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 ($\text{GABA}_A$) $\alpha_2$ gene in dorsal root ganglia (DRG) controls NPP.

Methods: After crush injury to the right sciatic nerve of female rats, the $\alpha_2$ 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 before the injury and ensuing 10 days ($n = 4$ to $10$). In vitro quantification of $\alpha_2$ GABA$_A$ protein and electrophysiological studies of GABA$_A$ 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 $\pm$ SD). Conversely, up-regulation of endogenous GABA via blockade of its uptake in DRG alleviates NPP.

Conclusion: The GABA$_A$ 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)

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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-α2 subunit because this subunit is abundantly present in DRG neurons. We performed behavioral assessments of NPP phenotype in conjunction with targeted GABA_A-α2 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-α2 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 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; (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, 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 α2 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-α2 GABA_A oligonucleotide (ASODNs) was 5’-TCCATCCCAAGCCCATCC-3’. The sequence of the MIS-α2 GABA_A oligonucleotide (MISODNs) was 5’-CTACCGCCTCTCTACAC-3’. The GABA uptake inhibitor NO-711 hydrochloride (Sigma-Aldrich, USA) was dissolved in appropriate sterile pH 7.4 buffer solution.
(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 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 lysis 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 2x 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_A 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_α_ receptor bands were normalized to actin used as a house-keeping protein.

**Electrophysiological Studies of GABA_α_ Currents.** To study GABA_α_ 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 MgCl₂, 2 mM CaCl₂, 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 MgCl₂, 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 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 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 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, 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 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 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 postcrush injury, we examined whether such injury modulates the expression of GABA_α_ receptors in L5 DRG neurons. Because adult rat DRG neurons preferentially express α₂β₂γ₂ subunits and α₂ is the important functional subunit, we focused on expression of the α₂ subunit. As shown in figure 2, expression of the GABA_α_ receptor α₂ subunit protein in L5 DRG on the ipsilateral (right) side was indistinguishable from that on the contralateral (left) side in both sham-operated
**α₂ Subunit of GABA<sub>A</sub> Receptors and Neuropathic Pain**

**PROTEIN EXPRESSION OF α<sub>2</sub> SUBUNIT**

**A**

**SHAM SURGERY**

Fig. 2. Crush injury to the sciatic nerve down-regulates expression of the α<sub>2</sub> subunit of γ-aminobutyric acid receptor type A (GABA<sub>A</sub>) 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<sub>2</sub> subunit of GABA<sub>A</sub> receptor in the L5 DRG on the ipsilateral side were indistinguishable from those on the contralateral side both at the baseline and on postoperative day (POD) 2 (n = 6 rats per data point) (A). However, in crush-injured animals (B), although the protein levels of α<sub>2</sub> subunit of GABA<sub>A</sub> 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 (approximately 30%) decrease in α<sub>2</sub> 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%.

![Image](https://example.com/image1)

If acute down-regulation of α<sub>2</sub> 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<sub>2</sub> subunit expression in sham-operated animals using selective knockdown AS technology should mimic the NPP phenotype. To test this hypothesis, we first confirmed the effectiveness of α<sub>2</sub> subunit knockdown in sham-operated animals using selective knockdown AS technology should mimic the NPP phenotype. To test this hypothesis, we first confirmed the effectiveness of α<sub>2</sub> subunit knockdown in sham-operated animals. We applied AS or mismatch ODNs for the α<sub>2</sub> 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 α<sub>2</sub> 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 rats per data point], whereas the α<sub>2</sub> 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 to 48h post-ODNs application (i.e., on POD 3 to 4). This down-regulation caused by direct L5 DRG application of ASTODN was restricted to the ipsilateral (right) DRG as shown in figure 3B. The application of MISODN also resulted in no change in α<sub>2</sub> subunit protein expression in ipsi-DRGs (fig. 3B, right panel). Taken together, this evidence suggests that direct DRG application induced α<sub>2</sub> subunit modulation that was restricted to the ipsilateral DRG. To validate further the down-regulation of α<sub>2</sub> subunits in DRG cells, we used a functional assay based on patch clamp recordings of...
GABA\textsubscript{\alpha\textsubscript{3}}-gated currents in small diameter (<30 \textmu 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\textsubscript{\alpha\textsubscript{3}} 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 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 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\textsubscript{\alpha\textsubscript{3}} \textalpha\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 \textalpha\textsubscript{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).

