precise molecular mechanisms contributing to the development and maintenance of peripheral neuropathy are still elusive.

Since the generation and propagation of action potentials in sensory neurons depend on the activity of voltage-gated sodium channels (VGSCs), it has been postulated that abnormal neuronal hyperexcitability due to altered regulation of VGSCs may play a key role in the molecular pathogenesis of painful neuropathy (10,11). Altered expression of VGSCs that are found in primary sensory neurons; e.g., NaV1.3, NaV1.7, NaV1.8 and NaV1.9; has been shown to associate at various degrees with human neuropathies (12-17) and animal neuropathy models (18-21). Among the peripheral VGSC isoforms, NaV1.8 is the predominant tetrodotoxin-resistant sodium channel expressed exclusively in primary sensory neurons with particularly high levels of expression in nociceptive neurons of small- and medium-sized soma diameters (22). NaV1.8 is involved in nociceptive signaling through up-regulation of channel expression and kinetics after tissue inflammation and its contribution to action potential propagation in nociceptive neurons (23,24). After peripheral nerve injury, NaV1.8 appears to be redistributed preferentially to nerve axons (9,25). Selective pharmacological inhibition of NaV1.8 function (26) or NaV1.8 expression in DRG using intrathecal administration of antisense oligodeoxynucleotides (27) has been shown to decrease mechanical allodynia and thermal hyperalgesia in rats with spinal nerve ligation (SNL). These reports underscore the role of NaV1.8 mRNA in the pathogenesis of pain behaviors. Here we tested this hypothesis by targeted suppression of NaV1.8 mRNA using short hairpin RNA (shRNA) delivery to lumbar dorsal root ganglion (DRG) neurons in vivo. We report that shRNA-derived NaV1.8 knockdown attenuated SNE-induced pain behaviors in rats after a distinct lag period. A series of mechanistic experiments suggested that the axonal accumulation of NaV1.8 mRNA, possibly through an injury-activated subcellular transport system, plays a causal role in the development of painful neuropathy.

**Experimental Procedures**

_Anomals_- Adult Sprague-Dawley rats weighing 200-300 g were used throughout. The animal experiments were performed in accordance with the guidance of the National Institutes of Health on animal care and University of California at Los Angeles Animal Research Committee.

_NaV1.8 shRNA plasmid DNA and lentiviral vector construction_- Four small interfering (si) RNA target sequences to NaV1.8 mRNA (Accession No. NM_017247) were designed: shRNA1 (nt 1203-1221); shRNA2 (3407-3425); shRNA3 (6009-6027); and shRNA4 (6033-6051). NaV1.8 shRNA1, 2 and 4 were previously published (29,30) and shRNA3 was newly designed (Dharmacon, Lafayette, CO). Nucleotide BLAST verified that siRNA sequences were complimentary only to the Nav1.8 mRNA. A random shRNA served as a negative control: GCAGCAACTGGACACGTGA. To create the short hairpin (sh) constructs, sense siRNA sequences were linked to their antisense sequences by a stem loop (TTCAAGAGA) (31). The 55-nt sense oligos and the 59 nucleotides of antisense oligos containing additional nucleotides at 5’ end for XhoI overhang were subcloned into pLL3.7 (Lentilox 3.7, MIT, Cambridge, MA) (Figure 1A). The shRNA-expressing vector contained mouse U6 promoter for shRNA expression and CMV promoter for enhanced green fluorescent protein (EGFP) expression. The pLL3.7-NaV1.8shRNA constructs were large-scale amplified and each lentiviral vector was produced by a triple transfection of three plasmids; pLL3.7-NaV1.8 shRNA, pΔ8.9, and pVSVG.

_Culture of DRG neurons_- DRG neuron cultures (32) were used to determine the efficiency
and specificity of shRNA constructs. Harvested rat lumbar DRG were transferred to ice-cold Hanks balanced salt solution (HBSS) containing 20% fetal bovine serum (FBS) and cut into small pieces. Tissues were washed with cold 20% FBS-HBSS and then HBSS before incubating in collagenase solution (1.25 mg collagenase P/5 ml HBSS and 0.2 mg DNase I/ml HBSS) for 75 min. Next, 1.25 mg/ml trypsin and 0.5 mg DNase I/ml in HBSS were added and incubated for 5 min at 37°C followed by wash with 20% FBS-HBSS followed by HBSS. After washing, the pellet was re-suspended in dissociation solution (12 mM MgSO4 and 0.2 mg DNase I /ml HBSS) and triturated with a silicone coated Pasteur pipette until the solution appeared uniformly cloudy. After centrifugation, the cell pellet was collected, re-suspended with 1000 ml DRG culture medium/well (10% FBS, 1% antibiotic-antimycotics, 0.5% of 1.5 mg/ml Uridine and 0.5% of 3.5 mg/ml floxuridine in MEM) and plated on a 6-well plate pre-coated with matrigel (Becton Dickinson, Franklin Lakes, NJ). Cells were subjected to experiments 24 hrs after seeding. The medium was changed every 2 days.

NaV1.8 shRNA lentiviral vectors (1 mg p24 = 5x10^7 infectious units in 293T cells/ml of MEM without FBS), polybrene (8 mg/ml), 1.5 mg/ml uridine (final v/v 0.5%) and 3.5 mg/ml floxuridine (final v/v 0.5%) were added to DRG cells and incubated for 24 hr. DRG cells were washed and supplemented with DRG culture medium. Cells were collected 48 hr after transduction for FACS, RT-PCR and immunocytochemistry analyses (see below).

