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Silencing the α_2 subunit of $GABA_A$ receptors in rat dorsal root ganglia reveals its major role in antinociception post-traumatic nerve injury

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Abstract

Background—Neuropathic pain is likely the result of repetitive high frequency bursts of peripheral afferent activity leading to long-lasting changes in synaptic plasticity in the spinal dorsal horn (DH). Drugs that promote GABA activity in the DH provide partial relief of neuropathic symptoms. We examined how *in vivo* silencing of the $GABA_A$ α_2 gene in DRG controls of NPP.

Methods—After crush injury to the right sciatic nerve of female rats, the α_2 $GABA_A$ antisense and mismatch oligodeoxynucleotides or NO-711 (a GABA uptake inhibitor) were applied to the L5 DRG. *In vivo* behavioral assessment of nociception was conducted prior to the injury and ensuing 10 days (n=4–10). *In vitro* quantification of α_2 $GABA_A$ protein and electrophysiology studies of $GABA_A$ currents were performed on acutely dissociated L5 DRG neurons at relevant time-points (n=6–14).

Results—NPP post-crush injury of a sciatic nerve in adult female rats coincides with significant down-regulation of the α_2 subunit expression in the ipsilateral DRG (about 30%). Selective down-regulation of α_2 expression in DRGs significantly worsens mechanical (2.55±0.75 to 5.16±1.16)

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and thermal (7.97±0.96 to 5.51±0.75) hypersensitivity in crush-injured animals and causes development of significant mechanical (2.33±0.40 to 5.00±0.33) and thermal (10.80±0.29 to 7.34±0.81) hypersensitivity in sham animals (data shown as MEAN±SD). Conversely, up-regulation of endogenous GABA *via* blockade of its uptake in DRG alleviates NPP.

Conclusions—The $GABA_A$ receptor in the DRG plays an important role in pathophysiology of NPP caused by sciatic nerve injury and represent promising target for novel pain therapies.

Keywords

crush injury; hyperalgesia; pain behavior; antisense technology; NO-711; rats

INTRODUCTION

The pathophysiology and treatment of traumatic peripheral nerve injury and ensuing neuropathic pain (NPP) remain a complex medical enigma.[1] Conventional pain management does not abolish the development of NPP nor does it provide complete or long-lasting relief. Increased excitability of dorsal root ganglia (DRG) neurons is important for the initiation and maintenance of central sensitization, an important contributor to the development of NPP.[2] More specifically, NPP caused by peripheral nerve injury could be the result of the repetitive, unopposed bursts of high frequency afferent activity from the periphery leading to a long-lasting increase in synaptic strength and changes in synaptic plasticity in the spinal dorsal horn (DH).[3,4]

Consequently, promotion of γ-aminobutyric acid (GABA) activity in the DH to activate inhibitory inputs and lessen high frequency stimulation in the spinal cord was beneficial in alleviating NPP symptoms in animal models of peripheral nerve injury.[5,6,7] However, beneficial effects of intrathecally administered GABAergic agents are transient,[8] suggesting that the DH may be an insufficient therapeutic target for modulating maladaptive, NPP-related circuitry. Although GABAergic modulation in the DH is important pathologically, the primary target for its modulation has to be carefully considered if the beneficial effects are to be long-lasting and without unwanted side effects.

The DRG plays a pivotal role in pain transmission from the periphery to the higher pain-processing regions and as such could be important for interventions aimed at suppressing high frequency afferent stimulation before it reaches the DH. Experimental evidence in animal models of NPP indicate that remodeling of different ion channels in soma of the DRG neurons can cause changes in excitability and alter normal sensory transmission in the DH.[9,10] Of special interest are the findings that the neuropathy caused by peripheral nerve injury is accompanied by a decreased expression of the $GABA_A$ receptor in sensory neurons.[11] While the role of ligand-gated $GABA_A$ channels in synaptic transmission of DH neurons in NPP is reasonably well established,[12] the role of $GABA_A$ related neuro-glia transmission in DRG remains poorly understood. $GABA_A$ receptors are located in DRG cells and when potentiated *in vivo*, may abolish NPP development and progression.[8] However, some *in vitro* data suggest that the activation of $GABA_A$ receptors in DRG may be excitatory and hence potentially responsible for painful behavior.[13] Considering the controversial reports regarding the role of GABA modulation in DRG neurons, we used the

traumatic sciatic nerve injury model to investigate the molecular mechanisms responsible for DRG-controlled GABA-mediated NPP with special emphasis on the $GABA_A$ α_2 subunit since this subunit is abundantly present in DRG neurons.[14] We performed behavioral assessments of NPP phenotype in conjunction with targeted $GABA_A$ α_2 down-regulation and direct DRG applications *in vivo*, which afforded higher selectivity and specificity. Our main hypothesis is that traumatic crush injury to the sciatic nerve results in down-regulation of the $GABA_A$ α_2 subunit in the corresponding DRG and that this downregulation plays an important role in NPP.

MATERIALS AND METHODS

ANIMALS AND CHEMICALS

In this study, we use adult female Sprague–Dawley rats (retired breeders, average weight from 200 to 250 g). The decision to focus on females was based on the following: 1) in previous studies, we showed that retired breeder female and adult male rats exhibit similar pain hypersensitivity after sciatic crush injury;[8] 2) we find that female rats are less aggressive and easier to handle during pain testing; 3) it has become recognized over the years that the majority of pain sufferers are women;[15,16] thus we consider studies of pain sensitivity in females important for the improvement of pain management; and 4) although the importance of the estrous cycle-dependent variability in nociceptive thresholds in females has been suggested, there are no conclusive and/or consistent reports confirming their cyclicity to be a major confounding factor for pain sensitivity.[17]

The α_2 $GABA_A$ antisense (AS) oligonucleotides and the mismatch control (MIS) oligodeoxynucleotides (ODNs) were purchased from Eurofins MWG Operon (Huntsville, Alabama, USA) and were made freshly from stock solutions, dissolved in appropriate buffer and were pH balanced (pH 7.4 to avoid DRG irritation) just before applying on L5 DRG. The sequence of the AS-α_2 $GABA_A$ oligonucleotide (ASODNs) was: 5′-TCCATCCCAAGCCCATCC-3′. The sequence of the MIS-α_2 $GABA_A$ oligonucleotide (MISODNs) was: 5′-CTACCGCACTCTCACCAC-3′. The GABA uptake inhibitor NO-711 hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in appropriate sterile pH 7.4 buffer solution.

IN VIVO STUDIES

1. Sciatic nerve crush injury model—All experimental protocols were approved by the University of Virginia Animal Care and Use Committee and were in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health). All possible efforts were made to minimize animals' suffering and the number of animals used while still permitting statistically valid conclusions to be drawn based on our previous experience.[8,18] Within each experiment, animals were randomly assigned to either the experimental or control conditions.). All experiments were performed in the laboratory of Vesna Jevtovic-Todorovic at the University of Virginia.

To induce neuropathic pain, we performed a crush injury to the right sciatic nerve. Rats were anesthetized with isoflurane (2% in air delivered *via* nose cone). After the right external

thigh and buttock area was shaved, the skin was prepared with antiseptic solution and alcohol, and a small incision was made. The *m. vastus lateralis* and *m. biceps femoralis* were separated bluntly at the mid-thigh level. At approximately 7 mm from the point of its emergence, the common sciatic nerve was mobilized and traumatized by a single 30-s crush with a serrated 5-inch curved artery hemostat that was compressed passed the first of three notches.[19] This approach enabled standardization of the technique and assured that the consistent force was applied in each animal. For the sham condition, the sciatic nerve was mobilized without crush injury. After surgery bupivacaine hydrochloride (0.25%) was injected in the skin surrounding the wound to minimize immediate incisional pain. Postoperatively, animals were housed in pairs in plastic cages with abundant supplies of water and food and were monitored closely and regularly.

The total loss of animals to behavioral follow up was approximately 15% due to the changes in their health status (i.e. trophic skin changes on their hind paws after crush injury to the sciatic nerve). The data from these rats were excluded from any further analysis.

2. Direct dorsal root ganglion application—We have established previously that the success rate in achieving hyperalgesia in the crush injury model is between 90%–95%, and that the animals reach stable hyperalgesic behavior at 48 hour after injury (by postoperative day 2 – POD 2).[8] Therefore, we initiated direct DRG application of either AS-α_2 $GABA_A$ (ASODNs) or MIS-α_2 $GABA_A$ oligonucleotides (MISODNs) on POD 2.[20] During direct DRG application of the oligonucleotides, anesthesia was maintained with 2% isoflurane delivered in air *via* nose cone. To minimize injury inflicted to each animal, we limited direct application to L5 DRG only. The choice of L5 DRG was based on the fact that 98–99% of all sciatic DRG neurons reside in the L4 - L5 DRGs (with the L3 and L6 DRGs contributing roughly 1.2% and 0.4%, respectively)[21,22,23,24,25] thus making L5 DRG a very important therapeutic target. To assess the local spread of the injectate we applied sodium fluorescein (500μg/100μl) directly onto L5 DRG (25–30 μl) and after 30 minutes assessed the presence of the fluorescence (Olympus SZX12 microscope; magnification 5×, Tokyo, Japan) in the L3, L4 and L5 DRGs. Once we have established that some spread occurs but only to L4 DRG and not to the ipsilateral L3 DRG or the corresponding segment of the spinal cord, we chose to perform all DRG application experiments on L5 DRG as follows: after making a midline incision at the L4–L6 spinal level, right para-spinal muscles were separated from the vertebrae and the L5 spinal nerve was tracked through the intervertebral foramen. A hole, 0.49-mm in diameter was drilled through the transverse process over the L5 DRG approximately 2 mm off the inferior edge of the transverse process, in the line with the course of the L5 spinal nerve. The drill bit and the 25-gauge (diameter 0.5 mm) needle used for application had a predetermined and limited length to prevent them from contacting the underlying ganglion. After slow injection of 25 μL of AS-α_2 $GABA_A$ (ASODNs), MIS-α_2 $GABA_A$ oligonucleotides (MISODNs), saline or 30 μL NO-711 hydrochloride solution into the hole, the inserted needle and microsyringe were left in place for at least 3 min to ensure complete delivery of the solution and to minimize possible extravasation outside the DRG site.

3. Behavioral assessment of heat nociception—The nociceptive response to heat stimulation was measured using a custom-built Hargreaves paw thermal stimulation system (UCSD, University Anesthesia Research and Development Group, La Jolla, CA, USA). Briefly, the system consists of a clear plastic chamber (10 × 20 × 24 cm) that sits on a clear, elevated glass floor and is temperature- regulated at 30 °C. Prior to the testing, each animal was placed in the testing room for about 30 min followed by acclimation in the plastic chamber for an additional 15 min. A radiant heat source mounted on a movable holder beneath the glass floor was positioned to deliver a thermal stimulus to the plantar side of the hind paw. When the animal withdraws the paw, a photocell detects interruption of a light beam reflection and the automatic timer shuts off. This method has a precision of ± 0.05 s for measurement of paw withdrawal latency (PWL) in seconds. To prevent thermal injury, the light beam is discontinued automatically at 20 s if the rat fails to withdraw its paw. Pain testing was done a couple of days before crush injury (baseline), immediately before crush injury (DAY 0) and every succeeding day for next ten days (POD 1 to POD 10).[8]

4. Behavioral Assessment of mechanical sensitivity—To measure mechanical sensitivity, rats were placed in a clear plastic cage with a wire-mesh-bottom, using an acclimation protocol as described above (*Behavioral assessment of heat nociception*). The plastic cage is large enough to permit the rat's freedom of movement while allowing investigators access to their paws. von Frey filaments (Stoelting, Wood Dale, IL, USA) were used to assess the mechanical threshold for paw withdrawal.[26] These filaments are designated as the log10 (milligram weight required to cause bending ×10). We have found that applying the 4.93 filament to the plantar surface of the foot causes a withdrawal response in female rats that results in an average of 1–2 paw withdrawal responses (PWRs) in 10 trials. Baseline withdrawal scores were determined in both paws, as well as neuropathic pain scores at POD 2 prior to injections of AS-α_2 $GABA_A$ (ASODNs) or MIS-α_2 $GABA_A$ oligonucleotides (MISODNs).

