Silencing the $\alpha_2$ Subunit of $\gamma$-Aminobutyric Acid Type A Receptors in Rat Dorsal Root Ganglia Reveals Its Major Role in Antinociception Posttraumatic Nerve Injury

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ABSTRACT

Background: Neuropathic pain (NPP) 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. Drugs that promote $\gamma$-aminobutyric acid (GABA) activity in the dorsal horn provide partial relief of neuropathic symptoms. The authors examined how in vivo silencing of the GABA receptor type A ($\alpha_2$ subunit) gene in dorsal root ganglia (DRG) controls NPP.

Methods: After crush injury to the right sciatic nerve of female rats, the $\alpha_2$ GABAA 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 before the injury and ensuing 10 days ($n = 4$ to 10). In vitro quantification of $\alpha_2$ GABAA protein and electrophysiological studies of GABA currents were performed on acutely dissociated L5 DRG neurons at relevant time points ($n = 6$ to 14).

Results: NPP postcrush injury of a sciatic nerve in adult female rats coincides with significant down-regulation of the $\alpha_2$ subunit expression in the ipsilateral DRG (approximately 30%). Selective down-regulation of $\alpha_2$ expression in DRGs significantly worsens mechanical ($2.55 \pm 0.75$ to $5.16 \pm 1.16$) and thermal ($7.97 \pm 0.96$ to $5.51 \pm 0.75$) hypersensitivity in crush-injured animals and causes development of significant mechanical ($2.33 \pm 0.40$ to $5.00 \pm 0.33$) and thermal ($10.80 \pm 0.29$ to $7.34 \pm 0.81$) hypersensitivity in sham animals (data shown as mean ± SD). Conversely, up-regulation of endogenous GABA via blockade of its uptake in DRG alleviates NPP.

Conclusion: The GABAA receptor in the DRG plays an important role in pathophysiology of NPP caused by sciatic nerve injury and represents promising target for novel pain therapies. (ANESTHESIOLOGY 2015; 123:654-67)

The he pathophysiology and treatment of traumatic peripheral nerve injury and ensuing neuropathic pain (NPP) remain a complex medical enigma. Conventional pain management neither does abolish the development of NPP nor does 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. 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). Consequently, promotion of $\gamma$-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. However, beneficial effects of intrathecally administered GABAergic agents...
are transient, suggesting that the DH may be an insufficient therapeutic target for modulating maladaptive, NPP-related circuitry. Although GABAergic modulation in the DH is pathologically important, 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 indicates that remodeling of different ion channels in some of the DRG neurons can cause changes in excitability and alter normal sensory transmission in the DH. Of special interest are the findings that the neuropathy caused by peripheral nerve injury is accompanied by a decreased expression of the GABA receptor type A (GABA$_A$) receptor in sensory neurons. Although the role of ligand-gated GABA$_A$ channels in synaptic transmission of DH neurons in NPP is reasonably well established, the role of GABA$_A$-related neuroglia 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. 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. 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$_A$ subunit because this subunit is abundantly present in DRG neurons. We performed behavioral assessments of NPP phenotype in conjunction with targeted GABA$_A$ $\alpha_2$ 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$_A$ $\alpha_2$ subunit in the corresponding DRG and that this down-regulation 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 250g). 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; (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; 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.

The $\alpha_2$ GABA$_A$ antisense (AS) oligonucleotides and the mismatch control (MIS) oligodeoxynucleotides (ODNs) were purchased from Eurofins MWG Operon (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-$\alpha_2$ GABA$_A$ oligonucleotide (ASODNs) was 5’-TCCATCCCAAGCCCATCC-3’. The sequence of the MIS-$\alpha_2$ GABA$_A$ oligonucleotide (MISODNs) was 5’-CTACCGCACTCTCAACC-3’. The GABA uptake inhibitor NO-711 hydrochloride (Sigma-Aldrich, USA) was dissolved in appropriate sterile pH 7.4 buffer solution.

**In Vivo Studies**

**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 the animals’ suffering, and the number of animals used while still permitting statistically valid conclusions to be drawn based on our previous experience. Within each experiment, animals were randomly assigned to either the experimental or control conditions.

To induce NPP, 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 buttck 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. 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.