**Fluorescence-activated cell sorting (FACS) analysis**- DRG cells were dissociated from the plate with 2 ml of trypsin solution. Cells were collected and re-suspended with cold phosphate-buffered saline (PBS). Cells were stained with 10 µg/ml of Isolectin-B4 (IB4) conjugated with AlexaFluor-647 (Invitrogen, Carlsbad, CA). EGFP and AlexaFluor-647 were measured by FACS (EPICS Elite ESP, Beckman Coulter, Fullerton, CA).

**Quantitative real time PCR**- Total RNA from DRG cultures was isolated and treated with DNase I (Ambion, Austin, TX). The steady state mRNA levels of NaV1.3, NaV1.6, NaV1.7, NaV1.8 and NaV1.9 were determined by Taqman-based real time PCR (RT-PCR): Nav1.3-Rn00565438_m1; Nav1.6-Rn00570506_m1; Nav1.7-Rn00581647_m1; Nav1.8-Rn_00568393_m1 and Nav1.9-Rn00570487_m1. The mRNA expression levels were normalized using the comparative C_T method.

**DRG culture immunocytochemistry**- DRG culture medium was decanted, cultures were rinsed with PBS at 37°C for 1 min and fixed with 3.7% formaldehyde for 10 min. Next, cells were treated with 0.2% TritonX for 5 min and background masking solution (ImageIT FX, Invitrogen) for 30 min, followed by incubation with primary antibody against rat C-terminal NaV1.8 peptide at 4°C overnight (1:200 dilution, Sigma), and followed by secondary antibody conjugated with AlexaFluor-647 (Invitrogen) at room temperature for 1 hours. After staining with 10 µg/ml AlexaFluor-594 conjugated with IB4 (Invitrogen) at room temperature for 30 min, slides were coverslipped with mounting medium (ProLong Gold, Invitrogen). A confocal laser-scanning microscope (Carl Zeiss LSM 310) was used to scan 1 µm focal layers of each specimen at 63x magnification. Individual sections were then digitally reconstructed and analyzed with digital software (ImageJ 1.43; NIH, Bethesda, MD).

**In vivo transfection with cationized gelatin (CG)/NaV1.8 shRNA plasmid DNA polyplexes**- CG/shRNA plasmid DNA polyplex was prepared as previously described (32). Briefly, 85 µl to 100 µl of CG/DNA polyplex injectates were prepared at 7.5:1 CG-to-DNA mass ratio containing 17 µg to 25 µg per injection. The CG/DNA polyplex injectates were incubated at 37°C for 30 min prior to injection.

Rats were anesthetized with isoflurane (2%), and the vector/DNA polyplex was slowly (~1 min) injected subcutaneously into the center of the plantar surface of the left hindpaw with 27-gauge needles. The needle was removed and the injection site was immediately sealed with liquid Band-Aid (Johnson & Johnson, New Brunswick, NJ).

**Detection of synthesized NaV1.8 siRNA molecules**- DRG and nerve tissues were harvested 2.5 days and 7 days after in vivo injection of CG/NaV1.8 shRNA polyplexes. Small-size RNA specimens were prepared from DRG and nerves (mirVaNa™ small RNA isolation kit, Ambion). Synthesized siRNA was detected by Taqman based stem–loop RT–PCR (33). RNA (100 ng) was used for reverse transcription with custom
stem-loop primer for siRNA3 with 100 mM dNTPs, 50 U/µl multiscribe reverse transcriptase, 10x reverse transcriptase buffer and 20 U/µl RNase inhibitor, followed by RT-PCR. 4.5S RNA served as an endogenous control. Spiked samples of siRNA3 molecules were obtained by adding known amounts of synthetic siRNA3 (500, 200, 100, 50, 10, and 1 pmole) to total RNA of rat DRG and nerves. The amount of mature siRNA3 from experimental tissues was based on comparison of C_T values between experimental and spiked samples using the standard curve.

In vivo efficiency of shRNA-derived NaV1.8 knockdown- DRG tissues were harvested 2.5 days after in vivo injection of CG/shRNA3 polyplex and acutely dissociated as described above. DRG culture was subjected to immunostaining with NaV1.8 antibody and GFP antibody, as well as IB4 staining. The NaV1.8 immunofluorescence intensity was determined in GFP+/IB4+ and GFP-/IB4+ neurons.

Sciatic nerve entrapment (SNE)- Surgical procedure for the SNE model was described previously (34,35). Briefly, in anesthetized rats, the left sciatic nerve was surgically exposed and 3 polyethylene cuffs (1 mm long, 2.28 mm outer diameter, and 0.76 mm inner diameter) were loosely fitted to the sciatic nerve proximal to the trifurcation of common peroneal, tibial and sural nerves (Figure 3A). Muscle and skin were separately closed.

Behavioral testing - For a set of control and SNE rats, daily measurements of hindpaw withdrawal thresholds to mechanical stimuli and withdrawal latencies to thermal stimuli were obtained in both naïve controls and SNE rats as described in detail previously (35).

In vivo transfection with CG/NaV1.8 shRNA plasmid DNA polyplexes- After the stable pain behavior was established; CG/shRNA plasmid DNA polyplex was injected to the ipsilateral hindpaw as described above.