IN VITRO STUDIES

1. Western blot analysis—For the collection of L5 DRGs, rats were anesthetized deeply with isoflurane and decapitated. Both ipsi-(right L5) and contra-(left L5) lateral DRGs were extracted rapidly and then frozen in liquid nitrogen and stored at − 80°C. The L5 DRGs were processed, either individually or pooled from 3 animals (depending on amount of tissue). When the sample was pooled from 3 animals, we regarded the sample as n=1 for the purpose of statistical analysis. Tissue samples were homogenized in a lysing buffer with complete protease inhibitor cocktail (Roche, Basel, Germany), sonicated and centrifuged at 4°C 12000 rpm for 10 min to remove cell debris. Supernatants were collected and protein concentrations were determined using the Lowry method. Samples were combined with 2× Laemmli buffer (Sigma-Aldrich, St. Louis, MO, USA), boiled for 5 minutes, loaded into a 10% polyacrylamide gel and electrophoresed. Separated proteins were transferred to nitrocellulose membranes and blocked with 3% bovine serum albumin (BSA) at room temperature for 1 hour. The membrane was incubated at 4°C overnight with primary antibodies for the $GABA_A$ α_2 receptor subunit (Alomone labs, Jerusalem, Israel) or the house-keeping protein actin (Sigma-Aldrich, St. Louis, MO, USA) at respective dilutions of 1:5000 and 1:10000. Appropriate horseradish peroxidase–conjugated secondary antibodies

anti-rabbit IgG (1:15000, Santa Cruz Biotechnology, Dallas, Texas, USA) were applied for 1 hour. Then, the membranes were developed using Super Signal ECL detection reagents (Thermo Scientific, Rockford, IL, USA) and band density was quantified using Syngene Gel documentation, G-box analysis software (Syngene, Cambridge, UK). Densities for $GABA_A$ α_2 receptor bands were normalized to actin used as a housekeeping protein.

2. Electrophysiology studies of $GABA_A$ currents—To study $GABA_A$ currents, we used acutely dissociated L5 DRG neurons from adult female Sprague-Dawley rats and standard whole-cell patch-clamp techniques as we described elsewhere.[27] An external solution containing GABA was applied directly onto the DRG cells using a manually-controlled custom built "sewer pipe" perfusion system. The tip of the perfusion pipette was placed within 200 μm of the recorded cell. Cells were clamped at –70 mV for all experiments. We used brief applications of GABA for 3–5 sec in order to avoid desensitization of GABA-gated currents. The extracellular recording solution contained (in mM): 140 NaCl, 4 KCl, 2 MgCl, 2 CaCl, 10 HEPES, 10 glucose, pH 7.3. The recording pipette was filled with a solution containing (in mM): 130 KCl, 1 EGTA, 5 $MgCl_2$, 40 HEPES, pH 7.3. Inward currents were evoked by applying 100 μM GABA in the external solution, and peak amplitudes of GABA-gated currents were measured in multiple cells from each of the rats. Peak values of GABA-evoked currents were divided by the cell capacitance to obtain current densities.

STATISTICAL ANALYSIS

All numerical data in the figures are presented as the means ± SD. The investigators were blinded to the treatment conditions of each animal (sham or crash-injured, ASODN or MISODN).

PWLs and PWRs were subjected to repeated-measures analysis of variance (ANOVA) containing two within-subjects factors: time of the session (prior to vehicle/test compound administration or daily post-treatment up to 10 days) and paw condition [right (crush-injured/treated) vs. left (sham-operated) paw]; where appropriate, relevant pairwise comparisons were conducted using the Tukey posthoc test.

To evaluate statistical differences in Western blot studies, the density values were analyzed using two-tail *t*-test where the left DRG (normalized at 100%) was used for comparison to the right DRG in each animal individually. Values of GABA current densities from all cells in our electrophysiology study were averaged per rat and thus "n" in our recordings represents the number of rats in each experimental group. The statistical analyses were performed using two-tail *t*-test or Mann-Whitney Rank Sum test if the data distribution failed normality testing. Statistical analyses were performed using GraphPad Prism®, (version 5.01, La Jolla, CA, USA) and Sigma Plot software (version 12.0, San Jose, CA, USA).

RESULTS

To verify the development of NPP posttraumatic nerve injury, we first established the presence of thermal hypersensitivity, an important feature of NPP, in animals with crush

injury to the sciatic nerve. We confirmed as previously reported[8] that compared with sham injury (Fig. 1A), crush injury to the right sciatic nerve causes significant decrease in PWLs in the ipsilateral paw as compared with the contralateral (unoperated) paw [$F(2,10)=84.366$, $p<0.001$] on postoperative day (POD) 2 (Fig. 1B), validating that thermal hypersensitivity develops rather quickly. Note the stable PWLs recordings at the baseline and immediately before crush injury (DAY 0) in paws of both sham and crush-injured rats (n=5 in sham and n=6 in crush-injured rats per data point).

To begin to understand the role of GABAergic modulation in the development of NPP post-crush injury, we examined whether such injury modulates the expression of $GABA_A$ receptors in L5 DRG neurons. Since adult rat DRG neurons preferentially express $\alpha_2\beta_3\gamma_2$ subunits[14,28] and α_2 is the important functional subunit, we focused on expression of the α_2 subunit. As shown in Figure 2, expression of the $GABA_A$ receptor α_2 subunit protein in L5 DRG on the ipsilateral (right) side was indistinguishable from that on the contralateral (left) side in both sham operated (A) [$t(5)=1.186$, $p=0.2888$; n=6 animals per data point] and crush injured (B) rats at the baseline [$t(13)=1.023$, $p=0.3248$; n=14 animals per data point] (left panels). However, on POD 2 (when significant thermal hyperalgesia was observed in crush-injured animals as shown in Fig. 1B) we noted a significant (about 30%) decrease in α_2 subunit expression in ipsilateral vs. contralateral L5 DRG in crush-injured animals [$t(7)=3.050$, $p=0.0186$; n=8 per data point] whereas there was no change in α_2 subunit expression in ipsilateral vs. contralateral L5 DRG of sham-operated animals [$t(5)=0.6038$, $p=0.5723$; n=6 per data point] (right panels).

If acute down-regulation of α_2 subunit expression in ipsilateral DRG plays an important role in the development of NPP in crush-injured animals, we reasoned that down-regulation of α_2 subunit expression in sham-operated animals using selective knock- down antisense technology should mimic the NPP phenotype. To test this hypothesis, we first confirmed the effectiveness of α_2 subunit knock-down in sham-operated animals. We applied antisense or mismatch ODNs for the α_2 subunit onto the right L5 DRG in sham animals on POD 2 once we confirmed that thermal hypersensitivity did not develop (see Fig. 1A). We noted that protein expression of the α_2 subunit in the right L5 DRG post-ASODN treatment was downregulated significantly compared with that in the left (untreated) L5 DRG (Fig. 3A, left panel) [$t(5)=14.20$, $p<0.001$; n=6 rats per data point] whereas the α_2 subunit protein expression post-MISODN treatment remained unchanged (Fig. 3A, right panel) [$t(5)=1.509$, $p=0.1917$; n=6 rats per data point] when examined 24–48 hrs post-ODNs application (i.e., on POD 3 to 4). This down-regulation caused by direct L5 DRG application of ASODN was restricted to the ipsilateral (right) DRG as shown in Fig. 3B. The application of MISODN also resulted in no change in α_2 subunit protein expression in ipsi- DRGs (Fig. 3B, right panel). Taken together, this evidence suggests that direct DRG application induced α_2 subunit modulation that was restricted to the ipsilateral DRG. To validate further the down-regulation of α_2 subunits in DRG cells, we used a functional assay based on patch-clamp recordings of $GABA_A$-gated currents in small diameter (less than 30 μm) acutely dissociated DRG cells from sham-operated, ASODN-treated rats and compared the findings with those from untreated (naïve) and MISODN-treated rats. All recordings were done using ipsilateral L5 DRGs. We recorded from smaller DRG cells since these are putative nociceptive neurons and previous studies have established that they express prominent $GABA_A$ receptor-

mediated inward currents.[29] Representative current traces (Fig. 3C, left panel) and summary of data from patch-clamp experiments (Fig. 3C, right panel) indicate that as compared with the naive (no treatment) (n=7 rats per data point from 17 cells) and MISODN (n=5 rats per data point from 22 cells), ASODN administration resulted in about a 3-fold decrease in GABA-gated current density in small DRG cells (*, $p<0.05$; Mann-Whitney Rank Sum Test) (6.07± 8.1 pA/pF, n=7 rats per data point from 32 cells) suggesting that selective knock-down of α_2 subunits in DRG cells results in down-regulation not only of its protein expression but also in functional $GABA_A$-gated currents. As expected, we found that there is no significant difference in GABA-gated current density between MISODN-treated animals (16.69 ±11.1 pA/pF) and naïve ones (17.5± 9.3 pA/pF; p=0.89, t-test). We also measured peak outward voltage-gated potassium currents in the same cells following recordings of GABA currents in order to establish whether ASODN treatment had any non-specific effects. DRG cells were held at a holding potential of −90 mV and stepped to test potential of 50 mV for 200 msec. The peak potassium current densities were not significantly different [$F(2,16)=1.509$, $p=0.2509$] between the ASODN-treated (192 ± 67 pA/pF, n=7 rats), MISODN-treated (241± 74 pA/pF, n=5 rats) and naïve animals (247 ± 53 pA/pF, n=7 rats) which argues against ASODN treatment having non-specific effects on DRG nociceptive neurons.

With the suggestion that the $GABA_A$ α_2 subunit may be an important target for NPP development, we examined whether ASODN treatment of sham animals would result in a NPP phenotype similar to that observed in crush-injured animals (Fig. 1B) where comparable down-regulation of α_2 subunit expression was reported (Fig. 2B, right panel). As shown in Figure 4, we examined thermal and mechanical hypersensitivity, two hallmark features of NPP, in rats in the sham surgery group after treatment with ASODNs (Fig. 4A) or MISODNs (Fig. 4B) on POD 2 when the lack of thermal hypersensitivity was confirmed. (The protocol was as described for biochemical and patch-clamp studies in Figure 3). We found statistically significant interaction between Time and Paw due to knock-down of the α_2 subunit with ASODNs [$F(11,44)=11.970$, $p<0.001$] which effectively induced thermal hyperalgesia in sham-operated rats as evidenced by significant decrease in PWLs on POD 3 through POD 5 (outlined with dashed rectangle) (n=5 per data point) [†††, $p<0.001$- before vs. after ASODNs treatment); ***, $p<0.001$; **, $p<0.01$ - PWLs in operated (R) paw vs. unoperated (L) paw at PODs 3, 4 and 5] (Fig. 4A). However, there was no change in PWLs in animals treated with MISODNs at any time point [$F(11,33)=0.837$, $p=0.606$] (n=4 per rats data point) (Fig. 4B).

