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Nociceptor Overexpression of $Na_V1.7$ Contributes to Chronic Muscle Pain Induced by Early-Life Stress

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Abstract

Adult rats previously submitted to neonatal limited bedding (NLB), a model of early-life stress, display muscle mechanical hyperalgesia and nociceptor hyperexcitability, the underlying mechanism for which is unknown. Since voltage-gated sodium channel subtype 7 ($Na_V1.7$) contributes to mechanical hyperalgesia in several preclinical pain models and is critical for nociceptor excitability, we explored its role in the muscle hyperalgesia exhibited by adult NLB rats. Western blot analyses demonstrated increased $Na_V1.7$ protein expression in L4–L5 dorsal root ganglia (DRG) from adult NLB rats, and antisense oligodeoxynucleotide (AS ODN) targeting $Na_V1.7$ alpha subunit mRNA attenuated the expression of $Na_V1.7$ in DRG extracts. While this AS ODN did not affect nociceptive threshold in normal rats it significantly attenuated hyperalgesia in NLB rats. The selective $Na_V1.7$ activator OD1 produced dose-dependent mechanical hyperalgesia that was enhanced in NLB rats, whereas the $Na_V1.7$ blocker ProTx-II prevented OD1-induced hyperalgesia in control rats and ongoing hyperalgesia in NLB rats. AS ODN knockdown of extracellular signal-regulated kinase 1/2, which enhances $Na_V1.7$ function, also inhibited mechanical hyperalgesia in NLB rats. Our results support the hypothesis that overexpression of $Na_V1.7$ in muscle nociceptors play a role in chronic muscle pain induced by early-life stress, suggesting that $Na_V1.7$ is a target for the treatment of chronic muscle pain.

Keywords

Maternal neglect; Nociceptor hyperexcitability; Neonatal limited bedding; Muscle Hyperalgesia; ERK1/2

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Introduction

Chronic musculoskeletal pain syndromes are extremely common and increase in incidence with age, affecting ~40% of Americans over the age of 65 years.[39] They are associated with a marked decrease in quality of life[48] and high economic cost, related to health care expenses, compensation and disability.[18] While several studies have shown that a history of early-life stress is an important risk factor for chronic musculoskeletal pain[14, 22, 37, 51, 56, 66], the mechanisms underlying this relationship remain poorly understood.

Voltage-gated sodium channels (Na_V) play an important role in nociceptor excitability and nociceptor-specific gene ablation has revealed their key contribution to nociception and pathological pain states.[13, 24] For instance, voltage-gated sodium channel subtype 7 ($Na_V1.7$, also called peripheral nerve type 1 [PN1]), which is widely expressed in sensory neurons, can amplify the impact of weak stimuli and act as a threshold channel in nociceptors.[13, 24, 47] Changes in $Na_V1.7$ expression, gating kinetics, and membrane trafficking may contribute to pathological pain.[24] For instance, enhanced expression[19, 35, 36, 63], or function[61, 64] of $Na_V1.7$, have been shown to increase both nociceptor excitability and nociceptive behavior. Importantly, several proalgesic mediators signal through extracellular signal-regulated kinase 1/2 (ERK1/2, also referred to as mitogen-activated protein kinase, 44/42MAPK), resulting in $Na_V1.7$ phosphorylation and modulation of its gating properties, to enhance nociceptor excitability.[61, 68] We have shown that adult rats submitted to a well-established model of early-life stress, neonatal limited bedding (NLB)[32], display persistent mechanical hyperalgesia, hyperalgesic priming and muscle nociceptor sensitization.[9, 26] Since skeletal muscle nociceptors express $Na_V1.7$[54], and tetrodotoxin, which blocks $Na_V1.7$, inhibits mechanical hyperalgesia in several preclinical models of muscle pain[10], we tested the hypothesis that muscle hyperalgesia in adult rats exposed as neonates to NLB is dependent on upregulation of nociceptor expression of $Na_V1.7$ and its modulation by ERK1/2.

Materials and methods

Animals

Behavioral experiments were performed on male and female adult Sprague-Dawley (Crl:CD, Charles River, Hollister, CA) rats, weighing 250–350 g. For the neonatal limited bedding (NLB) protocol, primiparous pregnant Sprague-Dawley (Crl:CD) female rats were purchased from the same provider. After delivery, dams were housed with their litter in standard cages on postnatal days 0–1. On postnatal day 2, some litters were assigned to the NLB protocol (see below). All animals were housed in the Laboratory Animal Resource Center of UCSF, under a 12-hours light/dark cycle (lights on 7 am–7 pm) and environmentally controlled conditions; ambient room temperature (21–23 °C), with food and water available *ad libitum*. Animal care and use in experiments conformed to National Institutes of Health guidelines. The UCSF Institutional Animal Care and Use Committee approved the experimental protocol. All efforts were made to minimize the number of animals used and their suffering.

Neonatal limited bedding (NLB) stress protocol

The details of our use of the NLB model have been described previously.[9, 26, 32] Briefly, beginning on postnatal day 2, dams and their pups were placed in cages fitted with a stainless steel mesh platform that was raised ~2.5 cm from the floor of the home cage, to allow collection of urine and feces. The nesting/bedding material provided consisted of one sheet of paper towel (~13 × 23 cm). Litters were otherwise undisturbed during postnatal days 2–9.

Antisense oligodeoxynucleotides

The contribution of $Na_V1.7$ and ERK to muscle mechanical nociceptive threshold in rats submitted to the NLB model was evaluated using intrathecal injections of antisense oligodeoxynucleotides (AS ODN). This method has been shown to inhibit the expression of specific proteins that contribute to a number of signaling pathways in nociceptors (for a review, see Stone and Vulchanova[62]).