DRG, nerve and hindpaw skin harvested from SNE rats- After completion of behavioral testing a set of SNE and shRNA-treated rats were anesthetized with pentobarbital (80 mg/kg) and perfused through the ascending aorta with 300 ml of 0.9% NaCl, followed by 300 ml of ice-cold freshly prepared 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The L4/L5 DRG and sciatic nerves proximal to the injury were harvested and post-fixed at 4°C for 2-4 hr. All DRG and sciatic nerves were cryoprotected through sucrose gradient concentrations in 0.1 M PB at 4°C overnight, and then embedded in the frozen mold using Tissue Freezing Medium (TFM™, Triangle Biomedical Sciences, Durham, NC). Specimens were cryosectioned at 25 µm (DRG) or 20 µm (sciatic nerve) and mounted on gelatin-coated pre-cleaned microscopic slides. To minimize variability between specimens, ipsilateral and contralateral tissues were processed simultaneously. Four specimens from ipsilateral and contralateral tissues were mounted on the same slide. For immunostaining, tissue sections were fixed in cold acetone (-20 °C) for 10 min, rinsed with tris-buffered saline (TBS) 4 times (3, 5, 7 and 7 min, respectively) and air-dried. Sections were incubated (30 min) in blocking solution (1% BSA + 2% normal donkey serum (NDS) + 0.2% Tween) and then incubated in mouse monoclonal antiserum targeting the C-terminal residues (1724-1956) of NaV1.8 (1:500 dilution, Neuromab, Davis, CA) at 4°C overnight. Then sections were washed and incubated in secondary antibody (AlexaFluor-488-conjugated donkey anti-mouse antiserum, Invitrogen) (1:500 dilution) for 1 hr at room temperature and then in IB4-AlexaFluor 594, 1:250 dilution) for 30 min at room temperature. All sections were coverslipped with mounting medium (Vectashield, Vector Laboratories, Burlingame, CA).

Images were acquired using a Leica confocal SP2 1P-FCS microscope (Leica Microsystems; Bannockburn, IL) and captured using the same parameter settings (i.e. gain, pinhole, thickness of scanned images). For each DRG and nerve, 4 sections were used for confocal scanning. Captured images were 512x512 pixels. ImageJ software was used for quantitative analysis. DRG neurons positive to IB4 and with clearly visible nuclei were included for analysis. Only cytoplasmic immunoreactivity of NaV1.8 in IB4+ DRG neurons was outlined for quantification.
making each region of interest correspond to the cytoplasmic profile of a single DRG neuron. Pixel intensities of image NaV1.8 immunoreactivity ranged from 0 (darkest) to 255 (lightest). The mean immunoreactivity for each cytoplasmic profile of DRG cells and nerves was converted to relative immunoreactivity (relative IR) using the formula (36); [(mean immunoreactivity value–MIN)/(MAX–MIN)]×100, where MAX and MIN are the maximum and minimum mean immunoreactivity values. Relative IR values were used to generate scatter plots. In addition, the mean immunoreactivity from DRG neuron and nerve sections was used for plotting bar graphs. DRG neurons were sorted based on size <700 µm² and 700-1200 µm². DRG neurons >1200 µm² were not included in the analyses due to the generally low levels of NaV1.8 expression.

Scn10a gene transcription- Total RNA was extracted from collected tissues (DRG, sciatic nerve and skin). To evaluate the transcriptional activity of Scn10A gene (encoding NaV1.8), NaV1.8 heteronuclear RNA (hnRNA, a.k.a. pre-mRNA) assay was performed as a surrogate measurement (37). Taqman-based primers were designed targeting the intron between exons 27 and 28 (5’TCATGGCTTGAGACACTGATTAGAC3’) and exon 28 (5’CAGTGACTTAAGGATTGCAGAAAACA3’). Total RNA (1 µg) from DRG of SNE-injured and shRNA-treated rats were reverse transcribed using a random hexamer primer and subjected to RT-PCR analysis using the hnRNA primer/probe set. The initial validation of hnRNA RT-PCR assay was performed in vitro using acutely dissociated DRG neuron cultures treated with different doses of NGF (0, 10, 50 or 100 ng/ml) for 48 hrs.

Quantitative real time PCR- The steady-state NaV1.8 mRNA levels of DRG, sciatic nerve and skin were determined using Taqman-based RT-PCR. GAPDH served as internal control. Data were expressed as a ratio of ipsilateral mRNA level over contralateral mRNA level within the same animals. In a separate experiment, the steady-state mRNA levels of NaV1.6, 1.7, 1.8 and 1.9 were determined in DRG and nerve contralateral and ipsilateral to SNE.

Statistical Analyses- Student’s t-test for 2-group comparison and one- or two-way analysis of variance (ANOVA) with Tukey’s multiple comparisons for multi-group comparison were used to analyze RT-PCR and immunohistochemistry data with p<0.05 accepted as statistically significant. Repeated measures ANOVA analysis was used to compare hindpaw sensitivity to mechanical and thermal stimuli in naïve and SNE-injured rats. Ipsilateral and contralateral hindpaw data were standardized by subtracting the ipsilateral and contralateral data points to get absolute difference. For each animal the baseline value (i.e. average standardized score, post-SNE or pre-injection) was subtracted from the corresponding value (absolute difference value) at each subsequent time point (post-injection) to adjust for any potential baseline difference between groups. The change from baseline means was compared parametrically using repeated measures ANOVA. For each outcome, estimated mean, SEM and p-value for between group comparisons across time were reported. 5% least significant difference (LSD) for within group comparison was calculated. The mean difference between any two-time points must be larger than LSD.

RESULTS

shRNA-mediated NaV1.8 knockdown in vitro. We designed four RNA interference knockdown vectors containing U6 promoter-driven shRNA and CMV promoter-driven EGFP reporter (Figure 1A). The efficacy and selectivity of NaV1.8 mRNA knockdown was determined in cultured DRG neurons after lentivirus-assisted transduction of the shRNA vectors. Transduction efficiency assessed by detection of EGFP in isoelectric B4 (IB4) positive neurons using FACS was determined to be 69.5% (data not shown). Significant NaV1.8 mRNA knockdown was achieved by shRNA3 and shRNA4 (Figure 1B). The effect of all shRNAs on other NaV isoforms (NaV1.3, NaV1.5, NaV1.6, NaV1.7 and NaV1.9) that share some sequence similarities with NaV1.8 was also examined. Off-target knockdown of NaV1.6 was observed with shRNA4, while shRNA1 decreased NaV1.3 mRNA (Figure 1B). Thus, effective and specific NaV1.8 knockdown was achieved only by shRNA3, which contained the 19-base-pair (bp) target sequence for short interfering (si) RNA within the coding region of NaV1.8 mRNA corresponding to the nucleotide
positions 6009-6027 (CCATCTAGCTCAATGCAAA; Accession No. NM_017247). The reduction of NaV1.8 protein expression by shRNA3 was measured and confirmed (p<0.05 from random shRNA) by immunohistochemistry of transfected IB4+ DRG neurons in culture (Figure 1C).