Similarly, when mechanical hypersensitivity was examined in sham-operated animals, we found that treatment with ASODNs (Fig. 4C) [$F(11,44)=18.696$, $p<0.001$; n=5 rats per data point], but not MISODNs (Fig. 4D) [$F(11,33)=0.287$, $p=0.984$; n=4 rats per data point] caused a significant increase in PWRs (outlined with dashed rectangle) on POD 3 through 8 (†††, $p<0.001$; ††, $p<0.01$; before and after ASODNs treatment), (***, $p<0.001$; PWRs in operated (R) paw vs. unoperated (L) paw).

The fact that ODN application to DRG *per se* does not cause changes in pain perception is confirmed by the lack of an effect of MISODNs on PWLs (Fig. 4B) or PWRs (Fig. 4D) in the ipsilateral paw.

Considering the fact that α_2 subunit down-regulation in ASODN-treated sham animals resulted in significant thermal and mechanical hyperalgesia, we reasoned that if maintaining $GABA_A$ α_2 subunit expression and function are important for NPP development post-crush injury, ASODN application in crush-injured animals may worsen thermal and mechanical hyperalgesia. To address this hypothesis, we administered the α_2 subunit ASODNs or MISODNs onto the ipsilateral DRG of injured animals on POD 2 after the thermal and mechanical hypersensitivity were confirmed (Figure 5) [***, p<0.001; PWLs in operated (R) paw vs. unoperated (L) paw on POD 2; ASODNs animals n=6, MISODNs animals n=5 per data point]. Overall statistical analysis showed significant interaction between Time and Paw after crush injury in both ASODNs (Fig. 5A) [F(11,55)=70.696, p<0.001] and MISODNs group (Fig. 5B) [F(11,44)=51.382, p<0.001] immediately following crush injury of right sciatic nerve. However, pairwise comparisons using the Tukey posthoc test showed that only ASODNs application caused significant worsening of thermal hypersensitivity in injured animals from PODs 3 to 6 (outlined with dashed rectangle) (†††, p<0.001; before vs. after ASODNs treatment) [***, p<0.001; PWLs in operated (R) paw vs. unoperated (L) paw throughout the entire post-operative period]. The 'recovery' of PWLs to pre-ASODN treatment levels (Fig. 5A) is to be expected considering that the half-life of the $GABA_A$ channel is estimated to be around 20 hours.[30,31] When mechanical hypersensitivity was assessed, we found similar pattern. Namely, although crush injury produced significant increase in PWRs in both ASODNs [F(11,55)=25.751, p<0.001] (Fig. 5C) and MISODNs groups [F(11,44)=37.424, p<0.001] (Fig. 5D), worsening of the mechanical hypersensitivity was observed only in ASODNs treated group from POD 3 through POD 6 (outlined with dashed rectangle) (ASODNs animals n=6 per data point, MISODNs animals n=5 per data point; ††, p=0.002 and †††, p<0.001; before vs. after ASODNs treatment). Again, the duration of the ASODN effect was similar to that described for thermal hypersensitivity (Fig. 5A and B) [***, p<0.001; PWLs in operated (R) paw vs. unoperated (L) paw throughout the entire post-operative period.]

To further validate the link between ASODN-induced worsening of NPP phenotype and down-regulation of α_2 subunits in DRG caused by crush injury (Fig. 2B, right panel), we used a functional assay based on patch-clamp recordings of $GABA_A$-gated currents in small diameter acutely dissociated DRG cells. All recordings were done on POD 3–4 using ipsilateral L5 DRGs. Representative current traces (Figure 5E) and a summary of the data from patch-clamp experiments (Figure 5F) indicate that when compared with the control (21.1±6.88 pA/pF, n=3 rats per data point, 7 cells), the ASODN treatment resulted in about 7-fold decrease in the GABA current density (2.71±3.25 pA/pF) in crush-injured rats (*, p< 0.05; Mann-Whitney Rank Sum Test, n=8 rats per data point, 13 cells) on POD 3–4, the time point at which we noted significant worsening of the pain phenotype in ASODN-treated crush-injured rats (Fig. 5A and C). Interestingly, in recordings from crush-injured rats we measured GABA-current densities similar to those from sham and naïve animals (Fig. 3C). This result likely represents a homeostatic compensatory response to crush injury-induced remodeling of $GABA_A$ receptors in DRGs. A similar phenomenon associated with peripheral nerve injury has been described in DH neurons where a decrease in GABA content is accompanied by an increase in postsynaptic $GABA_A$ currents.[32]

Collectively, these behavioral, biochemical and electrophysiological findings suggest that down-regulation of the $GABA_A$ α_2 subunit likely plays an important causal role in the development of NPP post-crush injury to the sciatic nerve.

If this conclusion is valid, we reasoned that α_2 subunit protein expression should remain down-regulated in crush injured animals even at later stages of the disease, considering the presence of the pain phenotype. To address this hypothesis, we chose a later day in the NPP progression – POD 10 - when NPP is well established in crush-injured animals (as shown in Fig. 5) and confirmed to be nonexistent in sham-operated animals (as shown in Fig. 4). We found no changes in α_2 subunit expression in ipsilateral L5 DRGs at POD 10 in sham-operated animals as compared with the contralateral L5 DRGs (Fig. 6A, n=6 rats) whereas we found a significant down-regulation of α_2 subunit expression in ipsilateral vs. contralateral L5 DRGs in crush-injured animals [about 35%, Fig. 6B; $t(5)=3.949$, $p=0.01$; n=6 animals]. The α_2 subunit protein down-regulation at POD 10 is very similar to that at POD 2 (as shown in Fig 2B, right panel), suggesting a protracted nature of α_2 subunit modulation in DRGs due to nerve crush injury. Based on this observation, we conclude that there is indeed an association between α_2 subunit protein down-regulation in DRG cells and the NPP phenotype.

To further test a potential causal link between $GABA_A$ receptor modulation (manifested as a down-regulation of α_2 subunit) and pain phenotype (manifested as thermal and mechanical hypersensitivity), we examined whether NPP could be alleviated if the GABA levels in L5 DRG are pharmacologically up-regulated with the use of a selective GABA uptake inhibitor. Previous biochemical studies have documented the existence of a glial-mediated GABA uptake system in sensory neurons of DRGs[33] and intracellular recordings from intact DRGs from naïve animals have shown that GABA-gated currents are enhanced with traditional pharmacological agents that inhibit GABA uptake.[34] Here we used the newer agent NO-711 as a specific blocker of the GABA transporter-1 (GAT-1) since it has been confirmed to maintain the extracellular GABA in CNS at higher levels[35]. As shown in Figure 7, we found that application of NO-711 at 50 μg in 30 μl of vehicle directly onto ipsilateral L5 DRG on POD 2 after thermal hyperalgesia was confirmed [***, $p<0.001$ baseline (B) vs. POD 2] induced a significant alleviation of thermal hyperalgesia [overall ANOVA $F(4,68)=8.332$; $p<0.001$], i.e. the PWLs in NO-711 treated animals (closed triangles) were significantly increased when compared to a vehicle treatment (closed squares) (††, $p<0.01$; †††, $p<0.001$; n=9 and 10 rats per data point in NO-711 and vehicle groups, respectively). The increase in PWLs also resulted in significant alleviation of thermal hyperalgesia on PODs 3 (#, $p<0.05$) and 4 (##, $p<0.01$) when compared to POD 2 immediately before the treatment. Note that the PWLs in vehicle-treated group remained significantly decreased throughout the testing period when compared to the baseline PWL recordings [***, $p< 0.001$; baseline (B) vs. any given POD]. The PWL recordings in the left (untreated paw) remained stable and similar to the baseline PWLs in both the vehicle and NO-711-treated groups (data not shown). These finding suggest that selective, pharmacological up-regulation of GABA levels in the ipsilateral DRG could compensate for the crush injury-induced downregulation of α_2 subunit expression by increasing the availability of its endogenous agonist, GABA.

To scrutinize the degree and the direction of spread of DRG drug applications to surrounding DRGs and the spinal cord, we applied 25–30 μl of sodium fluorescein on the right L5 DRG and after 30 min examined the intensity of fluorescein stain in L5 DRG and the neighboring ipsilateral L3 and L4 DRGs. As shown in Figure 8, there is no fluorescein labelling noted in the L3 DRG (A); however, we noted some fluorescein labeling in the L4 DRG (B) and substantially higher level of labelling in L5 DRG (C). Importantly, there was no spillage into the corresponding segment of the spinal cord (D). This suggests that the direct application onto the L5 DRG results in fairly contained distribution of an injectate with only limited spread to the ipsilateral L4 DRG.

DISCUSSION

Down-regulation of the $GABA_A$ α_2 subunit in the DRG plays an important role in NPP post-crush injury of a sciatic nerve in adult female rats. We base our conclusion on three important observations: 1) NPP detected post-crush injury of a sciatic nerve coincides with a significant down-regulation of the α_2 subunit expression; 2) when α_2 subunit expression and $GABA_A$ channel function are selectively knocked down in DRGs using specific ODNs *in vivo* antisense technology, we demonstrate worsening of NPP in crush-injured animals and *de novo* development of an “NPP-like state” in sham animals (with a duration of pain phenotype that correlates with the half-life of α_2 subunit turnover) and, 3) upregulation of GABA levels in DRG by pharmacological blockade of its uptake overcomes the effects of crush injury-induced down-regulation of the $GABA_A$ and alleviates NPP. Our data support the idea that the down-regulation of the $GABA_A$ system (specifically the α_2 subunit) in DRG neurons plays an important role in the pathophysiology of NPP after traumatic injury of the sciatic nerve.

NPP caused by peripheral nerve injury is the result of the repetitive bursts of high frequency afferent activity leading to long-lasting increases in synaptic strength and changes in synaptic plasticity in spinal DH.[3] The activation of inhibitory inputs and lessening of high frequency stimulation by promoting GABA activity in the spinal cord alleviates NPP symptoms in animal models of peripheral nerve injury.[6,7] However, beneficial effects of intrathecally administered GABAergic agents were only transient. Although GABAergic modulation in the spinal cord is important, timely pharmacological modulation of GABAergic function in the DRG completely abolishes the development of NPP in animals with peripheral nerve injury.[8] Those results suggest that the most promising target for GABAergic modulation could very well be the DRG since it is the immediate gateway between the injured afferent terminals and the higher pain centers. Here we validate this hypothesis using specific antisense-mediated knockdown of the α_2 subunit of $GABA_A$ receptors in DRGs.

A significant imbalance of GABA has been found in humans with NPP due to peripheral nerve injury.[36] Furthermore, it was suggested in the animal model of peripheral nerve injury that although there was a considerable depletion of GABA from its terminals in the spinal dorsal horn, there was no concomitant loss of GABA neurons.[37] Here we show that the restoration of GABA levels in the ipsilateral DRG using pharmacological blockade of GAT-1 results in alleviation of NPP, which would suggest that the maintenance of GABA

levels in both DH and DRG neurons is important in curtailing excessive neuronal stimulation from injured primary afferents. Hence an increase in availability of the endogenous agonist (GABA) may overcome the down-regulation in functional expression of $GABA_A$ receptor subunits. It is noteworthy that this observation was made using female retired breeders. The decision to use female rats was based on the fact that the majority of chronic pain sufferers are women thus necessitating better understanding of the mechanisms controlling their pain perception.[15,16] Having said that and despite the fact that our previous work with crush injury nerve model confirmed similar pain hypersensitivity in both retired breeder female and adult male rats,[8] the role of $GABA_A$ modulation in DRG neurons of male rats remains to be confirmed.