An AS ODN sequence, 5′- TGT TGG TCA GTA TGC TCG C −3′, was directed against a unique sequence of *Rattus norvegicus* $Na_V1.7$ alpha subunit mRNA. The corresponding GenBank accession number and ODN position within the cDNA sequence are NM_133289 and 2074 to 2092, respectively. The mismatch (MM) ODN sequence, 5′- T**A**T T**C**G TC**G** GT**C** TG**T** TC**A** C −3′, corresponds to the $Na_V1.7$ antisense sequence, with 6 bases mismatched (denoted by bold type). The AS ODN sequence, 5′- GCC GCC GCC GCC GCC AT −3′, was directed against a unique sequence of *R. norvegicus* ERK (MAPK) mRNA. A sense (SE) ODN sequence, 5′- ATG GCG GCG GCG GCG GC −3′, was used as a control. This AS ODN sequence is directed against the initiation codon of the translation start site of rat ERK1 and ERK2 (44/42 MAPK) mRNAs, which are conserved in humans, mice, and rats.[55] This AS ODN has been previously shown to produce selective knockdown of the expression of ERK1/2 (44/42 MAPK) protein in the spinal dorsal horn and to reduce nociceptive behavior in the rat.[16, 60]

A nucleotide BLAST search was performed to confirm that the mRNA sequences targeted by the AS ODN, or their MM, and SE controls, were not homologous to any other sequences in the rat database. The ODNs were synthesized and lyophilized by Invitrogen (Carlsbad, CA). Before use, ODNs were reconstituted in sterile 0.9% NaCl to a concentration of 10 μg/μl, aliquoted and stored at −20°C.

Intrathecal injections

Immediately before injection, AS, MM, or SE ODNs were diluted in sterile 0.9% NaCl and administered by intrathecal injection (120 μg/20 μl) once daily, for 3 days. ProTxII (Abcam, Cambridge, MA, USA), a toxin from the Peruvian green velvet tarantula (*Thrixopelma pruriens*) that selectively inhibits $Na_V1.7$[58], was diluted in 0.1% bovine serum albumin (BSA)-Dulbecco's phosphate buffer (DPBS), and a dose of 1 μg/20 μl was injected i.t., 30 min prior to nociceptive testing. Rats were briefly anesthetized with 2.5% isoflurane (Phoenix Pharmaceuticals, St. Joseph, MO) to facilitate i.t. injections, and a 29 G × ½" hypodermic needle was inserted into the subarachnoid space on the midline, between the L4

and L5 vertebrae. Proper intrathecal injections were confirmed by a sudden flicking of the rat's tail.[43]

Tissue harvesting and protein extraction

Control and NLB rats were euthanized by exsanguination while under deep isoflurane anesthesia. L4–L5 DRGs, which contain the somas of the sensory neurons innervating the gastrocnemius muscle[12, 30, 54], were quickly removed, snap frozen on dry ice, and stored at −80°C until further processing. DRGs were transferred into homogenization buffer [150 mM NaCl, 50 mM Tris-HCl pH 7.4, 2 mM EDTA, 2% sodium dodecylsulfate (SDS)] supplemented with a complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and homogenized with a sonicator. Proteins were solubilized by incubating the homogenate for 2 h at 25°C at 1400 r/min and then extracted by a 15 min centrifugation at 14,000 r/min. Protein concentration of the samples was determined using the micro bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) with BSA as a standard.

Western blot analysis

Changes in $Na_V1.7$ expression in lumbar DRGs were evaluated by Western blot analysis as previously described.[6–8] Briefly, 40 μg of protein per sample were mixed with 4X sample buffer (62.5 mM Tris-HCl pH 6.8, 3% SDS, 10% glycerol, 0.025% bromphenol blue), denatured by shaking for 10 min at 500 r/min at 90°C and electrophoresed on a 4%–15% precast polyacrylamide gel (Biorad, Hercules, CA) in 25 mM Tris buffer containing 192 mM glycine, and 0.1% SDS. Proteins were transferred onto a nitrocellulose (NC) membrane using the semidry method (transfer time 2 h at 1.5 mA*cm−2 with 47.9 mM Tris, 38.9 mM glycine, 0.038% SDS, and 20% methanol). The blotting membrane was saturated by shaking in Tris-buffered saline (pH 7.4) containing 5% BSA and 0.1% Tween 20 (antibody dilution buffer) for 1 h at room temperature (RT). The blot was probed with a rabbit anti-$Na_V1.7$ antibody (Cell signaling technology, #14573; 1:500 in antibody dilution buffer) or a rabbit anti- β-actin antibody (Abcam, #ab8227, 1:1000 in antibody dilution buffer), at 4°C overnight, rinsed with TBST (3 times at RT, 15 min each) and probed with a donkey anti-rabbit horseradish peroxidase conjugated antibody (GE healthcare, #NA934V; 1:2500 in antibody dilution buffer) for 2 h at RT. The Western blot was rinsed with TBST (3 × at RT, 15 min each), the $Na_V1.7$ immunoreactivity visualized with the enhanced chemiluminescence (ECL) detection kit (Pierce) and expressed as arbitrary units (a.u.) of immunoreactivity using β-actin as the reference housekeeping protein.

Intramuscular injections

We performed intramuscular (i.m.) injections of OD1 (Tocris Bioscience, Minneapolis, MN) a toxin from the Iranian yellow scorpion (*Odonthobuthus doriae*) that selectively activates $Na_V1.7$.[38] Rats were briefly anesthetized with 2.5% isoflurane to facilitate the injection (50 μl) of OD1 (dissolved in 0.1% BSA in DPBS [10–300 nM]) or vehicle, into the belly of the gastrocnemius muscle. The skin overlying the injection site was previously shaved and scrubbed with alcohol. Immediately after i.m. injections, the site at which the skin was punctured was marked with a fine-tip indelible ink pen, so that the mechanical nociceptive threshold at the underlying i.m. injection site in the muscle could be measured over time.

Measurement of muscle mechanical nociceptive threshold

Mechanical nociceptive threshold in the gastrocnemius muscle was quantified using a digital force transducer (Chatillon DFI2; Amtek Inc., Largo, FL) with a 7 mm-diameter probe.[9, 10, 26] Adult rats from litters previously submitted to the NLB model or kept undisturbed (naïve controls) were lightly restrained in an acrylic holder that allows for easy access to the hind limb and application of the transducer probe to the belly of the gastrocnemius muscle. The nociceptive threshold was defined as the force, in mN, required to elicit a flexion reflex. Baseline limb-withdrawal threshold was defined as the mean of 2–3 readings taken at 5-min intervals and one gastrocnemius muscle per rat was used in each experiment. Behavioral testing was performed blind to treatments.