In vivo administration of shRNA3 in naïve rats. We have previously developed an in vivo non-viral gene transfer protocol whereby subcutaneous injection of CG/plasmid DNA polyplex in the hindpaw resulted in its uptake, retrograde transport and subsequent expression in L4/L5 DRG neurons (Figure 2A) (32). This protocol was used to test the effects of the selected shRNA3 construct in vivo. The shRNA3 plasmid DNA was polyplexed with CG and subcutaneously injected into the rat hindpaw.

Based on previous evidence of reporter gene expression in DRG at 2.5 and 6 days after hindpaw injection (32), we wanted to determine if synthesis of the mature form of siRNA3 molecules (21 nucleotides with 3’overhangs) would follow a similar pattern. To determine the levels of synthesized siRNA molecules, we designed a set of stem-loop RT primer, forward/reverse primers and probe for Taqman-based RT-PCR. Standards were obtained by supplementing the total RNA samples of naïve DRG with 10, 50, 100, 200 or 500 pmol of synthetic siRNA3 and subjecting them to stem-loop RT-PCR. There was a linear correlation between the concentration of synthetic siRNA3 molecules and RT-PCR readings ($R^2 = 0.99$), resulting in the formulation of a simple conversion equation (Figure 2B). Using this method, ~120 pmol of mature siRNA3 was detected in DRG 2.5 days after the shRNA3 polyplex injection in naïve rats. The amount of mature siRNA3 increased two-fold in DRG collected 7 days after injection (Figure 2B). The amount of mature siRNA3 in the sciatic nerve was relatively low and did not significantly change between 2.5 to 7 days after injections.

We further examined the effect of shRNA3 injection on NaV1.8 immunoreactivity. L4/L5 DRG were harvested 2.5 days after injection of CG/NaV1.8 shRNA polyplex and acutely dissociated. DRG were cultured on matrigel-coated glass chamber slides and immunocytology with NaV1.8 antibody, GFP antibody and IB4 staining was performed. Analysis indicated that NaV1.8 immunoreactivity of GFP+/IB4+ DRG neurons was significantly reduced by ~40% as compared to GFP-/IB4- DRG neurons (Figure 2C).

In vivo administration of shRNA3 does not affect mechanical or thermal sensitivity in naïve rats.

In separate sets of naïve rats, daily behavioral testing of mechanical withdrawal thresholds and thermal withdrawal latency showed that neither mechanical nor thermal sensitivity was affected by unilateral injections of NaV1.8 shRNA or injections of the control random sequence shRNA (Figure 3B).

In vivo administration of shRNA3 or shRNA4 reverses SNE-induced neuropathy symptoms. Rats destined for SNE surgery were monitored daily for mechanical withdrawal thresholds and thermal withdrawal latencies. Once stable neuropathic pain symptoms were established, CG/shRNA3 polyplex was subcutaneously injected in the hindpaw of one randomly selected group (n=9) ipsilateral to SNE, and the other group received the CG/random shRNA injection (n=10). After a lag period of 3-4 days, the mechanical withdrawal thresholds and thermal withdrawal latencies in the shRNA3-treated group rapidly improved and reached their maximum levels 4-5 days after the shRNA3 injection (Figure 3C). The uninjured contralateral side was used to standardize the threshold and latency and the least significant difference (LSD) of the mean changes of threshold and latency from the baseline (prior to the first injection) was determined using the multiple comparisons one-way ANOVA test. The LSD of the mechanical withdrawal threshold was achieved during the 4-day period from 5 to 8 days after the injection, whereas the LSD of the thermal withdrawal latency was achieved earlier and lasted 6 days. After the return of pain behaviors to pre-injection levels (23 days post-SNE), a second injection achieved a similar magnitude of pain alleviation (Figures 3C). Importantly, no signs of anaphylactic reaction or inflammation at the injection site were detected in any of the rats.

The random shRNA-treated group, to our surprise, produced a short period of pain alleviation after the first and the second injections (Figure 3C). However, repeated measures ANOVA for the period between the first and second shRNA injections revealed that the effect
of shRNA3 was significantly different from the random shRNA for the mechanical withdrawal thresholds [group p = 0.0001; time p < 0.0001; group x time p < 0.0001] and the thermal withdrawal latencies [group p = 0.0370; time p = 0.0002; group x time p = 0.0006].

In a separate experiment, SNE-treated rats (n=8) were injected with shRNA4 polyplexed with CG. Similar to shRNA3, there was a significant attenuation of neuropathic pain symptoms after shRNA4 (Figure 3D). However, the onset of analgesic effects occurred faster with shRNA4. Maximum analgesic effects were observed after 2-3 days and lasted for 6 days (mechanical withdrawal thresholds) or 10 days (thermal withdrawal latencies). In addition, peak relief of mechanical allodynia symptoms was greater with shRNA4 (~75%) compared with shRNA3 (~50%).