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**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) \((n = 5 \text{ per data point})\) ♦️♦️♦️P < 0.001, before and after ASODN treatment; **P < 0.01, PWLs in operated \(R\) paw vs. unoperated \(L\) 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 MISODNs animals at any time point \((n = 4 \text{ per rats data point})\). (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 < 0.01, PWRs in operated \(R\) paw vs. unoperated \(L\) paw) \((n = 5 \text{ rats per data point})\). (D) In sham-operated animals post-MISODNs treatment, there was no change in mechanical sensitivity shown as stable PWRs recordings throughout the testing period \((n = 4 \text{ rats per data point})\).
on POD 3 through 8 (\(\dagger\dagger\dagger P < 0.001; \dagger\dagger P < 0.01; \) 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 \(\alpha_2\) subunit down-regulation in ASODN-treated sham animals resulted in significant thermal and mechanical hyperalgesia, we reasoned that if maintaining GABA\(_A\) \(\alpha_2\) 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 \(\alpha_2\) 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; \(\dagger\dagger\dagger P < 0.001; \) 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-ASODN treatment levels (fig. 5A) is to be expected considering that the half-life of the GABA\(_A\) 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 \(F(11,55) = 25.751, P < 0.001\) (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; \(\dagger\dagger P = 0.002\) and \(\dagger\dagger\dagger P < 0.001; \) 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) (***\(P < 0.001; \) PWRs 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 \(\alpha_2\) 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 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 naive 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\(_A\) \(\alpha_2\) 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 \(\alpha_2\) 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 \(\alpha_2\) 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 \(\alpha_2\) subunit expression in ipsilateral versus contralateral L5 DRGs in crush-injured animals (approximately 35%, fig. 6B; \(\text{t}(5) = 3.949, P = 0.01; n = 6\) animals). The \(\alpha_2\) 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 \(\alpha_2\) subunit modulation in DRGs due to nerve crush injury. Based on this observation, we conclude that there is indeed an association between \(\alpha_2\) 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 \(\alpha_2\) 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 naive 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) because it has been confirmed to maintain the extracellular GABA in central nervous system at higher levels. 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 < 0.01; baseline [B] vs. any given POD).

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 < 0.01; ††††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 < 0.01; baseline [B] vs. any given POD).

**Discussion**

Down-regulation of the GABA_\(\alpha_2\) 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 \(\alpha_2\) subunit expression; (2) when \(\alpha_2\) subunit expression...
and GABA$_{\alpha}$ 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 $\alpha_2$ subunit turnover); and (3) up-regulation of GABA levels in DRG by pharmacological blockade of its uptake overcomes the effects of crush injury–induced down-regulation of GABA levels in DRG by pharmacological blockade (image magnification of all tissue slices was $\times$5).

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. 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. 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 $\alpha_2$ subunit of GABA$_{\alpha}$ 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$_{\alpha}$ 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, the role of GABA$_{\alpha}$ 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 GABA$_{\alpha}$ receptors terminate in lamina I to III. They showed that GABA$_{\alpha}$ 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$_{\alpha}$-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 GABA$_{\alpha}$-expressing DRG neurons undergo similar down-regulation as the interneurons in DH.

![Fig. 8. Direct L5 dorsal root ganglion (DRG) application of sodium fluorescein](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/934313/)
Here, we focused on the $\alpha_2$ subunit of GABA$_\text{A}$ receptors. Based on polymerase chain reaction and protein expression studies, it is known that GABA$_\text{A}$ 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 $\alpha_2\beta_3\gamma_2$ subunits with the $\alpha_2$ subunit being the key functional component. Because knocking down the $\alpha_2$ 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 $\alpha_2$ subunit in DRG GABA$_\text{A}$ receptors is important for the development of pain. Specific targeting of $\alpha_2$ or $\alpha_3$ subunits in spinal DH neurons has been shown to modulate NPP caused by loose ligation of the sciatic nerve in mice, and preferential activation of the $\alpha_2$ subunit was highly effective in ameliorating NPP in GABA$_\text{A}$ receptor point-mutated knock-in mice ($\alpha_2$(H1101R)). 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 $\alpha_2$ and $\alpha_3$ subunits in spinal DH neurons and on the $\alpha_2$ subunit in DRG neurons.

A benefit of selective subunit modulation also could be appreciated in terms of different side effects. Previous studies showed that $\alpha_1$ but not $\alpha_2$ knock-in mice displayed an increase in duration and a decrease in latency of loss-of-righting reflex upon administration of benzodiazepines or barbiturates. Hence, although the role of each subunit remains to be fully understood, it is likely that selective up-regulation of the $\alpha_2$ 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 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. By being so snugly 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 postcrush 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$_\text{A}$ 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 down-regulation of the $\alpha_2$ subunit in affected DRG (and probably a down-regulation of GABA$_\text{A}$ 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$_\text{A}$ 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$_\text{A}$ 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$_\text{A}$ $\alpha_2$ 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 $\alpha_2$ 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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Competing Interests

The authors declare no competing interests.

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