Interactions between the GABA system of the DRG and the superficial layers of the spinal DH (layers I-III) have been reported previously.[28] In addition, sciatic nerve transection or loose ligation decreased the number of GABA-immunoreactive cells in lamina I-III of the rat lumbar DH.[32,38] Published data indicate that the majority of DRG neurons expressing $GABA_A$ receptors terminate in lamina I-III.[39] They showed that $GABA_A$ receptors located in corresponding DRG neurons can modulate transmitter release in the superficial lamina of DH, suggesting that there is a close and complex relationship between the GABAergic system in the DRG and in the spinal DH.

The significant down-regulation of the $\alpha 2$ subunit could be caused by a selective loss of $GABA_A$-expressing DRG neurons as has been reported in the ipsilateral DH post-peripheral nerve injury.[40] However, presently available evidence suggests that the number of DRG neurons remains roughly unchanged or may perhaps even increase after crush injury[41,42] thus making it unlikely that $GABA_A$-expressing DRG neurons undergo similar downregulation as the interneurons in DH.

Here we focused on the α_2 subunit of $GABA_A$ receptors. Based on PCR and protein expression studies it is known that $GABA_A$ receptors are present on DRG neurons,[28] (but also are functional and muscimol-sensitive,[43] with a unique subunit composition[14]. As DRG neurons are pseudo-unipolar and glutamatergic, it is unlikely that endogenous GABAergic signaling in DRGs is *via* neurotransmission. Adult rat DRG neurons preferentially contain $\alpha_2\beta_3\gamma_2$ subunits[14] with the α_2 subunit being the key functional component.[44] Since knocking down the α_2 subunit resulted in worsening of the pain phenotype in NPP animals and in a *de novo* pain phenotype in sham-operated animals, it appears that the α_2 subunit in DRG $GABA_A$ receptors is important for the development of pain. Specific targeting of α_2 or α_3 subunits in spinal DH $GABA_A$ receptors has been shown to modulate NPP caused by loose ligation of the sciatic nerve in mice and preferential activation of the α_2 subunit was highly effective in ameliorating NPP in $GABA_A$-receptor point-mutated knock-in mice [α_2(H101R)].[44] These findings together with ours suggest that the development of subtype-selective GABAergic agents for treatment of NPP could be a promising strategy, with particular focus on α_2 and α_3 subunits in spinal DH neurons and on the α_2 subunit in DRG neurons.

A benefit of selective subunit modulation also could be appreciated in terms of different side effects. Previous studies showed that α_1 but not α_2 knock-in mice displayed an increase in

duration and a decrease in latency of loss-of-righting reflex upon administration of benzodiazepines or barbiturates.[45] Hence, although the role of each subunit remains to be fully understood, it is likely that selective up-regulation of the α_2 subunit could be beneficial in alleviating NPP without causing undue sedation or locomotor impairments.

Our findings with DRG application of the GAT-1 inhibitor NO-711 suggest an important therapeutic benefit of targeted control of GABA availability in the DRG for the treatment of NPP. Considering that satellite glial cells (SGCs) in DRG play an important role in GABA uptake[33,34] and consequently in controlling GABA availability to the adjacent neurons, the question becomes whether SGCs in affected DRGs should be considered an important therapeutic target for controlling GABA-modulated neuronal excitability and central sensitization. For instance, inflammatory pain induced by injection of complete Freund's adjuvant into a mouse paw is accompanied by a significant increase in coupling of gap junctions between SGCs and possibly modulation of their GAT-1 function resulting in increased GABA availability.[46] By being so snuggly connected to the affected DRG neurons, SGCs can exert tight influence over every aspect of neuronal excitability. Although our study did not examine the specific SGC changes that may occur in DRG post-crush injury, our findings suggest that blocking the GAT-1 GABA uptake transporter (which is also located on the SCG) and increasing the availability of GABA in close proximity to an affected neuron could play an important role in alleviating NPP, even when the $GABA_A$ receptor subunit composition is down-regulated significantly.

Based on our findings and previously published reports we hypothesize that two important processes occur concomitantly. The first one involves acute downregulation of the α_2 subunit in affected DRG (and probably a down-regulation of $GABA_A$ receptors itself). The second one may involve the modulation of SGCs intercommunication and possibly modulation of their GAT-1 function and GABA uptake. Based on our findings (Figure 7) as well as those of others,[47] we propose that compensatory maintenance of $GABA_A$ current density in DRG neurons post-crush injury is a homeostatic response to curtail the barrage of action potential firing coming from the injured periphery in the presence of modulated GABA availability. This compensatory response appears to be insufficient to prevent thermal hypersensitivity. However, with the pharmacological modulation of GAT-1 uptake and increase in GABA availability around the affected neurons, the upregulation of $GABA_A$ current may reach the critical compensatory point necessary to provide a complete abolishment of NPP development and progression. Further functional, biochemical and electrophysiological studies are needed to confirm this chain of events.

In conclusion, we demonstrate that peripheral nerve injury resulting in NPP in adult female rats is accompanied by a significant downregulation of $GABA_A$ α_2 subunit in the ipsilateral DRG neurons and that further down-regulation of this subunit results in worsening of pain. If indeed selective subunit modulation of GABAergic DRG receptors is critical for the development of symptoms and signs of NPP, pharmacological or gene targeting strategies aimed at the α_2 subunit in GABAergic DRG neurons may prove to be of great benefit in the treatment of this intractable and debilitating disease.

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REFERENCES

1. Cohen SP, Mao J. Neuropathic pain: mechanisms and their clinical implications. BMJ. 2014; 348:1–12.
2. Julius D, Basbaum AI. Molecular mechanisms of nociception. Nature. 2001; 413:203–210. [PubMed: 11557989]
3. Coderre TJ, Katz J, Vaccarino AL, Melzack R. Contribution of central neuroplasticity to pathological pain: review of clinical and experimental evidence. Pain. 1993; 52:259–285. [PubMed: 7681556]
4. Yaksh TL, Chaplan SR. Physiology and pharmacology of neuropathic pain. Anesthesiol Clin N Am. 1997; 15:335–353.
5. Yaksh TL. Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: effects of modulatory receptor systems and excitatory amino acid antagonists. Pain. 1989; 37:111–123. [PubMed: 2542867]
6. Sivilotti L, Woolf CJ. The contribution of GABAA and glycine receptors to central sensitization: disinhibition and touch-evoked allodynia in the spinal cord. J Neurophysiol. 1994; 72:169–179. [PubMed: 7965003]
7. Kaneko M, Hammond DL. Role of spinal gamma-aminobutyric acidA receptors in formalin-induced nociception in the rat. J Pharmacol Exp Ther. 1997; 282:928–938. [PubMed: 9262360]
8. Naik AK, Pathirathna S, Jevtovic-Todorovic V. GABA(A) receptor modulation in dorsal root ganglia in vivo affects chronic pain after nerve injury. Neuroscience. 2008; 154:1539–1553. [PubMed: 18554816]
9. Dib-Hajj SD, Black JA, Waxman SG. Voltage-gated sodium channels: therapeutic targets for pain. Pain Med. 2009; 10:1260–1269. [PubMed: 19818036]
10. Jacus MO, Uebele VN, Renger JJ, Todorovic SM. Presynaptic Cav3.2 channels regulate excitatory neurotransmission in nociceptive dorsal horn neurons. J Neurosci. 2012; 32:9374–9382. [PubMed: 22764245]
11. Obata K, Yamanaka H, Fukuoka T, Yi D, Tokunaga A, Hashimoto N, Yoshikawa H, Noguchi K. Contribution of injured and uninjured dorsal root ganglion neurons to pain behavior and the changes in gene expression following chronic constriction injury of the sciatic nerve in rats. Pain. 2003; 101:65–77. [PubMed: 12507701]
12. Liu W, Liu Z, Liu L, Xiao Z, Cao X, Cao Z, Xue L, Miao L, He X, Li W. A novel human foamy virus mediated gene transfer of GAD67 reduces neuropathic pain following spinal cord injury. Neurosci Lett. 2008; 432:13–18. [PubMed: 18180106]
13. Aptel H, Hilaire C, Pieraut S, Boukhaddaoui H, Mallié S, Valmier J, Scamps F. The Cav3.2/alpha1H T-type Ca2+ current is a molecular determinant of excitatory effects of GABA in adult sensory neurons. Mol Cell Neurosci. 2007; 36:293–303. [PubMed: 17716912]
14. Ma W, Saunders PA, Somogyi R, Poulter MO, Barker JL. Ontogeny of GABAA receptor subunit mRNAs in rat spinal cord and dorsal root ganglia. J Comp Neurol. 1993; 38:337–359. [PubMed: 7509352]
15. Unruh AM. Gender variations in clinical pain experience. Pain. 1996; 65:123–167. [PubMed: 8826503]
16. Berkley KJ. Sex differences in pain. Behav Brain Sci. 1997; 20:371–380. [PubMed: 10097000]
17. Mogil JS, Chanda ML. The case for the inclusion of female subjects in basic science studies of pain. Pain. 2005; 117:1–5. [PubMed: 16098670]