Effect of NaV1.8 shRNA3 injection on NaV1.8 protein expression. DRG and sciatic nerves were harvested during maximal suppression of neuropathy symptoms 12 days after the second NaV1.8 shRNA3 injection. Analysis of NaV1.8-immunoreactivity (NaV1.8-ir) in DRG sections co-stained with IB4 (38) revealed a reduction of NaV1.8-ir in clusters of IB4+ neurons (Figure 4A). Scatter plot of NaV1.8-ir intensity measured from a total of 857 IB4+ DRG neurons demonstrated that there are essentially 2 clusters; a high intensity cluster between 45% and 60%; and a low intensity cluster less than 40% (Figure 4B). The high intensity cluster was composed of all groups, whereas the low intensity cluster contained predominantly the SNE/shRNA3 group. The low intensity cluster accounted for 9.8% - 18.2% neurons in the SNE/NaV1.8 shRNA group compared to only 1.5% of neurons from the SNE/random shRNA group. The average NaV1.8 immunoreactivity was significantly decreased in the SNE/shRNA3 group compared to SNE/random shRNA group of small (<700 µm²) and medium (700-1200 µm²) size IB4+ DRG neurons (Figure 4D). The expression of NaV1.8 in larger diameter neurons (>1200 µm²) was not included in this analysis due to their generally low level NaV1.8 expression that could confound the measurements of decreases in NaV1.8 protein from shRNA3 injection.

Sciatic nerve sections revealed a robust increase of NaV1.8-ir in the SNE/random shRNA group, confirming previous findings of SNE-induced increases in NaV1.8-ir (28). By contrast, NaV1.8-ir in SNE-injured nerve was markedly reduced by the shRNA3 injection (Figure 4A). Scatter plot of a total of 261 randomly selected microscopic fields of sciatic nerve illustrates the uniform increases in ipsilateral NaV1.8-ir and its reduction by the shRNA3 injection to the range comparable with that of the contralateral (uninjured) sciatic nerve (Figure 4C). The average NaV1.8-ir was significantly higher in the SNE-injured/random shRNA-injected group than the uninjured contralateral group. Injection of shRNA3 effectively attenuated the increased NaV1.8-ir, although falling short of completely normalizing the average NaV1.8-ir to the level of uninjured contralateral sciatic nerves (Figure 4E).

Effect of shRNA3 injection on NaV1.8 gene expression. We first determined if SNE- and shRNA-induced changes in NaV1.8 were due to altered transcription of the Scn10A gene, which encodes NaV1.8. Due to difficulties experienced with a conventional nuclear run-off assay, we used an alternative method (37), in which heterogeneous nuclear RNA (hnRNA) or pre-mRNA of NaV1.8 was measured using primers designed across the intron-exon boundaries of the Scn10A gene (Figure 5A). Measurements in the DRG of SNE rats (n = 4) revealed similar levels of NaV1.8 hnRNA among all groups suggesting that neither SNE nor shRNA injections affected the Scn10A gene transcription (Figure 5B). Because NaV1.8 hnRNA was not detected in sciatic nerve (Figure 5B), local non-neuronal cells were unlikely to be the source of NaV1.8 mRNA in sciatic nerves. In a separate group (n=4) of SNE-treated rats without shRNA injections, the steady-state NaV1.8 mRNA in DRG was not affected by SNE, whereas NaV1.8 mRNA, but not NaV1.6, NaV1.7 or NaV1.9 mRNA, was significantly increased in the affected nerve (Figure 5C), confirming previous findings (28).

Next, we addressed the effect of the shRNA3 treatment on the steady-state NaV1.8 mRNA. L4/L5 DRG and sciatic nerve tissues were harvested from naïve (n = 4) or SNE-injured (n = 4) rats after shRNA3 or random shRNA injections. Naïve rat tissues were obtained 11 days after shRNA injections. Tissues from SNE rats were harvested 11 days after the second shRNA3 injection, when alleviation of SNE-induced pain symptoms was still evident (Figures 5D).
Compared to naïve rats, SNE decreased the steady-state NaV1.8 mRNA levels in the DRG. Surprisingly, NaV1.8 mRNA levels in the DRG were not affected by shRNA3 injection as compared to the random shRNA injection (Figure 5D). However, the increased NaV1.8 mRNA level by SNE injury in the nerve was significantly attenuated by the shRNA3 treatment to the levels observed in the naïve nerve (Figure 5D).

DISCUSSION

The present study demonstrated that NaV1.8 knockdown using peripheral administration of shRNA in the SNE-injured rats resulted in significant reduction of neuropathic pain behaviors. This confirms and extends previous studies in which pharmacological blockade of NaV1.8 function or expression was shown to decrease mechanical allodynia and thermal hyperalgesia in experimental neuropathies. The peripheral neuropathy induced by SNE is highly comparative to that of chronic constriction injury (CCI), which uses chromic gut suture materials instead of polyethylene cuffs (25,39,40). SNE was demonstrated to produce consistent pain behaviors (34,41), a bona fide transient loss of varicosities in nociceptive fibers (42), and increases in evoked excitability of sciatic nerve compound action potentials (28). The increased excitability of the injured sciatic nerve likely contributes to the ectopic activity in the injured nerve and the exaggerated afterdischarge of wide dynamic range neurons in the spinal cord evoked by mechanical cutaneous stimulation in this model (43). The hyperexcitability and ectopic burst discharge of primary sensory neurons are widely considered to be the major contributors to pain symptomatology of peripheral neuropathy models. Although the behavioral symptoms of the SNE/CCI models are superficially similar to the model of segmental deafferentation induced by L5/L6 spinal nerve ligation (SNL) (44), these models differ in many important respects. In contrast to the SNE/CCI models, where injured and uninjured axons co-mingle in the sciatic nerve, the tight ligation (deafferentation) of L5/L6 spinal nerve segments in the SNL model results in complete segregation of the deafferented neuronal somata in L5/L6 DRG and the “uninjured” somata and axons of L4 DRG neurons. The phenotypic alterations in SNL include large decreases in NaV1.8 mRNA, protein, and function in the deafferented L5 DRG and large increases in NaV1.8 mRNA, protein, and function in the “uninjured” L4 DRG (45), concomitant with large increases in NaV1.8 protein, function and resistance to tetrodotoxin (TTX) blockade of action potential conduction in the “uninjured” sciatic nerve axons (9,27). By contrast, the SNE/CCI produce modest decreases in NaV1.8 mRNA and protein within L4/L5 DRG (25,28,29) and increases in immunohistochemical detection of axonal NaV1.8 protein (27,28). Functionally, only modest increases in resistance to TTX conduction block are observed after SNE (28). Moreover, the demonstrated increases in sciatic nerve NaV1.8 mRNA after SNE (28) and the lack of nerve NaV1.8 mRNA increases after the L5 spinal nerve ligation (unpublished observations) suggest differences in the molecular mechanisms of axonal NaV1.8 accumulation in the two models.