**Direct Dorsal Root Ganglion Application.** We have established previously that the success rate in achieving hyperalgesia in the crush injury model is between 90 and 95% and that the animals reach stable hyperalgesic behavior at 48 h after injury (by postoperative day [POD] 2). Therefore, we initiated direct DRG application of either ASODNs or MISODNs on POD 2. 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 to 99% of all sciatic DRG neurons reside in the L4 to L5 DRGs (with the L3 and L6 DRGs contributing roughly 1.2 and 0.4%, respectively), thus making L5 DRG a very important therapeutic target. To assess the local spread of the injectate, we applied sodium fluorescein (500 μg/100 μl) directly onto L5 DRG (25 to 30 μl) and after 30 min assessed the presence of the fluorescence (magnification ×5, Olympus SZX12 microscope, 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 to L6 spinal level, right paraspinal 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 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 ASODNs, 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.

**Behavioral Assessment of Heat Nociception.** The nociceptive response to heat stimulation was measured using a custom-built Hargreaves paw thermal stimulation system (University of California, San Diego, University Anesthesia Research and Development Group, USA). In brief, 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. Before testing, each animal was placed in the testing room for approximately 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 10 days (POD 1 to POD 10).

**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 in Behavioral Assessment of Heat Nociception section). 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, USA) were used to assess the mechanical threshold for paw withdrawal. 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 one to two paw withdrawal responses (PWRs) in 10 trials. Baseline withdrawal scores were determined in both paws as well as NPP scores at POD 2 before injections of ASODNs or MISODNs.

**In Vitro Studies**

**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 three animals (depending on the amount of tissue). When the sample was pooled from three 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, Germany), sonicated, and centrifuged at 4°C 12,000 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), boiled for 5 min, loaded into a 10% polyacrylamide gel, and electrophoresed. Separated proteins were transferred to nitrocellulose membranes and blocked with 3% bovine serum albumin at room temperature for 1 h. The membrane was incubated at 4°C overnight with primary antibodies for the GABA<sub>A</sub> α<sub>2</sub> receptor subunit (Alomone Labs, Israel) or the house-keeping protein actin (Sigma-Aldrich) at respective dilutions of 1:5,000 and 1:10,000. Appropriate horseradish peroxidase–conjugated secondary antibodies anti-rabbit immunoglobulin G (1:15,000; Santa Cruz Biotechnology, USA) were applied for 1 h. Then, the membranes were developed using Super Signal ECL detection reagents (Thermo Scientific, USA), and band density was quantified using Syngene Gel documentation, G-box analysis software (Syngene, United Kingdom). Densities for...
GABA<sub>2</sub> receptor bands were normalized to actin used as a house-keeping protein.

**Electrophysiological Studies of GABA<sub>2</sub> Currents.** To study GABA<sub>2</sub> currents, we used acutely dissociated L5 DRG neurons from adult female Sprague–Dawley rats and by using standard whole cell patch clamp techniques. 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 to 5 s to avoid desensitization of GABA-gated currents. The extracellular recording solution contained 140 mM NaCl, 4 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM HEPES, and 10 mM glucose, at pH 7.3. The recording pipette was filled with a solution containing 130 mM KCl, 1 mM EGTA, 5 mM MgCl<sub>2</sub>, and 40 mM HEPES, at 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 crush-injured animal, ASODN, or MISODN).

Paw withdrawal latencies and PWRs were subjected to repeated-measures ANOVA containing two within-subjects factors: time of the session (before vehicle/test compound administration or daily posttreatment 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 post hoc test.

To evaluate the statistical differences in Western blot studies, the density values were analyzed using two-tailed <i>t</i> 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 electrophysiological 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-tailed <i>t</i> test or Mann–Whitney rank sum test if the data distribution failed normality testing. Statistical analyses were performed using GraphPad Prism<sup>®</sup> (version 5.01, USA) and SigmaPlot Software (version 12.0, 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<sup>8</sup> 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 [<i>F</i>(2,10) = 84.366, <i>P</i> < 0.001] on 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 (<i>n</i> = 5 in sham and <i>n</i> = 6 in crush-injured rats per data point).