Statistical analysis

Group data are expressed as mean ± SEM of n independent observations. Comparable numbers of male and female rats were used in each experiment and all data were combined and analyzed by condition. Statistical comparisons were made using Prism 9.0 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between treatments were made by means of Student's t-test, one, two or three-way repeated measures analysis of variance (ANOVA) followed by Bonferroni, Šídák's or Dunnet's multiple comparisons tests. P values < 0.05 were considered statistically significant.

Results

Increased expression of $Na_V1.7$ in NLB rats

Compared to control (naïve) rats, male (control: 2521 ± 4.1 mN [n=42] *vs* NLB: 2218.7 ± 4.8 mN [n=27], $P < 0.001$), and female (control: 2435 ± 5.6 mN [n=33] *vs* NLB: 2185.3 ± 6.4 mN [n=32], $P < 0.001$) NLB rats exhibited, as adults, a significantly lower mechanical nociceptive threshold (i.e., muscle hyperalgesia) (Fig. 1A). In control rats a lower mechanical nociceptive threshold was observed in females compared to males ($P < 0.001$, Fig. 1A). This sex difference, albeit of small magnitude, was also observed in NLB rats ($P < 0.001$, Fig. 1A).

To test the hypothesis that $Na_V1.7$ plays a role in the muscle hyperalgesia exhibited by NLB rats, we assessed its protein expression in L4–L5 DRG extracts from NLB and control rats. Western blot analysis of extracts from NLB rats demonstrated a marked increase (~29%) in $Na_V1.7$ immunoreactivity (7.4 ± 0.4 a.u., [n = 6]), compared to extracts from control rats (5.7 ± 0.6 a.u., [n = 6], Student's *t*-test, $P = 0.026$, Fig. 1B,C).

Antisense knockdown of $Na_V1.7$

To evaluate the involvement of nociceptor $Na_V1.7$ in mechanical hyperalgesia displayed by NLB rats, an AS ODN targeting $Na_V1.7$ mRNA was designed and its effects on the respective protein expression assessed. Western blot analysis of L4–L5 DRG extracts from rats submitted to the AS treatment demonstrated a marked decrease (~44%) in $Na_V1.7$ expression (1.76 ± 0.18 a.u., [n = 6]), compared to the MM treated group (3.14 ± 0.46 a.u., [n = 6], Student's t-test, $P = 0.0129$, Fig. 2A,B). We next determined whether $Na_V1.7$ expression plays a role in the baseline mechanical nociceptive threshold. Compared to

baseline (i.e., pre $Na_V1.7$ ODN), no significant difference in nociceptive threshold was observed between control rats treated with AS (2506.2 ± 12.9 mN *vs* 2487.3 ± 19.2 mN; [n=9], $P > 0.05$, Fig. 2C), or MM (2506.4 ± 13.9 mN *vs* 2486 ± 14.2 mN; [n=6], $P > 0.05$, Fig. 2B) ODN. In contrast, NLB rats treated with AS (2180.1 ± 16.4 mN *vs* 2417.1 ± 10.5 mN; [n=8], $P < 0.001$), but not MM (2210.7 ± 11.3 mN *vs* 2193.5 ± 15.7 mN; [n=10], $P > 0.05$) ODN, exhibited a significant increase in nociceptive threshold (Fig. 2D). Three-way ANOVA was performed on data in **C** and **D**: Time (pre-post) × NLB × NaV1.7 antisense $P<0.0001$, indicating NLB rats are different from control due to over-expression of NaV1.7.

$Na_V1.7$ activator OD1

To assess the direct involvement of $Na_V1.7$ in nociceptor terminals in muscle nociception, we studied the effect of i.m. injections of OD1, a selective $Na_V1.7$ activator. The change in nociceptive threshold induced by the highest dose of OD1 studied reached a peak 20 min after injection (vehicle: 2424.8 ± 20.2 mN [−1.9 ± 0.7%, n=8] *vs* OD1: 1641.6 ± 32.4 mN [−33.7 ± 1.6%, n=7]; $P < 0.001$, Fig. 3A). This hyperalgesia was still significant 2 hours after injection (vehicle: 2445.3 ± 14.4 mN [−1 ± 0.8%, n=8] *vs* OD1: 1819.7 ± 38.7 mN [−26.6 ± 1.3%, n=7]; $P < 0.001$, Fig. 3A). Compared to vehicle, OD1 (10, 30, 100, 300 nM/50 μl) produced a significant (n=6/8 group, $P < 0.001$), dose-dependent (r2 = 0.7732, $P < 0.001$) decrease in mechanical nociceptive threshold (Fig. 3B).

Since NLB rats display $Na_V1.7$-dependent muscle hyperalgesia, we compared OD1-induced hyperalgesia in NLB and control rats. Measurements of nociceptive threshold taken 30 min after a 100 nM/50 μl injection show a significant enhancement in OD1-induced muscle hyperalgesia in NLB (baseline: 2219.7 ± 16.1 mN *vs* post OD1: 1555.3 ± 31.6 mN [−29.9 ± 1.1%, n=6]), compared to control (baseline: 2479.3 ± 17.6 *vs* post OD1: 1860.8 ± 91.9 mN [−22.6 ± 3.5%, n=8]; $P = 0.0165$, Fig. 3C) rats. Readings taken 60 min after OD1 also revealed a significant increase in OD1-induced muscle hyperalgesia in NLB (post OD1: 1612.3 ± 23.3 mN [−27.4 ± 0.9%, n=6]), compared to control (baseline: 2479.3 ± 17.6 vs post OD1: 1906.9 ± 28.7 mN [−23.1 ± 1.0%, n=8]; $P = 0.021$, Fig. 3C) rats. Three-way ANOVA for Time (pre-post) × NLB × OD1 shows that OD1 effect does not differ with NLB treatment over time, $P=0.5630$.