Targeted gene delivery to the sciatic nerve via subcutaneous injection of CG/DNA polyplex. We used a simple gene transfer protocol of subcutaneous CG/DNA polyplex injection in the hindpaw (32) to deliver shRNA plasmid DNA targeting NaV1.8 mRNA to sciatic nerve, which resulted in retrograde transfection and synthesis of mature siRNA molecules in L4/L5 DRG (Figure 2B). Unlike other studies using intrathecal injections of antisense oligo-deoxynucleotides (9,27) or epidural injections of siRNA targeting NaV1.8 (29), the expression of plasmid DNA by injection of CG/DNA polyplex is limited to the DRG and sciatic nerve innervating the subcutaneous injection site (32). In this study, approximately 10% of small and medium DRG cells were found positive for the reporter GFP. Puigdellivol-Sanchez et al. (2002) estimated only 20-30% of DRG neurons to innervate the hindpaw (46). Therefore, the relevant transfection efficiency of the present method may exceed 40%.

Significant alleviation of mechanical allodynia and thermal hyperalgesia in SNE-injured rats was achieved with a distinct latent period of 3-4 days after hindpaw injection of shRNA3 specific to NaV1.8 (Figure 3C). Following neuronal uptake, the injected CG/DNA polyplex may undergo retrograde axonal transport along the sciatic nerve (10-15 cm) before reaching the soma in the DRG. The rate of microtubule-mediated retrograde...
axonal transport along the sciatic and spinal nerves has been estimated to be 10-25 cm/day (47,48). Thus, CG/DNA polyplex should reach DRG neuronal nuclei within a day and the mature siRNA should be synthesized. Indeed, the synthesis of mature siRNA as well as measurable NaV1.8 knockdown in DRG neurons was evident 2.5 days after the NaV1.8 shRNA injection but doubled by 7 days (Figure 2B). Therefore, the latent period of 3-4 days is likely due to the continued accumulation of the siRNA and resultant NaV1.8 knockdown.

In rats injected with shRNA4, the late onset pain alleviation appeared to be consistent with the pain alleviation pattern of shRNA3, but the effect was more significant (Figure 3D). Because both shRNA3 and shRNA4 were shown to decrease NaV1.8 mRNA levels, the late onset pain alleviation may likely be due to NaV1.8 knockdown, and the robust pain alleviation by shRNA4 may directly relate to its larger knockdown efficiency (Figure 1B). It was noted, however, that significant pain relief was faster with shRNA4. Because shRNA4 also affected NaV1.6 (Figure 1B), it is tempting to speculate that the knockdown of NaV1.6 could contribute to the earlier onset of pain relief. Further studies are needed.

The U6 promoter in our shRNA constructs is widely used in mammalian expression systems; however, when applied in vivo, its transcriptional activity was shown to be silenced after 1~2 weeks (49). Therefore, the gradual return of pain symptoms after shRNA injection may primarily be due to the silencing of promoters in the expression plasmid.

In the random shRNA-injected group, we unexpectedly observed small decreases in neuropathy symptoms of relatively short duration (Figure 3C). Suspecting an off-target effect, we repeated a BLAST search using the 19 nucleotides (nt) of the random sequence siRNA; however, none of mRNA species in the database were detected. It is possible that the seed sequence (2nd-7th nt) of random siRNA could behave as a miRNA and promote translational inhibition of the off-target gene(s) (50). Within the scope of this study, possible off-target molecules were not determined.

NaV1.8 expression in DRG and sciatic nerve after SNE injury. Decreases in expression of NaV1.8 in the DRG were demonstrated after SNL (27), CCI (25,29), as well as SNE (28), while expression of NaV1.8 in peripheral nerve increases (9,25,28). The present study confirmed these previous reports (Figure 4) and also demonstrated the accumulation of NaV1.8 mRNA in sciatic nerve ipsilateral to SNE injury (Figure 5C).

A recent study suggested that chronic nerve compression (CNC) resulted in the up-regulation of NaV1.8 immunoreactivity in Schwann cells (51). Notably, the CNC model does not induce mechanical allodynia or thermal hyperalgesia, instead resulting in progressive decreases in mechanical sensitivity (52). In our study, the lack of detectable NaV1.8 hnRNA in the SNE-injured nerve (Figure 5B) strongly suggests that the source of NaV1.8 mRNA is not the non-neuronal cells, such as Schwann cells, but the axons themselves.