To begin to understand the role of GABAergic modulation in the development of NPP postcrush injury, we examined whether such injury modulates the expression of α<sub>2</sub> subunits in L5 DRG neurons. Because adult rat DRG neurons preferentially express α<sub>1</sub>, β<sub>2</sub>, γ<sub>2</sub> subunits<sup>14,28</sup> and α<sub>2</sub> is the important functional subunit, we focused on expression of the α<sub>2</sub> subunit. As shown in figure 2, expression of the GABA<sub>2</sub> receptor α<sub>2</sub> subunit protein in L5 DRG on the ipsilateral (right) side was indistinguishable from that on the contralateral (left) side in both sham-operated...
For the \( \alpha_2 \) subunit onto the right L5 DRG in sham animals on POD 2 once we confirmed that thermal hypersensitivity did not develop (fig. 1A). We noted that protein expression of the \( \alpha_2 \) subunit in the right L5 DRG post-ASODN treatment was down-regulated significantly compared with that in the left (untreated) L5 DRG (fig. 3A, left panel) \( [t(5) = 14.20, P < 0.001; n = 6 \text{ rats per data point}] \), whereas the \( \alpha_2 \) subunit protein expression post-MISODN treatment remained unchanged (fig. 3A, right panel) \( [t(5) = 1.509, P = 0.1917; n = 6 \text{ rats per data point}] \) when examined 24 to 48h post-ODNs application \( (i.e., \text{ on POD 3 to 4}) \). This down-regulation caused by direct L5 DRG application of ASODN was restricted to the ipsilateral (right) DRG as shown in figure 3B. The application of MISODN also resulted in no change in \( \alpha_2 \) subunit protein expression in ipsi-DRGs \( (i.e., \text{ on POD 3 to 4}) \). This down-regulation caused by direct L5 DRG application of ASODN was restricted to the ipsilateral (right) DRG as shown in figure 3B. The application of MISODN also resulted in no change in \( \alpha_2 \) subunit protein expression in ipsi-DRGs (fig. 3B, right panel). Taken together, this evidence suggests that direct DRG application induced \( \alpha_2 \) subunit modulation that was restricted to the ipsilateral DRG. To validate further the down-regulation of \( \alpha_2 \) subunits in DRG cells, we used a functional assay based on patch clamp recordings of
GABA_\text{A}^-\text{gated currents in small diameter (<30 \text{\mu m}) acutely dissociated DRG cells from sham-operated, ASODN-treated rats and compared the findings with those from untreated (naive) and MISODN-treated rats. All recordings were done using ipsilateral L5 DRGs. We recorded from smaller DRG cells because these are putative nociceptive neurons, and previous studies have established that they express prominent GABA_\text{A}^- receptor-mediated inward currents.\textsuperscript{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 three-fold decrease in GABA-gated current density in small DRG cells (P < 0.05) (n = 7 rats per data point from 32 cells). n.s. = nonsignificant.

![Fig. 3. Direct in vivo L5 dorsal root ganglion (DRG) application of antisense oligodeoxynucleotides (ASODNs) but not mismatch oligodeoxynucleotides (MISODNs) causes significant down-regulation of \( \alpha_2 \) subunit protein expression in the treated DRG but not in the contralateral L5 DRG in sham animals. Targeted knockdown of \( \alpha_2 \) subunit in the right L5 DRG post-ASODN treatment resulted in approximately 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 \( \alpha_2 \) subunit protein expression (A, right panel) (n = 6 rats per data point). The protein determination was performed 24 to 48 h postoligodeoxynucleotides (ODNs) application (i.e., on postoperating day 3 to 4). (B) Topographically selective \( \alpha_2 \) subunit protein down-regulation is shown in the representative gels. (C) Representative current traces (left panel) and summary of data from patch clamp experiments (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 three-fold decrease in \( \gamma \)-aminobutyric acid (GABA)-gated current density in small DRG cells (P < 0.05) (n = 7 rats per data point from 32 cells). n.s. = nonsignificant.

With the suggestion that the GABA_\text{A}^-\text{\( \alpha \text{\textsubscript{2}} \}} subunit may be an important target for NPP development, we examined whether ASODN treatment of sham animals would result in an NPP phenotype similar to that observed in crush-injured animals (fig. 1B) where comparable down-regulation of \( \alpha_2 \) 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 fig. 3). We found statistically significant interaction between time and paw due to knockdown of the α2 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).