$Na_V1.7$ blocker ProTx-II

We next explored the effect of intrathecal administration of ProTx-II, a selective $Na_V1.7$ blocker. To evaluate the effect of ProTx-II on $Na_V1.7$-dependent mechanical hyperalgesia, we studied its effect on the mechanical hyperalgesia induced by i.m. injection of the $Na_V1.7$ activator OD1, in normal rats. Rats were injected i.t. with ProTx-II (1 μg/20 μl), or vehicle (0.1% BSA-DPBS), and i.m. with OD1 (100 nM, 50 μl) into the right gastrocnemius muscle. Intrathecal ProTx-II, but not its vehicle, significantly inhibited the reduction of mechanical nociceptive threshold (i.e., mechanical hyperalgesia) produced by i.m. OD1, measured 30 min (ProTx-II: 2383.5 ± 4.7 mN [-8.8 ± 0.5%] *vs* vehicle: 1926.6 ± 13.5 mN [−26 ± 1.3%]; n = 6/group, $P < 0.001$), or 60 min (ProTx-II: 2358.7 ± 3.7 mN [−3.7 ± 0.5%] *vs* 1827.7 ± 23.3 mN [−25.7 ± 0.8%]; n = 6/group, $P < 0.001$) after injection (Fig. 4A).

Next, we evaluated the effect of i.t. ProTx-II on the mechanical hyperalgesia exhibited by NLB rats. Intrathecal ProTx-II, but not vehicle, significantly increased muscle nociceptive threshold (i.e., inhibited the mechanical hyperalgesia) in NLB rats, at 30 min (ProTx-II: 2414.8 ± 7 mN [10.4 ± 0.4%, n=9] *vs* vehicle: 2195.3 ± 12.5 mN [0.9 ± 0.7%, n=8]; $P < 0.001$), or 60 min (ProTx-II: 2404.7 ± 15.4 mN [9.9 ± 0.7%, n=9] *vs* vehicle: 2193.9 ± 16.5 mN [0.8 ± 0.7%, n=8]; $P < 0.001$), after i.t. injection (Fig. 4B).

Antisense knockdown of ERK1/2

Evidence indicates that posttranscriptional modifications of $Na_V1.7$, such as phosphorylation by pERK1/2, enhance nociceptor excitability[61], likely contributing to hyperalgesia. Thus, to determine if ERK phosphorylation of $Na_V1.7$ contributes to hyperalgesia in NLB rats, we knocked down nociceptor ERK1/2 expression by means of AS ODN directed against ERK1/2 mRNA. In control rats, ERK1/2 AS ODN treatment did not significantly modify the nociceptive threshold (post AS: 2475.5 ± 14.1 mN, [n=6] *vs* post SE: 2470.7 ± 14.1 mN, [n=6], $P > 0.05$, Fig. 5A). While there were no significant differences at baseline (pre AS: 2229.7 ± 9 mN *vs* pre SE: 2210.3 ± 7.5 mN), NLB rats that received i.t. AS (2430.2 ± 12.8 mN, [n=6]) exhibited a significant increase in nociceptive threshold compared to the SE treated (2207.2 ± 6.5 mN, [n=6], $P < 0.001$) group (Fig. 5B). Three-way ANOVA analysis shows a significant interaction of Time (pre-post) × NLB × MAPK antisense, $P<0.0001$, indicating a role for MAPK mediating hyperalgesia in NLB rats.

Discussion

Although early-life stress is associated with the development of chronic musculoskeletal pain in adults[22, 37, 56, 66], the underlying mechanism remains unclear, precluding the development of rational therapies. The finding that increased expression of $Na_V1.7$ in nociceptors plays a central role in the hyperalgesia exhibited by NLB rats provides insight into how early-life stress can produce musculoskeletal pain syndromes.

As previously reported[9, 26], the NLB protocol produced persistent muscle mechanical hyperalgesia in adult rats, consistent with reports of visceral hyperalgesia also induced by NLB.[27, 53] These observations are in good agreement with somatic[57] and visceral[21] mechanical hyperalgesia induced by maternal separation, which further supports the view that early-life stress produces life-long enhancement of nociceptive processing. We also observed that the sex difference in muscle nociceptive threshold (i.e. lower threshold in females) observed in control rats[29] is reduced in NLB rats (see Fig. 1A). Of note, testosterone levels, which contribute to the higher nociceptive threshold observed in male rats[5, 67] are reduced after exposure to chronic stress[50, 72], and thus might also be reduced by NLB, an hypothesis that warrants further investigation.

NLB[26] and water avoidance stress[20] induce muscle nociceptor hyperexcitability, observed *in vivo* as decreased mechanical threshold and increased conduction velocity of group III (Aδ) and group IV (C) fibers innervating the gastrocnemius muscle. This nociceptor hyperexcitability has also been observed *in vitro*, as a marked increase in the number of action potentials evoked by a depolarizing pulse, in DRG neurons cultured from mice exposed to water avoidance stress.[31] Similarly, DRG neurons from rats exposed to diverse

stressors, including water avoidance stress, also display reduced rheobase and increased number of action potentials in response to depolarizing currents.[70] Thus, the finding that DRGs from NLB rats exhibit enhanced expression of $Na_V1.7$ provides a mechanistic explanation for the nociceptor hyperexcitability and mechanical hyperalgesia observed in this model. Importantly, water avoidance stress also enhances the expression of $Na_V1.7$ in DRG neurons in a stress hormone-dependent manner[49, 71], and increases nociceptor responsiveness to mechanical stimulation[20], suggesting that nociceptor hyperexcitability induced by stress is dependent on enhanced expression of $Na_V1.7$. We therefore assessed whether increased expression of $Na_V1.7$ plays a role in the muscle hyperalgesia displayed by NLB rats.