18. Naik AK, Latham JR, Obradovic A, Jevtovic-Todorovic V. Dorsal root ganglion application of muscimol prevents hyperalgesia and stimulates myelin protein expression after sciatic nerve injury in rats. Anesth Analg. 2012; 114:674–682. [PubMed: 22190549]
19. Bridge PM, Ball DJ, Mackinnon SE, Nakao Y, Brandt K, Hunter DA, Hertl C. Nerve crush injuries--a model for axonotmesis. Exp Neurol. 1994; 127:284–290. [PubMed: 8033968]
20. Zhang JM, Homma Y, Ackerman WE, Brull SJ. Topical application of acidic bupivacaine to the lumbar ganglion induces mechanical hyperalgesia in the rat. Anesth Analg. 2001; 93:466–471. [PubMed: 11473881]
21. Brunner R, Zimmermann P, Klussmann FW. Localization neurophysiological properties of motoneurones of the m triceps surae of the rat after retrograde labelling with Evans Blue. Cell Tissue Res. 1980; 212:73–81. [PubMed: 7438197]
22. Swett JE, Wikholm RP, Blanks RH, Swett AL, Conley LC. Motoneurons of the rat sciatic nerve. Exp Neurol. 1986; 93:227–252. [PubMed: 3732460]
23. Asato F, Butler M, Blomberg H, Gordh T. Variation in rat sciatic nerve anatomy: implications for a rat model of neuropathic pain. J Peripher Nerv Syst. 2000; 5:19–21. [PubMed: 10780679]
24. Swett JE, Torigoe Y, Elie VR, Bourassa CM, Miller PG. Sensory neurons of the rat sciatic nerve. Exp Neurol. 1991; 114:82–103. [PubMed: 1915738]
25. Rigaud M, Gemes G, Barabas ME, Chernoff DI, Abram SE, Stucky CL, Hogan QH. Species and strain differences in rodent sciatic nerve anatomy: implications for studies of neuropathic pain. Pain. 2008; 136:188–201. [PubMed: 18316160]
26. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods. 1994; 53:55–63. [PubMed: 7990513]
27. Jagodic MM, Pathirathna S, Joksovic PM, Lee W, Nelson MT, Naik AK, Su P, Jevtovic-Todorovic V, Todorovic SM. Upregulation of the T-type calcium current in small rat sensory neurons after chronic constrictive injury of the sciatic nerve. J Neurophysiol. 2008; 99:3151–3156. [PubMed: 18417624]
28. Liu J, Wolfe D, Hao S, Huang S, Glorioso JC, Mata M, Fink DJ. Peripherally delivered glutamic acid decarboxylase gene therapy for spinal cord injury pain. Mol Ther. 2004; 10:57–66. [PubMed: 15233942]
29. Fabbro A, Nistri A. Chronic NGF treatment of rat nociceptive DRG neurons in culture facilitates desensitization and deactivation of GABAA receptor-mediated currents. Br J Pharmacol. 2004; 142:425–434. [PubMed: 15148248]
30. Peris J, Jung BJ, Resnick A, Walker P, Malakhova O, Bokrand Y, Wielbo D. Antisense inhibition of striatal GABAA receptor proteins decreases GABA-stimulated chloride uptake and increases cocaine sensitivity in rats. Brain Res Mol Brain Res. 1998; 57:310–320. [PubMed: 9675429]
31. Lyons HR, Gibbs TT, Farb DH. Turnover and down-regulation of GABA(A) receptor alpha1, beta2S, and gamma1 subunit mRNAs by neurons in culture. J Neurochem. 2000; 74:1041–1048. [PubMed: 10693935]
32. Scholz J, Broom DC, Youn DH, Mills CD, Kohno T, Suter MR, Moore KA, Decosterd I, Coggeshall RE, Woolf CJ. Blocking caspase activity prevents transsynaptic neuronal apoptosis and the loss of inhibition in lamina II of the dorsal horn after peripheral nerve injury. J Neurosci. 2005; 25:7317–7323. [PubMed: 16093381]
33. Schon F, Kelly JS. Selective uptake of (3H)beta-alanine by glia: association with glial uptake system for GABA. Brain Res. 1975; 86:243–257. [PubMed: 234775]
34. Gallagher JP, Nakamura J, Shinnick-Gallagher P. Effects of glial uptake and desensitization on the activity of gamma-aminobutyric acid (GABA) and its analogs at the cat dorsal root ganglion. J Pharmacol Exp Ther. 1983; 226:876–884. [PubMed: 6310083]
35. Smith CG, Bowery NG, Whitehead KJ. GABA transporter type 1 (GAT-1) uptake inhibition reduces stimulated aspartate and glutamate release in the dorsal spinal cord in vivo via different GABAergic mechanisms. Neuropharmacology. 2007; 53:975–981. [PubMed: 17981306]
36. Mertens P, Ghaemmaghami C, Bert L, Perret-Liaudet A, Sindou M, Renaud B. Amino acids in spinal dorsal horn of patients during surgery for neuropathic pain or spasticity. Neuroreport. 2000; 11:1795–1798. [PubMed: 10852247]

37. Miletic G, Draganic P, Pankratz MT, Miletic V. Muscimol prevents long-lasting potentiation of dorsal horn field potentials in rats with chronic constriction injury exhibiting decreased levels of the GABA transporter GAT-1. Pain. 2003; 105:347–353. [PubMed: 14499453]

38. Castro-Lopes JM, Tavares I, Coimbra A. GABA decreases in the spinal cord dorsal horn after peripheral neurectomy. Brain Res. 1993; 620:287–291. [PubMed: 8369960]

39. Labrakakis C, Tong CK, Weissman T, Torsney C, MacDermott AB. Localization and function of ATP and GABAA receptors expressed by nociceptors and other postnatal sensory neurons in rat. J Physiol. 2003; 549:131–142. [PubMed: 12665615]

40. Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ. Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. J Neurosci. 2002; 22 6724-3671.

41. Muratori L, Ronchi G, Raimondo S, Geuna S, Giacobini-Robecchi MG, Fornaro M. Generation of new neurons in dorsal root Ganglia in adult rats after peripheral nerve crush injury. Neural Plast. 2015; 2015:860546. [PubMed: 25722894]

42. Swett JE, Hong CZ, Miller PG. Most dorsal root ganglion neurons of the adult rat survive nerve crush injury. Somatosens Mot Res. 1995; 12:177–189. [PubMed: 8834296]

43. Sukhotinsky I, Ben-Dor E, Raber P, Devor M. Key role of the dorsal root ganglion in neuropathic tactile hypersensibility. Eur. J Pain. 2004; 8:135–143. [PubMed: 14987623]

44. Knabl J, Witschi R, Hösl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy JM, Rudolph U, Möhler H, Zeilhofer HU. Reversal of pathological pain through specific spinal GABAA receptor subtypes. Nature. 2008; 451:330–334. [PubMed: 18202657]

45. Harris RA, Osterndorff-Kahanek E, Ponomarev I, Homanics GE, Blednov YA. Testing the silence of mutations: Transcriptomic and behavioral studies of GABA(A) receptor α1 and α2 subunit knock-in mice. Neurosci Lett. 2011; 488:31–35. [PubMed: 21056629]

46. Dublin P, Hanani M. Satellite glial cells in sensory ganglia: their possible contribution to inflammatory pain. Brain Behav Immun. 2007; 21:592–598. [PubMed: 17222529]

47. Oyelese AA, Kocsis JD. GABAA-receptor-mediated conductance action potential waveform in cutaneous, muscle afferent neurons of the adult rat: differential expression response to nerve injury. J. Neurophysiol. 1996; 76:2383–2392. [PubMed: 8899611]

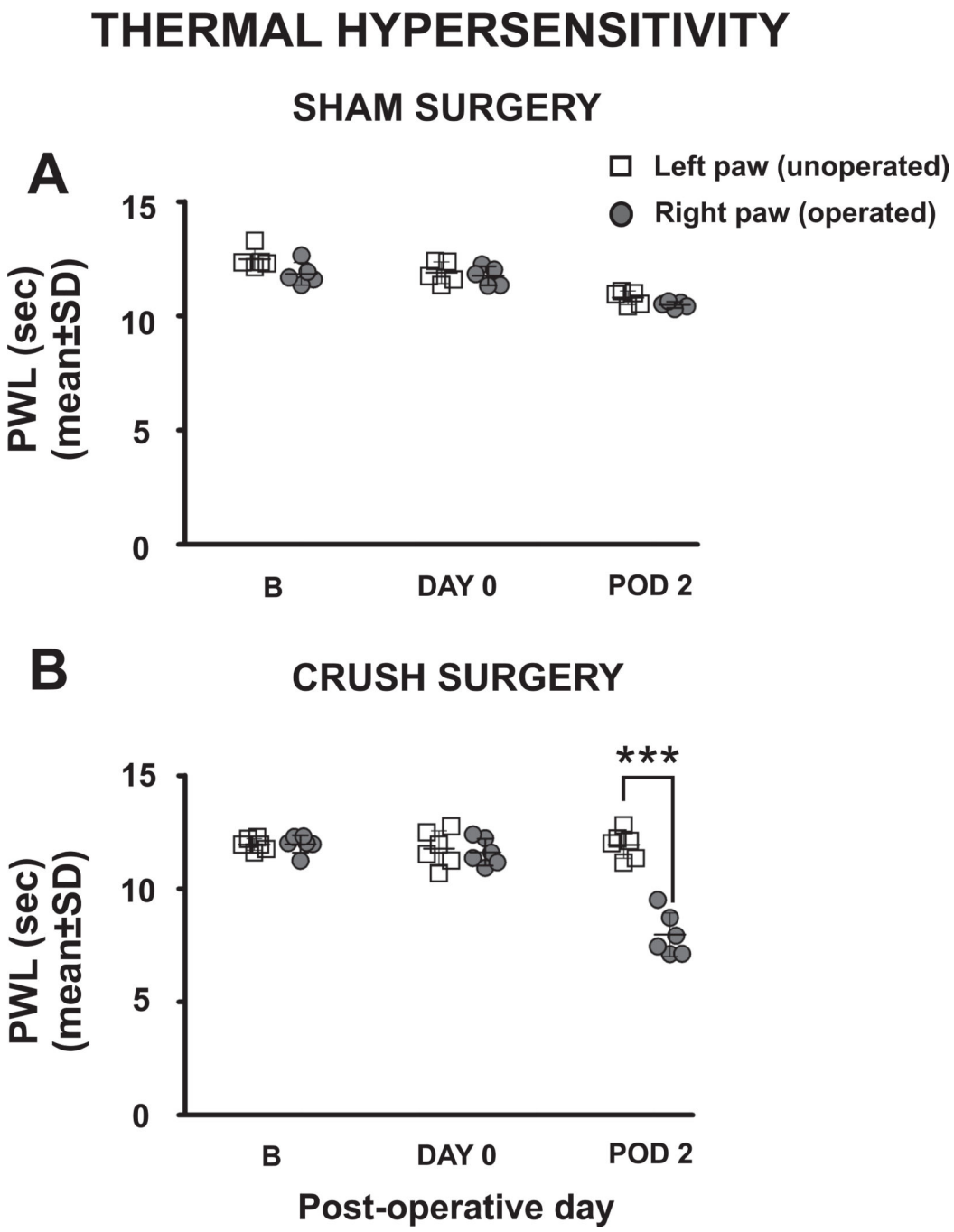

Figure 1.

Crush injury to the sciatic nerve induces thermal sensitivity in the ipsilateral but not contralateral paw in adult female rats. When paw withdrawal latencies (PWLs) were measured in sham injured animals (A) there was no change in the ipsilateral (right) paw compared with the contralateral (left) paw at any time point [at baseline (B), DAY 0 (before surgery) and post-operative day (POD) 2] (n=5 rats per data point). However, when PWLs were measured in crush-injured animals, (B) there was a significant decrease in PWLs in the ipsilateral paw when compared to the contralateral paw (***, $p<0.001$) on POD 2 (Fig. 1B)

validating quick development of thermal hypersensitivity. Note the stable PWLs recordings at the baseline and immediately before crush injury (DAY 0) in both paws of sham and crush-injured rats (n=6 rats per data point).