**Fig. 4.** Selective knockdown of the α2 subunit in the ipsilateral L5 dorsal root ganglion 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 (AS) oligodeoxynucleotides (ASODN) treatment on postoperative day (POD) 2 (marked with an arrow as AS) after the lack of thermal hypersensitivity was confirmed. The knockdown of the α2 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 (MIS) 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 MISODN 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* < 0.001, ††*P* < 0.001, before and after ASODNs treatment, **P* < 0.01, 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).
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 α2 subunit down-regulation in ASODN-treated sham animals resulted in significant thermal and mechanical hyperalgesia, we reasoned that if maintaining GABAα2 subunit expression and function are important for NPP development postcrush injury, ASODN application in crush-injured animals may worsen thermal and mechanical hyperalgesia. To address this hypothesis, we administered the α2 subunit ASODNs or MISODNs onto the ipsilateral DRG of injured animals on POD 2 after the thermal and mechanical hypersensitivity was confirmed (fig. 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 groups (fig. 5B) [F(11,44) = 51.382, P < 0.001] immediately after crush injury of right sciatic nerve. However, pairwise comparisons using the Tukey post hoc 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 postoperative period). The “recovery” of PWLs to pre-ASODNs treatment levels (fig. 5A) is to be expected considering that the half-life of the GABAα2 channel is estimated to be approximately 20 h.30,31 When mechanical hypersensitivity was assessed, we found similar pattern. Namely, although crush injury produced significant increase in PWRs in both ASODNs [P < 0.001] 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. 5, A and B) (***P < 0.001; PWLs in operated [R] paw vs. unoperated [L] paw throughout the entire postoperative period).

To further validate the link between ASODN-induced worsening of NPP phenotype and down-regulation of α2 subunits in DRG caused by crush injury (fig. 2B, right panel), we used a functional assay based on patch clamp recordings of GABAα2-gated currents in small diameter acutely dissociated DRG cells. All recordings were done on POD 3 to 4 using ipsilateral L5 DRGs. Representative current traces (fig. 5E) and a summary of the data from patch clamp experiments (fig. 5F) indicate that when compared with the control (21.1 ± 6.88 pA/pF; n = 3 rats per data point, seven cells), the ASODN treatment resulted in about seven-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 to 4, the time point at which we noted significant worsening of the pain phenotype in ASODN-treated crush-injured rats (fig. 5, A 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 GABAα2 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 GABAα2 currents.32

Collectively, these behavioral, biochemical, and electrophysiological findings suggest that down-regulation of the GABAα2 subunit likely plays an important causal role in the development of NPP postcrush injury to the sciatic nerve.

If this conclusion is valid, we reasoned that α2 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 α2 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 α2 subunit expression in ipsilateral versus contralateral L5 DRGs in crush-injured animals [approximately 35%, fig. 6B; t(5) = 3.949, P = 0.01; n = 6 animals]. The α2 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 α2 subunit modulation in DRGs due to nerve crush injury. Based on this observation, we conclude that there is indeed an association between α2 subunit protein down-regulation in DRG cells and the NPP phenotype.

To further test a potential causal link between GABAα2 receptor modulation (manifested as a down-regulation of α2 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

Anesthesiology 2015; 123:654-67

661 Obradović et al.
Here, we used the newer agent NO-711 as a specific blocker of the GABA transporter-1 (GAT-1) because it has been confirmed to maintain the extracellular GABA in central nervous system 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 [fig. 7B] vs. POD 2) induced a significant alleviation of thermal hyperalgesia [overall ANOVA F(4,68) = 8.332; P < 0.001], that is, the PWLs in NO-711-treated animals (closed triangles) were
significantly increased when compared with 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 with POD 2 immediately before the treatment. Note that the PWLs in vehicle-treated group remained significantly decreased throughout the testing period when compared with the baseline PWL recordings (**P < 0.001; baseline [B] vs. any given POD).

**Discussion**

Down-regulation of the GABA<sub>α2</sub> subunit in the DRG plays an important role in NPP postcrush injury of a sciatic nerve in adult female rats. We base our conclusion on three important observations: (1) NPP-detected postcrush injury of a sciatic nerve coincides with a significant down-regulation of the α<sub>2</sub> subunit expression; (2) when α<sub>2</sub> subunit expression significantly increased when compared with 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 with POD 2 immediately before the treatment. The PWLs in vehicle-treated group remained significantly decreased throughout the testing period when compared with the baseline PWL recordings (**P < 0.001; baseline [B] vs. any given POD).