Since $Na_V1.7$ is present in muscle nociceptors[54] and intrathecal AS ODN is an effective strategy to decrease $Na_V1.7$ mRNA expression in DRG neurons[44], we explored this approach to evaluate the role of $Na_V1.7$ in hyperalgesia in NLB rats. While the antisense treatment produced significant knockdown of $Na_V1.7$ protein, it did not modify the mechanical nociceptive threshold in normal (control) rats. Our findings are consistent with many previous reports in which knockdown[25, 59, 69] or inhibition of $Na_V1.7$[11, 15, 25, 41, 45, 46, 52, 65, 69] has no effect on baseline nociceptive threshold, but attenuates hyperalgesia in several pain models (e.g., post-surgical, inflammatory, cancer, burn, diabetic, chemotherapy or nerve injury neuropathic). One study[44], using high dose (1 mg, i.t.) modified AS ODN for $Na_V1.7$, reported an increased baseline mechanical nociceptive responses; this discrepancy may reflect the higher (60%) long-lasting (at least 4 weeks) $Na_V1.7$ protein knockdown reached in this study. However, even a relatively smaller knockdown of $Na_V1.7$ that produces no change in nociceptive baseline, is sufficient to produce antihyperalgesic and/or antiallodynic effects.[25, 69] In good agreement with this, the same AS ODN produced a marked increase in mechanical threshold in NLB rats, indicating a central role for $Na_V1.7$ in persistent muscle hyperalgesia induced by early-life stress. Of note, our results show increased expression of $Na_V1.7$ in the DRG, where transcription and likely translation of this target occurs[1], being transported to both central and peripheral projections. In both locations, $Na_V1.7$ contributes not only to the initiation and the upstroke phase of the nociceptor action potential, but also influences synaptic transmission in the dorsal horn of the spinal cord as well as peripheral neuropeptide release in the skin[2]. Since $Na_V1.7$ is expressed in a defined subset of type IV (C-fibers) innervating the gastrocnemius muscle[54], and transcription of $Na_V1.7$ does not occur in the dorsal horn of the spinal cord[4], the antinociceptive effect produced by intrathecal treatment with a selective $Na_V1.7$ blocker (ProTx-II) can only be explained by action on $Na_V1.7$(+) muscle nociceptors peripherally activated by OD1. These observations are further supported by the work of others showing that in models of painful neuropathy[36] and incisional pain[35], which produce up-regulation of NaV1.7 expression in DRG, intrathecal ProTx-II attenuates pain behavior. $Na_V1.7$ is crucial for setting the nociceptor action potential threshold and defines normal excitability of nociceptors[42]. We acknowledge, however, that taking into account the recent discovery of $Na_V1.7$ protein transfer from primary afferent sensory neurons to second-order neurons of the dorsal horn of the spinal cord[4], we cannot rule out an effect of intrathecal treatments on spinal $Na_V1.7$.

To further characterize the role of $Na_V1.7$ in NLB-induced muscle hyperalgesia we explored the effect of OD1, a selective activator of this ion channel. An α-scorpion toxin, OD1, impairs $Na_V1.7$ fast inactivation, increases peak current at all voltages, induces persistent current[38], and produces spontaneous nociceptive behavior (licking, flinching, lifting and shaking of the injected hind paw) upon intraplantar injection.[17, 23] Consistent with the role of $Na_V1.7$ on OD1-induced spontaneous pain its co-administration with selective (e.g., GpTx-1) and non-selective (e.g., tetrodotoxin) $Na_V1.7$ inhibitors, fully prevents OD1-induced spontaneous nociceptive behavior, whereas this nociceptive response is markedly attenuated in $Na_V1.7$ knockout mice.[23] We found that intramuscular OD1 produced a dose-dependent, long-lasting mechanical hyperalgesia in control rats. Importantly, this hyperalgesia was enhanced in NLB rats, supporting the prominent role of $Na_V1.7$ in NLB-induced chronic muscle pain. In line with these observations, intrathecal ProTx-II, a treatment that produces marked antihyperalgesic effects on $Na_V1.7$-dependent models of postsurgical[35] or neuropathic[34, 65] pain, not only prevented OD1-induced hyperalgesia in control rats but also markedly attenuated ongoing hyperalgesia in NLB rats.

NLB rats display an ongoing muscle hyperalgesia mediated by the pro-inflammatory cytokine interleukin 6 (IL-6), as shown by the marked antihyperalgesic effect of knockdown of the subunit glycoprotein 130[9], which interacts with the IL-6 receptor and is key for IL-6 intracellular signaling.[28] Of note, muscle mechanical hyperalgesia induced by intramuscular IL-6 depends on ERK.[40] And, mechanical hyperalgesia induced by epinephrine is sensitive to local injection of U0126, an inhibitor of ERK upstream activator, MEK[3], consistent with the view that the ERK1/2 signaling pathway plays an important role in nociceptor sensitization and mechanical hyperalgesia induced by diverse proalgesic mediators.[33] Moreover, several lines of evidence show that direct phosphorylation of $Na_V1.7$ by pERK1/2 induces nociceptor sensitization and hyperalgesia.[61, 68] Indeed, inhibition of ERK1/2 decreases excitability in sensory neurons by inhibiting phosphorylation of $Na_V1.7$ and modulating its gating properties.[61] Thus, it was not surprising that knockdown of ERK1/2 by intrathecal antisense markedly attenuated the mechanical hyperalgesia displayed by NLB rats. Of note, this intervention was devoid of effect in control rats, suggesting that activation of ERK1/2 triggered by algogens, such as IL-6, might contribute to the NLB pain phenotype by sensitizing $Na_V1.7$. Whether IL-6 also contributes to the increased expression of $Na_V1.7$ observed in NLB rats remains to be established.

Conclusion

In summary, persistent muscle mechanical hyperalgesia induced by early-life stress is mediated by increased expression of nociceptor $Na_V1.7$. Antisense ODN attenuation of nociceptor expression of $Na_V1.7$ or pharmacological blockade of $Na_V1.7$ inhibited muscle hyperalgesia observed in NLB rats. A contribution of ERK1/2 to this mechanical hyperalgesia was also observed, likely due to its sensitizing effect on $Na_V1.7$. These observations provide a novel mechanism for musculoskeletal pain after exposure to early-life adversity, suggesting that targeting $Na_V1.7$ could be a useful strategy for the treatment of stress-induced muscle pain.

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Disclosures:

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Perspective:

We demonstrate that early-life adversity, induced by exposure to inconsistent maternal care, produces chronic muscle hyperalgesia, which depends, at least in part, on increased expression of $Na_V1.7$ in nociceptors.