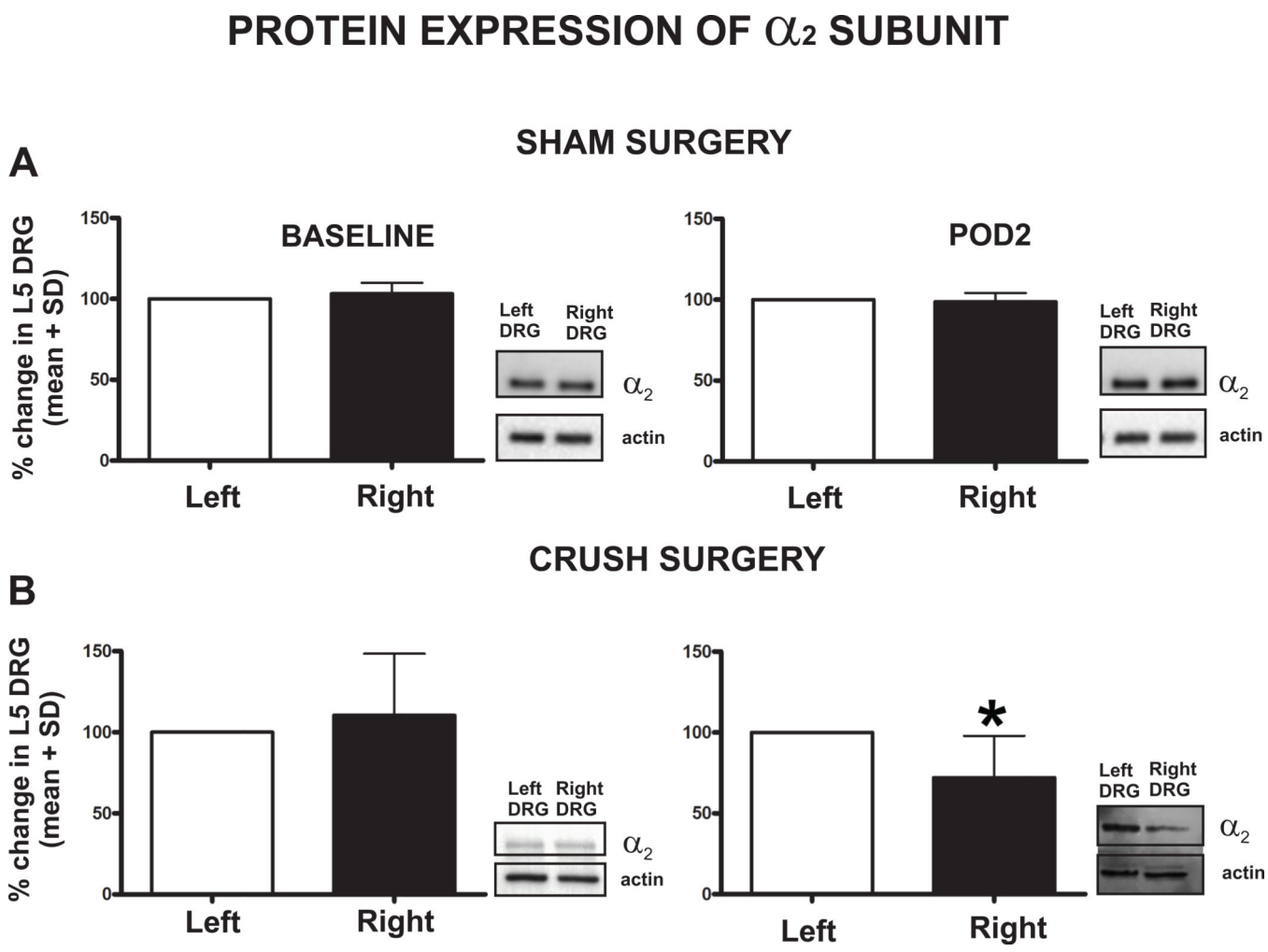

Figure 2.

Crush injury to the sciatic nerve down-regulates expression of the α_2 subunit of $GABA_A$ receptors in the ipsilateral (Right) but not the contralateral (Left) L5 dorsal root ganglion (DRG) neurons in adult female rats. In sham animals, the protein levels of α_2 subunit of $GABA_A$ receptor in the L5 DRG on the ipsilateral side were indistinguishable from those on the contralateral side both at the baseline and on post-operative day (POD) 2 (n=6 rats per data point) (A). However, in crush-injured animals (B), although the protein levels of α_2 subunit of $GABA_A$ receptor in the L5 DRG on the ipsilateral side were indistinguishable from those on the contralateral side at the baseline (n=14 rats per data point), on POD 2 there was a significant (about 30%) decrease in α_2 subunit expression in the ipsilateral L5 DRGs (n=8 per data point) (*, $p<0.05$). The protein expression in the ipsilateral DRG in each animal is presented as a percent change as compared with its corresponding contralateral DRG that was set at 100%.

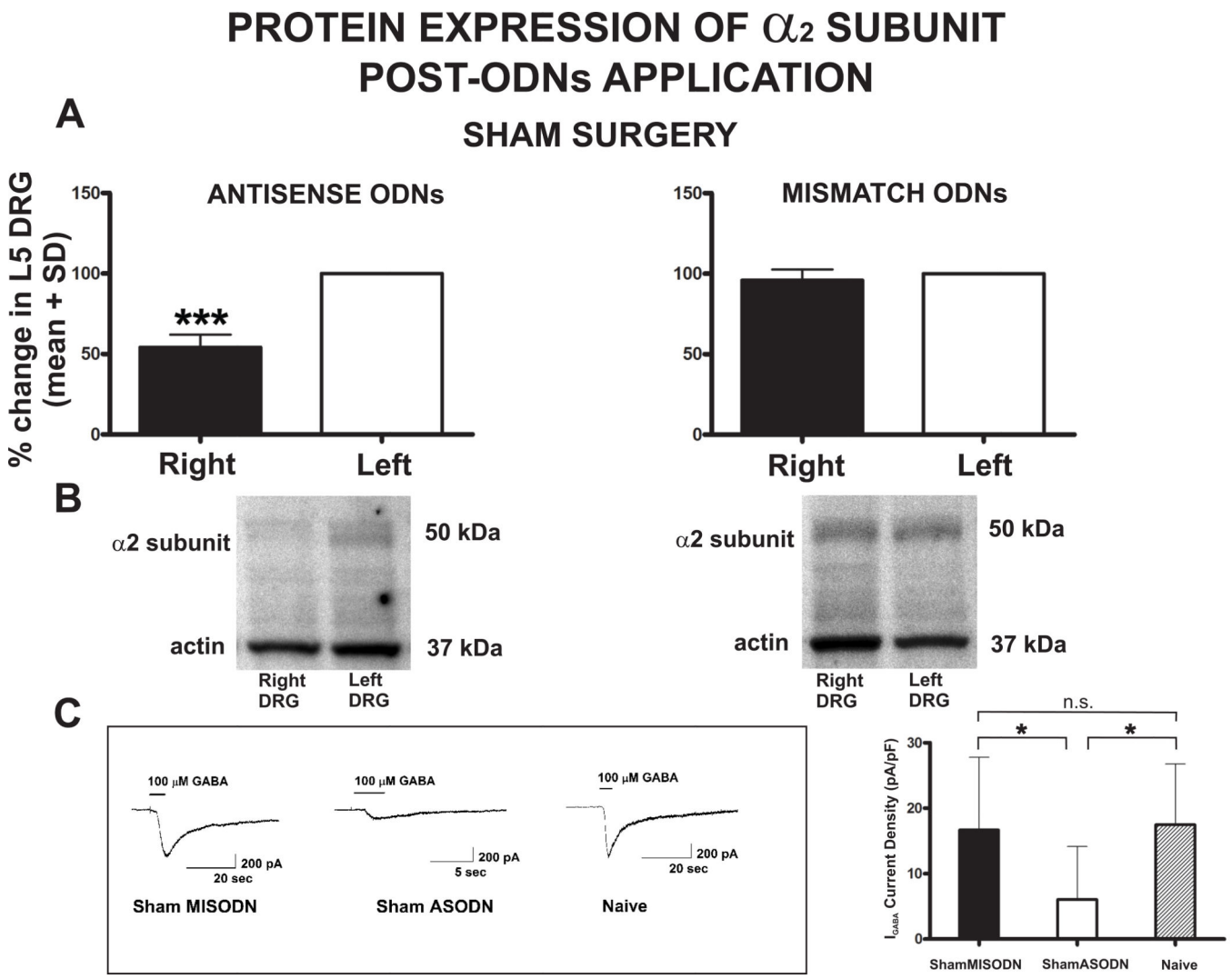

Figure 3.

Direct *in vivo* L5 dorsal root ganglion (DRG) application of antisense oligodeoxynucleotides (ASODNs) but not mismatch oligodeoxynucleotides (MISODNs) causes significant down-regulation of α_2 subunit protein expression in the treated DRG but not in the contralateral L5 DRG in sham animals. Targeted knock-down of α_2 subunit in the right L5 DRG post-ASODN treatment resulted in about 50% decrease in its protein expression as compared with untreated (left) L5 DRG (A, left panel) (***, $p<0.001$) (n=6 rats per data point). As expected, MISODN treatment had no effect on the α_2 subunit protein expression (A, right panel) (n=6 rats per data point). The protein determination was performed 24–48 hrs post-ODNs application (i.e. on post-operating day 3 to 4). (B) Topographically selective α_2 subunit protein down-regulation is shown in the representative gels. (C) Representative current traces (Fig. 3C, left panel) and summary of data from patch-clamp experiments (Fig. 3C, right panel) indicate that as compared with no treatment (n=7 rats per data point from 17 cells) and MISODN treatment (n=5 rats per data point from 22 cells), ASODN administration resulted in about a 3-fold decrease in GABA-gated current density in small DRG cells (*, $p<0.05$) (n=7 rats per data point from 32 cells).

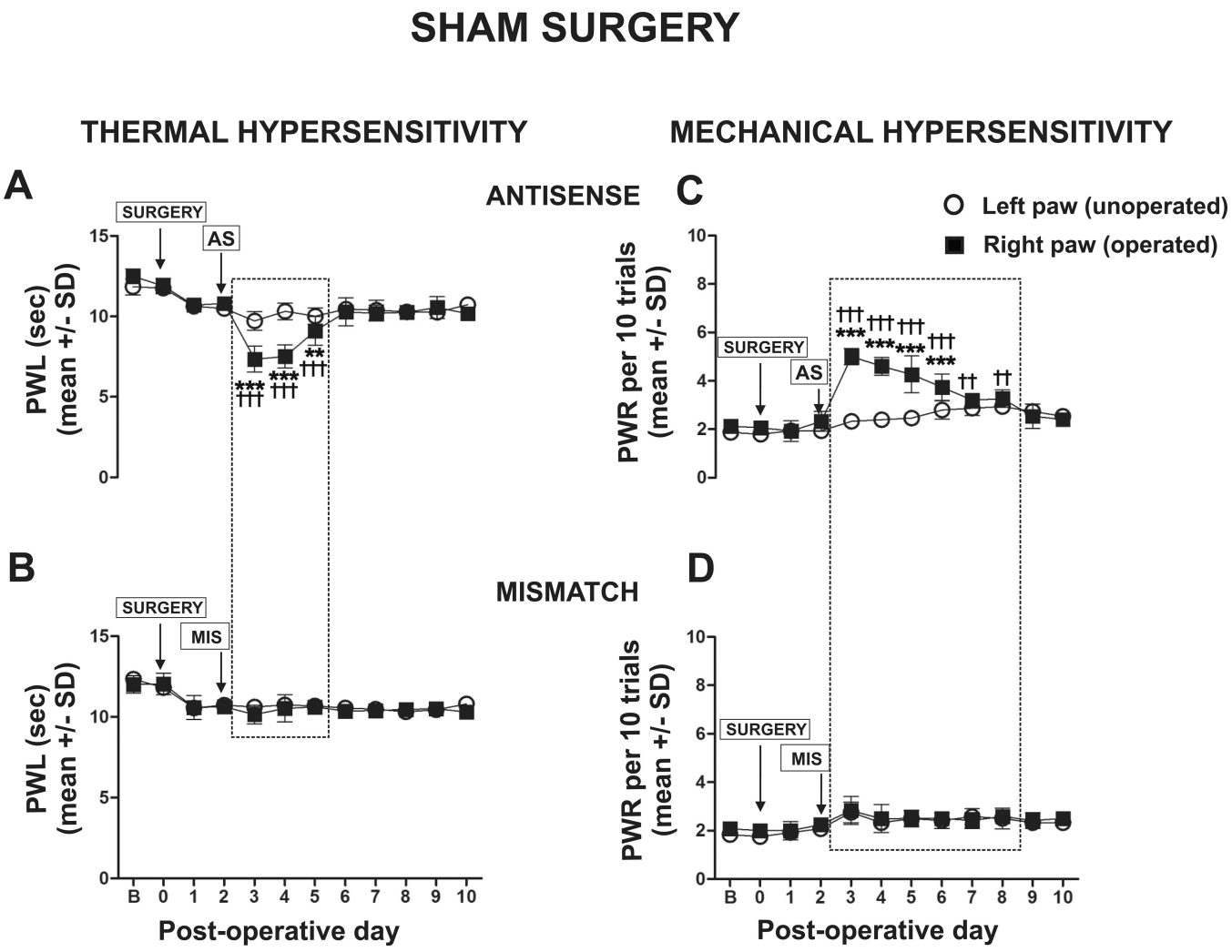

Figure 4.