The present study further addressed if increases in NaV1.8 transcription from the Scn10A gene in DRG contributed to the axonal NaV1.8 mRNA accumulation. The NaV1.8 hnRNA level was not influenced by SNE-injury (Figure 5B) and thus de novo Scn10A gene transcription should not have a direct mechanistic role in the accumulation of axonal NaV1.8 mRNA. Also, we previously determined that the accumulation of axonal NaV1.8 was not due to the increase of the mRNA half-life evaluated by poly-A tail elongation (28).

It has been established that mRNAs are actively transported to subcellular sites (53). For example, subcellular localization of β-actin mRNA occurs during neuronal regeneration facilitated by the binding of zip code protein (ZBP1) to its 3'UTR (54). Therefore, it is reasonable to postulate that NaV1.8 mRNA may be post-transcriptionally transported to the SNE-injured axons from their DRG somata (Figure 6).

Increased transport of NaV1.8 mRNA to axons in the absence of increased somatic mRNA production (Figure 5B) might be expected to result in decreased steady-state levels of somatic NaV1.8 mRNA ipsilateral to the SNE treatment. We have previously observed a trend of decreased somatic NaV1.8 mRNA ipsilateral to SNE and significant decreases in somatic NaV1.8 immunoreactivity (28). A similar trend of decreased somatic NaV1.8 mRNA may be seen in
DRG ipsilateral to SNE compared to contralateral or na"ive rat DRG in Figure 5D. It must also be noted that the amount of NaV1.8 mRNA in the axons is approximately 1/10 to 1/50 of the DRG NaV1.8 mRNA (Figure 5C). Therefore, relatively large increases in axonal NaV1.8 mRNA may result from relatively small decreases in the amount of somatic NaV1.8 mRNA.

Since all the necessary molecular components for protein translation exist in peripheral axons (55,56), the accumulated axonal NaV1.8 mRNA may, in part, contribute to the increased functional expression of NaV1.8 in the injured nerve. The present study further demonstrated that injection of shRNA3 achieved a normalization of SNE-induced NaV1.8 mRNA in the nerve during the time (Figure 5D) when suppression of pain symptoms was maximal (Figures 3C). From these observations, NaV1.8 mRNA in the nerve, not in the DRG, appears to play a more relevant role to the pathogenesis of this painful neuropathy.

**Mechanism of NaV1.8 knockdown and neuropathic pain alleviation.**

Most of the mature siRNA3 was detected in the DRG (Figure 2B) suggesting that the somata of sensory neurons in the DRG may be the main site of siRNA-derived NaV1.8 mRNA degradation. If so, the attenuation of pain symptoms would occur after the depletion of axonally-transported NaV1.8 mRNA within the somata of DRG neurons and subsequent decreases in axonal NaV1.8 synthesis (Figure 6). However, it has also been well documented that RNAi machinery exists in the peripheral nerve axons (57,58). Therefore, siRNA-mediated degradation of NaV1.8 mRNA could also occur in peripheral axons.

Maximum relief of mechanical allodynia with shRNA3 in the present study was ~50% and was less effective than the relief of thermal hyperalgesia which reached nearly 100% (Figure 3C). Mechanical stimulation activates low threshold mechanoreceptors (e.g., Meissner’s corpuscle and Merkel disk receptors) on the encapsulated terminals of Aα- or Aβ-fibers (59). These fibers terminate subcutaneously at the epidermal-dermal junction and are thickly myelinated. By contrast, thinly myelinated Aδ-fibers and unmyelinated C-fibers have bare nerve endings and both highly innervate the glabrous skin of hindpaw. While Aδ-fibers are high threshold mechanoreceptors and constitute the afferent portion of the reflex arc that results in withdrawal from noxious and mechanical stimuli, C-fibers are polymodal and respond to thermal (both heat and cold), mechanical and chemical stimuli (59). Subcutaneously injected CG/DNA polyplex may be better endocytosed into bare nerve endings of Aδ-fiber and C-fiber than into the encapsulated terminals of Aα- and Aβ-fibers. Since NaV1.8 is primarily synthesized in Aδ and C nociceptors (60), the nearly complete NaV1.8 knockdown observed in the sciatic nerve tissue may reflect the preferential CG/shRNA uptake by these neurons, reflected in the nearly complete alleviation of thermal hyperalgesia.

**Conclusions.** Taken together, this study demonstrated that NaV1.8 in the affected sciatic nerve, not in DRG, plays a significant role in the development and maintenance of painful neuropathy symptoms. We propose that the molecular mechanism of SNE-induced neuropathy includes injury-induced post-transcriptional NaV1.8 mRNA transport, axonal accumulation of NaV1.8 mRNA, and its local protein translation leading to the increased NaV1.8 functional expression in injured nerve. This study further suggests that axonal NaV1.8 mRNA may be an attractive therapeutic target for painful neuropathy, and the subcutaneous injection of CG/DNA polyplex at the pain site may present a novel therapeutic modality.

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Figure 1. Efficiency and specificity of RNA interference-derived knockdown of NaV1.8.
A: Third generation self-inactivating long terminal repeat (SIN-LTR) vector construct (Lentilox 3.7) carrying shRNA3 driven by the U6 promoter and the GFP reporter gene driven by the CMV promoter. B: RT-PCR for the steady state mRNA levels of NaV1.3, NaV1.6, NaV1.7, NaV1.8 and NaV1.9 in acutely dissociated DRG neurons in vitro after transduction of lentiviral vectors carrying NaV1.8 shRNA 1, 2, 3, 4 or control random shRNA. *, p < 0.05 against random shRNA. C: NaV1.8 immunoreactivity of DRG neurons after shRNA3 and random shRNA transduction. *, p < 0.05.
Figure 2. RNA interference-derived knockdown of NaV1.8 in DRG neurons in vivo