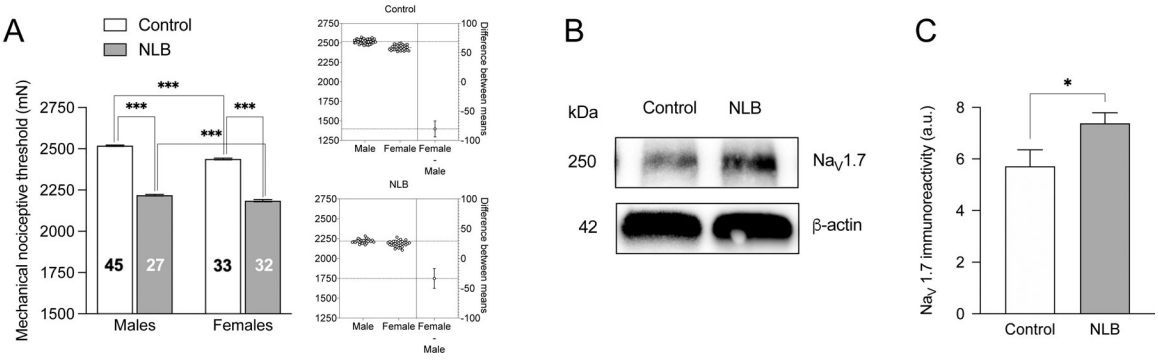

Figure 1. Enhanced Na$_V$1.7 expression associated with muscle mechanical hyperalgesia in NLB rats.

A. Consistent with previous reports[9, 26], NLB rats exhibit, as adults, decreased mechanical nociceptive threshold in the gastrocnemius muscle (~20% lower) compared to naïve control rats. Two-way ANOVA showed significant effects for condition (i.e, control or NLB, $F_{1,72}$ = 2682, $P < 0.001$), sex ($F_{1,58}$ = 124.3, $P < 0.001$) and interaction ($F_{1,58}$ = 22.36, $P < 0.001$). Bonferroni's post hoc test revealed significant differences in baseline between control and NLB rats. Of note, NLB rats exhibited a reduced sex difference in muscle mechanical nociceptive threshold. Inset figures show estimation plots; **B**. Representative Western blot from L4–L5 DRG extracts from control and NLB rats. The calculated molecular weight of Na$_V$1.7 is 226 kDa (according to UniProt KB database entry O08562). β-actin, was used as a loading control (molecular weight of 42 kDa according to UniProt KB database entry P60711); **C**. Semi-quantitative Western blot analysis demonstrates a significant increase in Na$_V$1.7 immunoreactivity in protein extracts from NLB rats compared to control rats. Numbers in the bars indicate sample size for each group. ***$P < 0.001$

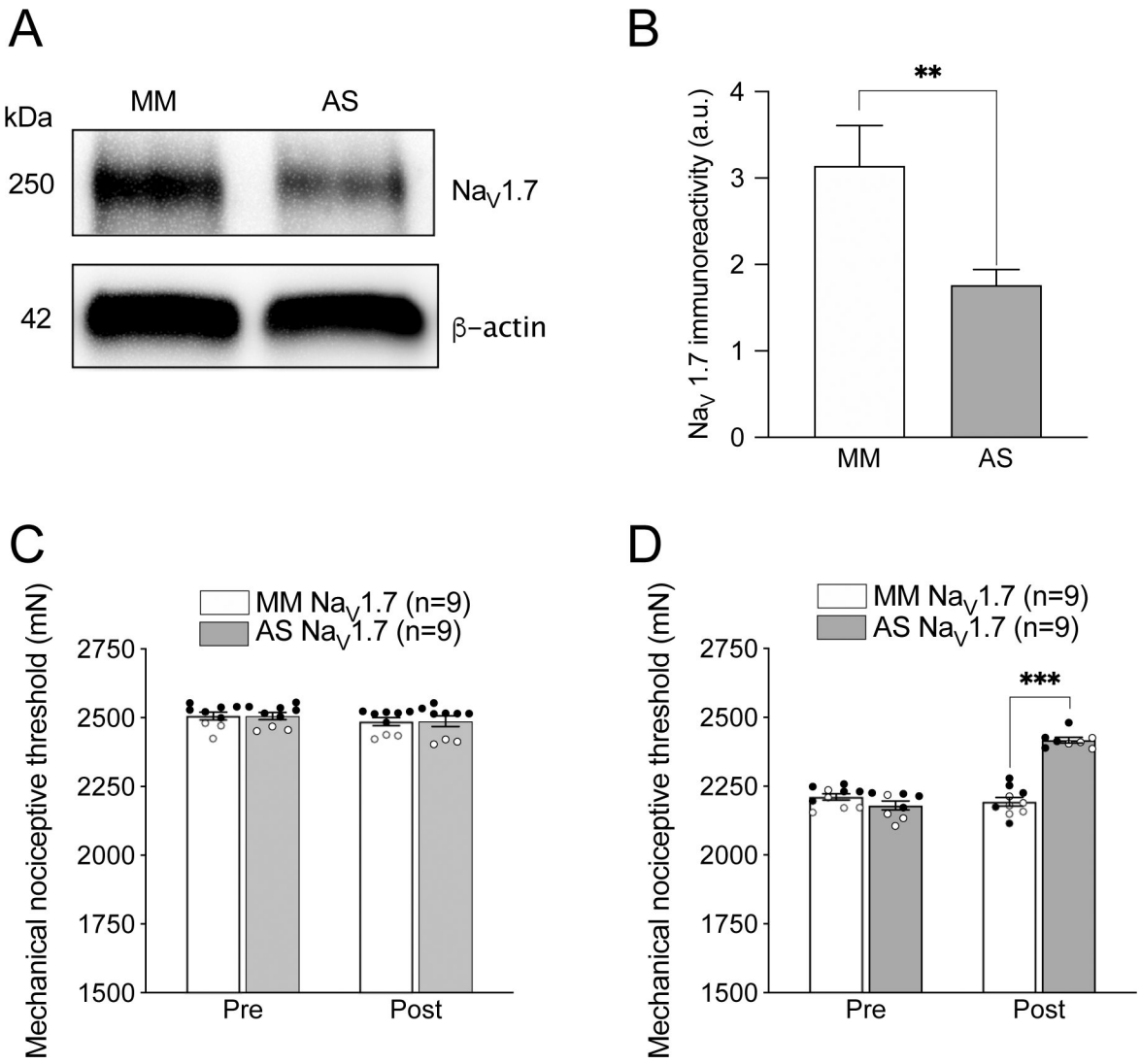

Figure 2. Effect of AS ODN directed against $Na_V1.7$.