Selective knock-down of the α_2 subunit in the ipsilateral L5 dorsal root ganglion (DRG) neurons of sham animals mimics the neuropathic pain phenotype caused by crush injury of a sciatic nerve in adult female rats. (A) Thermal withdrawal latencies (PWLs) were measured in rats that had sham surgery (at day 0, marked with an arrow as SURGERY) followed by antisense oligodeoxynucleotides ASODN treatment on post-operative day (POD) 2 (marked with an arrow as AS) after the lack of thermal hypersensitivity was confirmed. The knock-down of the α_2 subunit with ASODNs resulted in a significant decrease in PWLs on POD 3 through 5 (outlined with dashed rectangle) (n=5 per data point) [†††, $p<0.001$, before and after ASODN treatment); ***, $p<0.001$; **, $p<0.01$, PWLs in operated (Right paw) paw vs. unoperated (Left paw) paw at PODs 3, 4 and 5]. (B) Thermal PWLs were measured in rats that had sham surgery followed by mismatch oligodeoxynucleotides MISODN treatment on POD 2 (marked with an arrow as MIS) after the lack of thermal hypersensitivity was confirmed. There was no change in PWLs in MISODNs animals at any time point (n=4 per rats data point). (C) Mechanical hypersensitivity in sham-operated animals post-ASODN treatment measured as a significant increase in paw withdrawal responses (PWRs) was noted on POD 3 through 8 (outlined with dashed rectangle) [(††, $p<0.01$, †††, $p<0.001$,

before and after ASODNs treatment) (***, $p<0.001$, PWRs in operated (Right paw) paw vs. unoperated (Left paw) paw)] (n=5 rats per data point). (D) In sham-operated animals post-MISODNs treatment there was no change in mechanical sensitivity shown as stable PWRs recordings throughout the testing period (n=4 rats per data point).

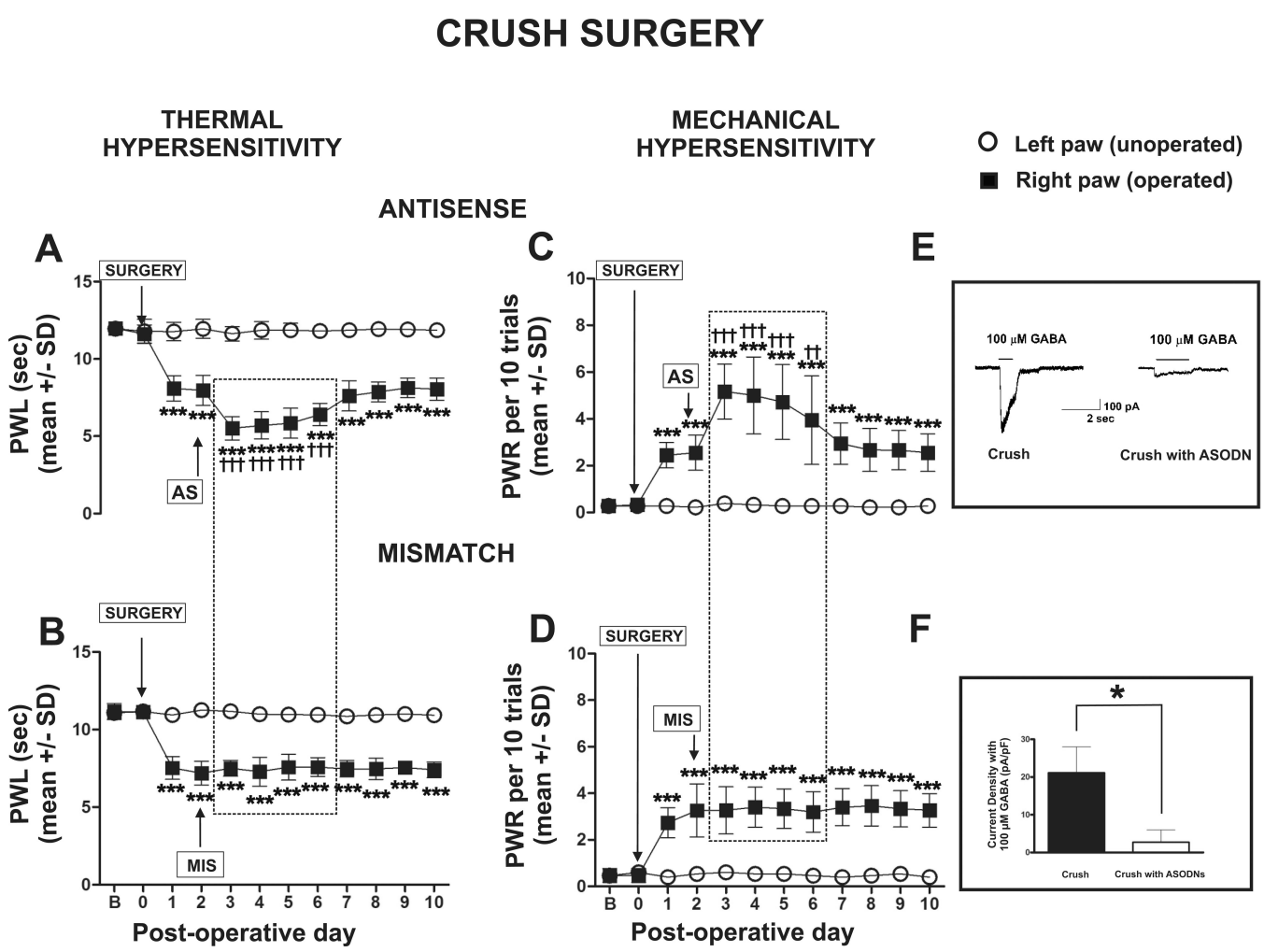

Figure 5.

Selective knock down of the α_2 subunit in the ipsilateral L5 dorsal root ganglion (DRG) neurons worsens neuropathic pain phenotype caused by crush injury of a sciatic nerve in adult female rats.

(A) Paw withdrawal latencies (PWLs) were measured in rats after sciatic crush injury (at day 0, marked with an arrow as SURGERY) followed by antisense oligodeoxynucleotides ASODN treatment on post-operative day (POD) 2 (marked with an arrow as AS) after thermal hypersensitivity was confirmed [***, $p<0.001$; PWLs in operated (Right paw) vs. unoperated (Left paw) on POD2] (n=6 animals). The knock-down of the α_2 subunit with ASODNs resulted in significant further decrease in PWLs on PODs 3 to 6 (outlined with dashed rectangle) (†††, $p<0.001$; before and after ASODNs treatment) [***, $p<0.001$; PWLs in operated (Right paw) vs. unoperated (Left paw) throughout the entire post-operative period]. (B) Thermal PWLs were measured in rats after sciatic crush injury followed by mismatch oligodeoxynucleotides MISODN treatment on POD 2 (marked with an arrow as MIS) after thermal hypersensitivity was confirmed. There was no further change in PWL caused by MISODN treatment at any time point [***, $p<0.001$; PWLs in operated (Right paw) vs. unoperated (Left paw) throughout the entire post-operative period] (n=5 animals per data point). (C) Mechanical hypersensitivity in crush-injured animals, demonstrated as a

significant increase in paw withdrawal responses (PWRs), was confirmed on POD 2 before ASODN treatment was applied [***, $p<0.001$; PWLs in operated (Right paw) vs. unoperated (Left paw)]. A further increase in PWRs was noted on POD 3 through POD 5 (outlined with dashed rectangle) [(††, $p<0.01$ and †††, $p<0.001$; before and after ASODNs treatment, n=6 per time point). (D) In crush-injured animals post-MISODN treatment there was no further change in mechanical sensitivity as shown by stable PWR recordings throughout the testing period (n=5 rats per data point) [(***, $p<0.001$, PWRs in operated (Right paw) vs. unoperated (Left paw)]. (E) Inward GABA-gated current traces from representative DRG cells in the crush-injured control group (left trace) and the crush-injured group treated with direct DRG application of ASODN (right trace). Horizontal bars indicate duration of GABA application. (F) Bar graphs of averages from multiple experiments similar to the one depicted in panel E of this figure indicate that GABA-gated current densities in the ASODN-treated group (open bar, n=8, 13 cells) are about 7-fold decreased (*, $p<0.05$) as compared with the control group (filled bar, n=3 rats, 7 cells). Bars represent average values of GABA-gated current densities per rat and lines are SD.

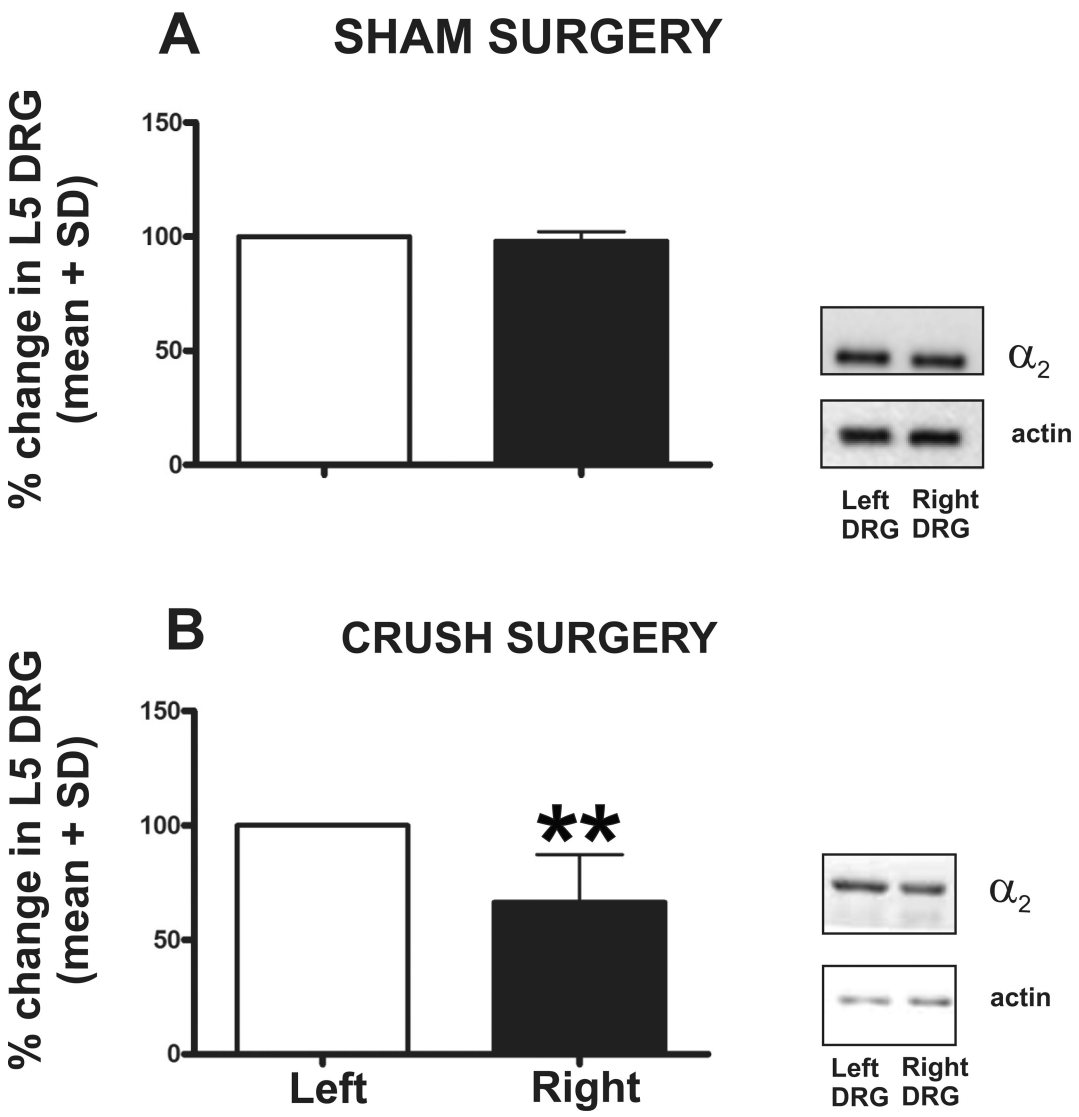

Figure 6.