**Fig. 6.** The crush injury–induced knockdown of the α<sub>2</sub> subunit protein expression in the ipsilateral L5 dorsal root ganglion (DRG) is long lasting. (A) In sham animals, there is no difference in α<sub>2</sub> subunit expression in the ipsilateral L5 DRG as compared with the contralateral L5 DRG at postoperative day (POD) 10 (n = 6 rats per data point). (B) In crush-injured animals, there is a significant down-regulation of the α<sub>2</sub> subunit expression in the ipsilateral L5 DRG compared with the contralateral one at POD 10 (approximately 30%; **P < 0.01; n = 6 rats per data point).

**Fig. 7.** Blockade of γ-aminobutyric acid uptake in dorsal root ganglion alleviates thermal hyperalgesia postcrush 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 postoperative 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, that is, the PWLs in NO-711-treated animals (closed triangles) were significantly increased when compared with a vehicle treatment (closed squares) (†††P < 0.001; †††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 with POD 2 immediately before the treatment. The PWLs in vehicle-treated group remained significantly decreased throughout the testing period when compared with the baseline PWL recordings (**P < 0.001; baseline [B] vs. any given POD).
and GABA<sub>α</sub> channel function are selectively knocked down in DRGs using specific ODNs in vivo AS 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<sub>2</sub> subunit turnover); and (3) up-regulation of GABAα levels in DRG by pharmacological blockade of its uptake overcomes the effects of crush injury–induced down-regulation of the GABA<sub>α</sub> and alleviates NPP. Our data support the idea that the down-regulation of the GABA<sub>α</sub> system (specifically the α<sub>2</sub> subunit) in DRG neurons plays an important role in the pathophysiology of NPP after traumatic injury of the sciatic nerve.

Neuropathic pain 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. 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.15,16 However, beneficial effects of intrathecal 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. Those results suggest that the most promising target for GABAergic modulation could very well be the DRG because it is the immediate gateway between the injured afferent terminals and the higher pain centers. Here, we validate this hypothesis using specific AS-mediated knockdown of the α<sub>2</sub> subunit of GABA<sub>α</sub> receptors in DRGs.

A significant imbalance of GABA has been found in humans with NPP due to peripheral nerve injury. 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 DH, there was no concomitant loss of GABA neurons. 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<sub>α</sub> 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. 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,5,6 the role of GABA<sub>α</sub> 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 to III) have been reported previously. In addition, sciatic nerve transaction or loose ligation decreased the number of GABA-immunoreactive cells in lamina I to III of the rat lumbar DH. Published data indicate that the majority of DRG neurons expressing GABAα receptors terminate in lamina I to III. They showed that GABAα 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 α2 subunit could be caused by a selective loss of GABAα-expressing DRG neurons as has been reported in the ipsilateral DH post peripheral nerve injury. However, presently available evidence suggests that the number of DRG neurons remains roughly unchanged or may perhaps even increase after crush injury, thus making it unlikely that GABAα-expressing DRG neurons undergo similar down-regulation as the interneurons in DH.
Here, we focused on the α2 subunit of GABA_A receptors. Based on polymerase chain reaction and protein expression studies, it is known that GABA_A receptors are present on DRG neurons but also are functional and muscimol sensitive. With a unique subunit composition, as DRG neurons are pseudounipolar and glutamatergic, it is unlikely that endogenous GABAergic signaling in DRGs is via neurotransmission. Adult rat DRG neurons preferentially contain α2βγ3β2 subunits and are unable to transmit pain phenotype in sham-operated animals, and in a de novo pain phenotype in sham-operated animals, it appears that the α2 subunit in DRG GABA_A receptors is important for the development of pain. Specific targeting of α2 or α1 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 α2 subunit resulted in the inhibition of GABAergic neurons in DRG postcrush injury, our findings suggest that blocking the α2 subunit in affected DRG (and possibly 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 (fig. 7) as well as those of others, we propose that compensatory maintenance of GABA_A current density in DRG neurons postcrush 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 up-regulation 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 down-regulation of GABA_A α2 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 α2 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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Competing Interests

The authors declare no competing interests.

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