A. Representative Western blot analysis of L4–L5 DRG extracts from rats injected daily i.t. with 120 μg/20 μl of AS or MM ODNs for 3 consecutive days revealed significant down-regulation of $Na_V1.7$ in the AS ODN compared to MM ODN group (see results section for details). β-actin was used as a housekeeping gene product; **B**. Comparison of the protein expression by Western blotting demonstrated a significant decrease in $Na_V1.7$ in DRG extracts from rats treated with AS ODN, compared to the MM ODN treated group; **C.** AS and MM ODN treatments were devoid of effect on baseline muscle mechanical nociceptive threshold in control (naïve) rats. Two-way repeated measures ANOVA showed only significant effect for time ($F_{1,16} = 9.103$, $P < 0.001$), but not for treatment ($F_{1,16} = 0.0007$, $P = 0.9788$), or interaction ($F_{1,16} = 0.0142$, $P = 0.9065$); **D.** $Na_V1.7$ AS ODN treatment inhibits muscle hyperalgesia in NLB rats. Two-way repeated measures ANOVA showed significant effects for treatment ($F_{1,16} = 35.36$, $P < 0.001$), time ($F_{1,16} = 97.89$, $P < 0.001$), and interaction ($F_{1,16} = 130.9$, $P < 0.001$). Bonferroni's post hoc test revealed significant differences in baseline between AS and MM groups at ODN treatment (Post)

time point. Three-way ANOVA was performed on data in **C** and **D**: Time (pre-post) × NLB × NaV1.7 antisense $F_{1, 64} = 37.43$ $P<0.0001$, indicating NLB rats are different from control due to over-expression of NaV1.7.

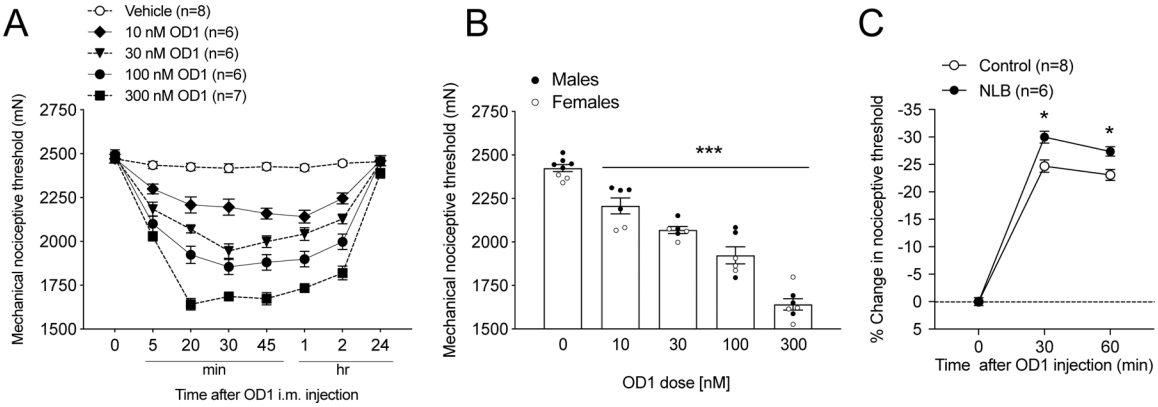

Figure 3. Effects of $Na_V1.7$ activator OD1 on muscle mechanical nociceptive threshold.

A. Time course of changes in the local mechanical nociceptive threshold of control rats injected i.m. with different doses of OD1 or vehicle. Two-way repeated measures ANOVA showed significant effects for treatment ($F_{4,28} = 72.41$, $P < 0.001$), time ($F_{7,196} = 396.6$, $P < 0.001$), and interaction ($F_{28,196} = 38.92$, $P < 0.001$). Bonferroni's post hoc test revealed significant differences between vehicle and OD1-treated rats over a period of 0.5 to 2 h after injection (solid symbols, $P < 0.001$); **B.** Injection of OD1 into the right gastrocnemius muscle produced dose-dependent decrease in mechanical nociceptive threshold as measured 20 min after injection. One-way repeated measures ANOVA showed significant effect for treatment ($F_{4,28} = 83.09$, $P < 0.001$). Dunnett's multiple comparisons test revealed significant differences between vehicle (0) and OD1 doses; **C.** Comparison of nociceptive effects of OD1 (100 nM/50 µl) in control and NLB rats at baseline (0), 30 and 60 min after i.m. injection. Two-way repeated measures ANOVA showed significant effects for treatment ($F_{1,10} = 10.12$, $P = 0.0097$), time ($F_{2,20} = 752$, $P < 0.001$), and interaction ($F_{2,20} = 5.382$, $P = 0.013$). NLB rats displayed increased hyperalgesia to OD1 compared to control rats 30 min after the injection, as revealed by Bonferroni's multiple comparisons test. *$P < 0.05$; ***$P < 0.001$. Three-way ANOVA for Time (pre-post) × NLB × OD1 shows that OD1 effect does not differ with NLB treatment over time ($F_{1, 22} = 0.3449$, P=0.5630).