The crush injury-induced knock-down of the α_2 subunit protein expression in the ipsilateral L5 dorsal root ganglion DRG is long-lasting. (A) In sham animals, there is no difference in α_2 subunit expression in the ipsilateral L5 DRG as compared with the contralateral L5 DRG at post-operative day (POD) 10 (n=6 rats per data point). (B) In crush-injured animals there is a significant downregulation of the α_2 subunit expression in the ipsilateral L5 DRG compared with the contralateral one at POD 10 (about 30%; **, $p<0.01$; n=6 rats per data point).

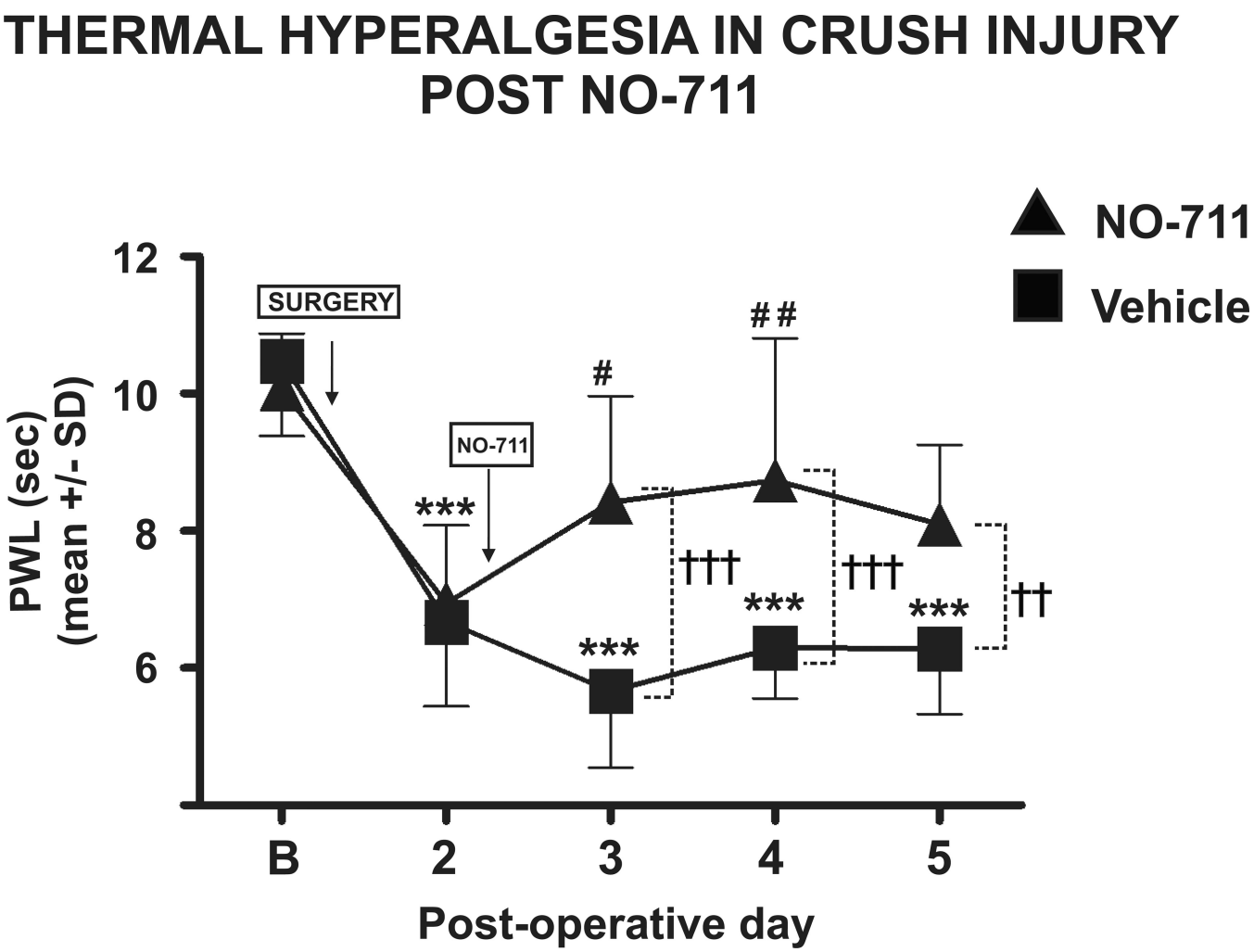

Figure 7.

Blockade of GABA uptake in dorsal root ganglion (DRG) alleviates thermal hyperalgesia post-crush sciatic nerve injury. Thermal paw withdrawal latencies (PWLs) were measured in rats after sciatic crush injury (marked with an arrow as SURGERY) and thermal hyperalgesia was confirmed with a significant decrease in PWLs on post-operative day (POD) 2 [***, $p<0.001$ baseline (B) vs. POD 2]. Post- NO-711 treatment (marked with an arrow as NO-711), there was significant alleviation of thermal hyperalgesia, i.e. the PWLs in NO-711 treated animals (closed triangles) were significantly increased when compared to a vehicle treatment (closed squares) (††, $p<0.01$; †††, $p<0.001$; n=9 and 10 rats per data point in NO-711 and vehicle groups, respectively). The increase in PWLs also resulted in significant alleviation of thermal hyperalgesia on PODs 3 (#, $p<0.05$) and 4 (##, $p<0.01$) when compared to POD 2 immediately before the treatment. The PWLs in vehicle-treated group remained significantly decreased throughout the testing period when compared to the baseline PWL recordings (***, $p< 0.001$; baseline (B) vs. any given POD).

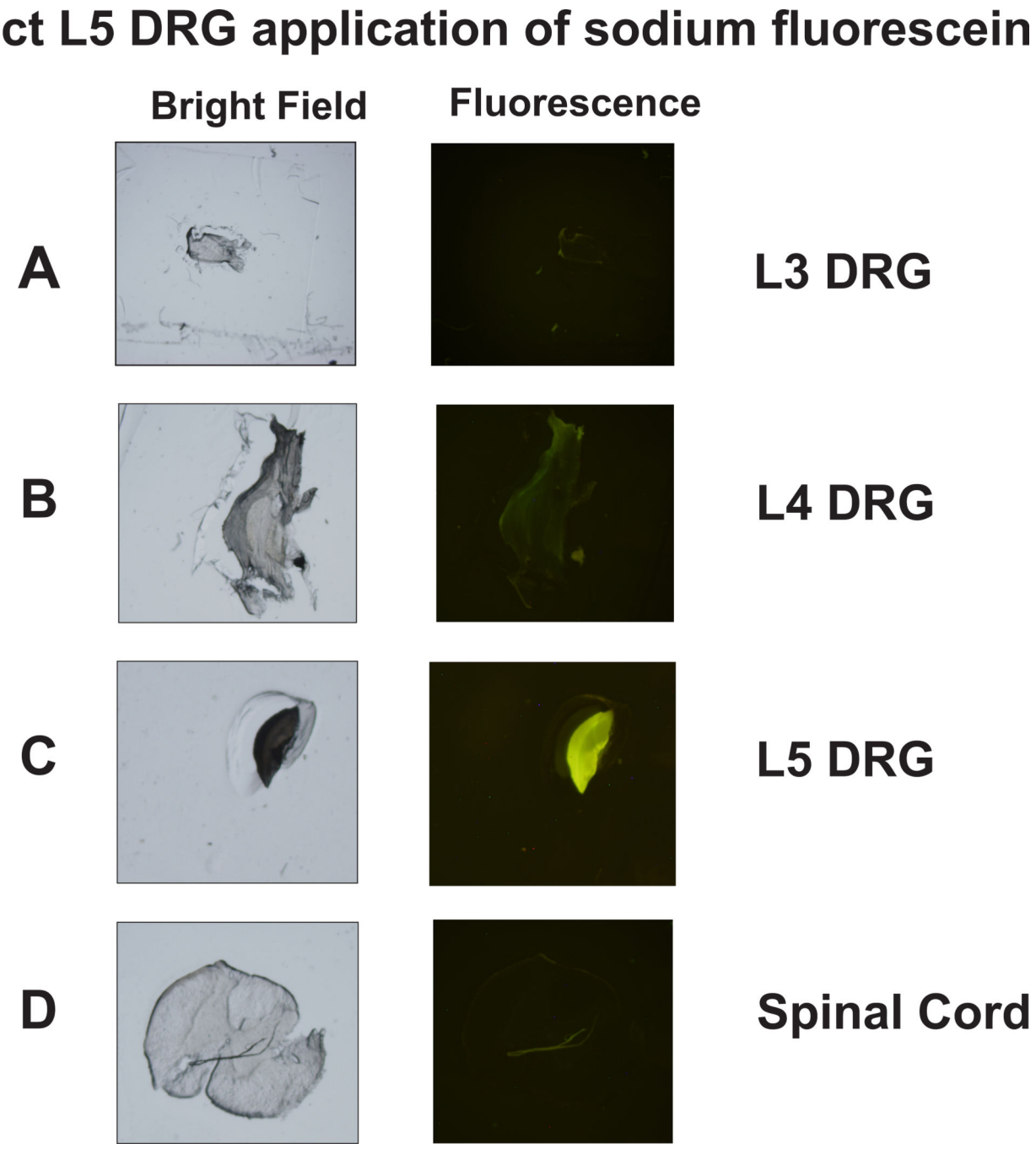

Figure 8.

Direct L5 dorsal root ganglion DRG application of sodium fluorescein results in very limited spread to the ipsilateral DRGs. When sodium fluorescein was applied onto the L5 DRG at 25–30 μl and the intensity of fluorescein epifluorescence was assessed 30 minutes later, we noted a lack of labelling in the L3 DRG (A). While there was some epifluorescence detected in the ipsilateral L4 DRG (B), substantially higher level of labelling was noted in the L5 DRG (C). Importantly, there was no spillage into the corresponding segment of the spinal

cord shown as the complete absence of epifluorescence (image magnification of all tissue slices was 5×).