A: Diagram of the retrograde gene transfer protocol using cationized gelatin (CG) and plasmid DNA polyplex via subcutaneous peripheral injection of the rat hindpaw. This protocol was used to deliver shRNAs in vivo. B: Quantitative measurement of the mature siRNA synthesized from shRNA plasmid DNA using stem-loop primer. The standard curve of relative siRNA3 expression corresponded to the known amount of siRNA3 added in DRG total RNA (left). Synthesized mature siRNA3 in DRG and nerve 2.5 days and 7 days after shRNA3 injection in naïve rats (n=4, right). *, p < 0.05. C: NaV1.8 immunocytochemistry of IB4-labeled DRG neurons acutely dissociated 2.5 days after injection of the CG/NaV1.8 shRNA3 polyplex. Significantly reduced NaV1.8 immunoreactivity was observed in GFP+/IB4+ neurons, compared to that in GFP-/IB4+ neurons. *, p < 0.05.
Figure 3. Reversal of SNE-induced neuropathy symptoms after the injection of NaV1.8 shRNA or random shRNA.

A: Diagram of SNE-induced neuropathic pain model representing location of polyethylene cuffs. In some experiments, L4/L5 DRG and sciatic nerve tissues proximal to the SNE site were harvested. B: Hindpaw withdrawal thresholds (mean ± S.E.M) to mechanical stimuli and hindpaw withdrawal latencies (mean ± S.E.M) to thermal stimuli for the ipsilateral and contralateral sides of two groups of naïve rats (n = 8 each) injected (day 0) with shRNA3 or random shRNA. C: Changes in hindpaw withdrawal thresholds to mechanical stimuli (left) and withdrawal latencies to thermal stimuli (right) after unilateral SNE surgery (day 0) and after shRNA3 or random shRNA injections (day 8 and day 23). Data are presented as mean ± S.E.M (n=9 shRNA3, n=10 random shRNA). D: Changes in hindpaw withdrawal thresholds to mechanical stimuli (left) and withdrawal latencies to thermal stimuli (right) after unilateral SNE surgery (day 0) and after shRNA4 injection (day 8). Data are presented as mean ± S.E.M (n=8) of differences between ipsilateral and contralateral thresholds and latencies. *, p < 0.05 (repeated measures ANOVA).
Figure 4. NaV1.8 immunostaining in DRG and nerve tissues harvested from SNE rats injected with either shRNA3 or random shRNA.
A: NaV1.8 immunoreactivity and IB4 staining in representative ipsilateral/contralateral sections from L4/L5 DRG and sciatic nerves proximal to the SNE site. Note the decreased NaV1.8 immunoreactivity in DRG and nerve after shRNA3 injections compared to random shRNA injections. B: Scatter plot of relative NaV1.8 immunofluorescence intensity of IB4+ L4/L5 DRG neurons ipsilateral to shRNA3 injection (red triangles: n = 297 cells, 4 rats), random shRNA injection (green circles: n = 258 cells, 4 rats) and contralateral DRG (blue diamonds: n = 302 cells, 8 rats). C: Scatter plot of relative NaV1.8 immunofluorescence intensity of nerve sections ipsilateral to shRNA3 injection (red triangles: n = 81 areas, 27 sections), ipsilateral to random shRNA injection (green circles: n = 102 areas, 34 sections) and in contralateral nerve (blue diamonds: n = 78 areas, 26 sections). D: Average NaV1.8 immunofluorescence intensity of IB4+ DRG neurons sorted by cell area < 700 µm² and 700-1200 µm² (mean ± S.E.M.). *, p < 0.05. E: Average NaV1.8 immunofluorescence intensity of nerve sections. *, p < 0.05.
Figure 5. Effects of SNE and shRNA3 treatment on NaV1.8 gene transcription and steady state mRNA levels in DRG and nerve.

A: Diagram of relevant exon structures Scn10A encoding NaV1.8 and the locations of PCR primers, targeting NaV1.8 mRNA (forward primer in exon 14 and reverse primer in exon 15) and NaV1.8 heterogeneous nuclear RNA (hnRNA) (forward primer-intron, reverse primer-exon 28). B: Scn10A gene transcription was measured by NaV1.8 hnRNA in SNE-treated rats with NaV1.8 shRNA (n=4) or random shRNA (n=4) injection. Data are presented with the contralateral tissue to SNE of the random shRNA group as the standard. The transcriptional activity of Scn10a in DRG was not affected by SNE injury and the injection of NaV1.8 shRNA. There was no NaV1.8 hnRNA detected in the nerve tissues as well as in the food pad tissue where cationized gelatin/plasmid DNA polyplex was injected. C: mRNA levels of NaV1.6, 1.7, 1.8 and 1.9 in the DRG and sciatic nerve of the SNE rat model. The relative mRNA level was normalized by the housekeeping gene expression in DRG. *, p < 0.05. D: Steady state NaV1.8 mRNA levels in DRG and sciatic nerve harvested from naïve or SNE-injured rats treated with shRNA3 (black bars) or random shRNA (white bars). RT-PCR data were compared using the untreated contralateral tissue as the standard in each group (n=4). *, p < 0.05.
Figure 6. A hypothetical molecular mechanism of painful neuropathy involving axonal NaV1.8 mRNA.

A: NaV1.8 mRNA is largely localized in DRG in the uninjured neurons. B: SNE injury induces axonal accumulation of NaV1.8 mRNA, likely through active mRNA transport, not by the increase of gene transcription or axonal mRNA half-life. C: Retrograde transport of NaV1.8 shRNA facilitates RNAi-derived NaV1.8 mRNA degradation in DRG, which eventually normalizes axonal NaV1.8 mRNA, leading to the attenuation of neuropathic pain symptoms after a distinct lag period.