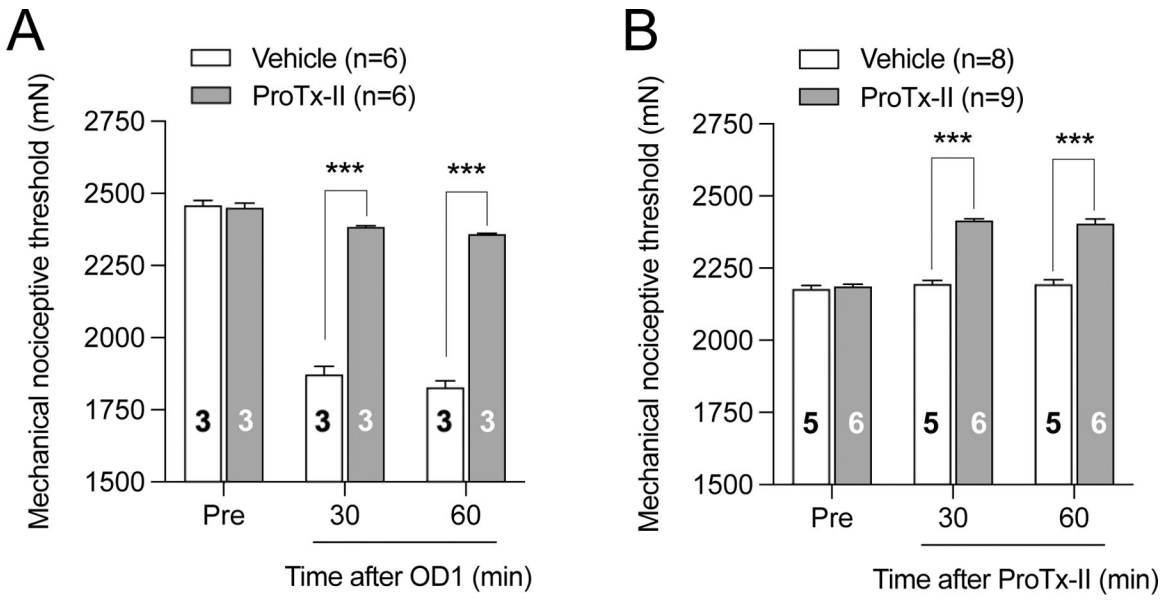

Figure 4. Effects of $Na_V1.7$ inhibitor ProTx-II on muscle mechanical hyperalgesia.

A. Control rats were injected i.t. with ProTx-II (1 μg/20 μl), or vehicle (20 μl), followed by i.m. OD1 (100 nM/50 μl), and readings taken 30 and 60 min after OD1 injection. Two-way repeated measures ANOVA showed significant effects for treatment ($F_{1,10} = 287.5$, $P < 0.001$), time ($F_{2,20} = 502.2$, $P < 0.001$), and interaction ($F_{2,20} = 295.6$, $P < 0.001$). Bonferroni's post hoc test revealed significant differences in nociceptive threshold between vehicle and ProTx-II treated rats 30 and 60 min after OD1 injection; **B.** NLB rats were injected i.t. with ProTx-II (1 μg/20 μl), or vehicle (20 μl), and readings taken 30 and 60 min after injection. Two-way repeated measures ANOVA showed significant effects for treatment ($F_{1,15} = 150.4$, $P < 0.001$), time ($F_{2,30} = 82.09$, $P < 0.001$), and interaction ($F_{2,30} = 59.93$, $P < 0.001$). Compared to vehicle, ProTx-II significantly attenuated the mechanical hyperalgesia exhibited by NLB rats 30 and 60 min after i.t. injection of ProTx-II, as revealed by Bonferroni's multiple comparisons test. Numbers in bars indicate number of females included in each group. ***$P < 0.001$

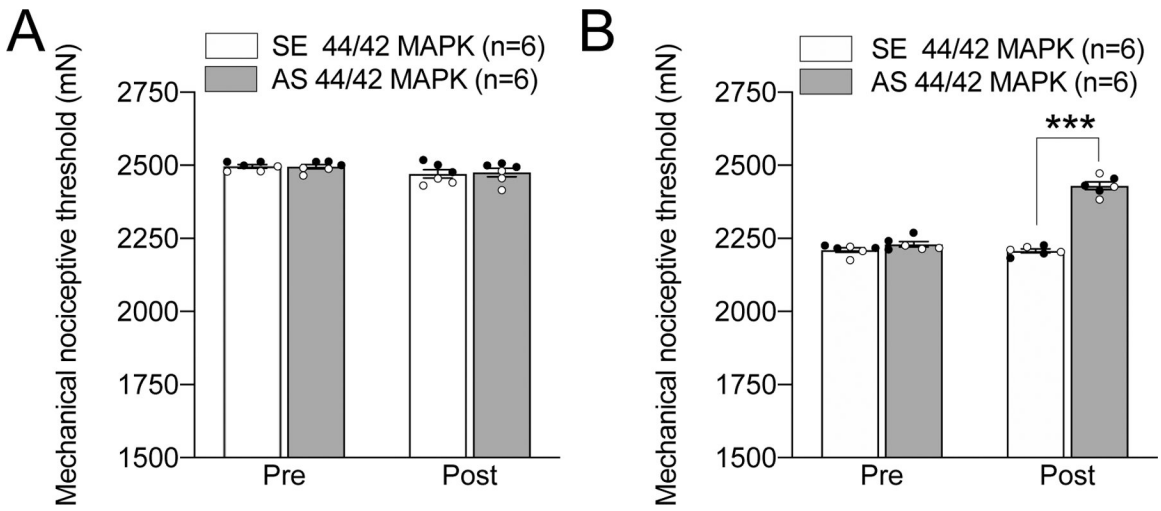

Figure 5. Contribution of ERK1/2 (44/42 MAPK) to early-life stress-induced muscle mechanical hyperalgesia.

(**A**) Effect of AS ODN targeting ERK1/2 mRNA on muscle mechanical nociceptive threshold did not produce significant changes in mechanical nociceptive threshold in control rats (two-way repeated measures ANOVA: treatment [$F_{1,10} = 0.01497$, $P = 0.905$], time [$F_{1,10} = 8.757$, $P = 0.0143$], interaction [$F_{1,10} = 0.1709$, $P = 0.688$]); (**B**) In contrast, the same AS ODN treatment in NLB rats. Two-way repeated measures ANOVA showed significant effects for treatment ($F_{1,10} = 140.1$, $P < 0.001$), time ($F_{1,10} = 147$, $P < 0.001$), and interaction ($F_{1,10} = 156.6$, $P < 0.001$). Bonferroni's post hoc test revealed significant differences in baseline between AS and SE groups after ODN treatment (*Post* time point). Three-way ANOVA analysis shows a significant interaction of Time (pre-post) × NLB × MAPK antisense $F_{1, 20} = 77.96$, P<0.0001.