



Published in final edited form as:

J Neurochem. 2015 June ; 133(6): 857–869. doi:10.1111/jnc.13103.

Blocking the GABA transporter GAT-1 ameliorates spinal GABAergic disinhibition and neuropathic pain induced by paclitaxel

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Abstract

Paclitaxel is a chemotherapeutic agent widely used for treating carcinomas. Patients receiving paclitaxel often develop neuropathic pain and have a reduced quality of life which hinders the use of this life-saving drug. In this study, we determined the role of GABA transporters in the genesis of paclitaxel-induced neuropathic pain using behavioral tests, electrophysiology, and biochemical techniques. We found that tonic GABA receptor activities in the spinal dorsal horn were reduced in rats with neuropathic pain induced by paclitaxel. In normal controls, tonic GABA receptor activities were mainly controlled by the GABA transporter GAT-1 but not GAT-3. In the spinal dorsal horn, GAT-1 was expressed at presynaptic terminals and astrocytes while GAT-3 was only expressed in astrocytes. In rats with paclitaxel-induced neuropathic pain, the protein expression of GAT-1 was increased while GAT-3 was decreased. This was concurrently associated with an increase of global GABA uptake. The paclitaxel-induced attenuation of GABAergic tonic inhibition was ameliorated by blocking GAT-1 but not GAT-3 transporters. Paclitaxel-induced neuropathic pain was significantly attenuated by the intrathecal injection of a GAT-1 inhibitor. These findings suggest that targeting GAT-1 transporters for reversing disinhibition in the spinal dorsal horn may be a useful approach for treating paclitaxel-induced neuropathic pain.

Keywords

taxol; nociception; neurotoxicity; γ -aminobutyric acid; IPSCs; patch clamp

Introduction

Paclitaxel (taxol) is a potent anti-tumor drug used for the treatment of carcinomas in a wide range of organs including lung, breast, ovaries, prostate and others. The clinical application of this life-saving agent is hampered by paclitaxel-induced neuropathic pain (P-INP).

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The authors declare that there are no conflicts of interest.

Currently, effective treatments for P-INP are not available. It is known that pathological pain originates from aberrant neuronal activities along the pain signalling pathway, including peripheral nociceptors, neurons in the spinal dorsal horn and supraspinal pain centres. Homeostasis between excitatory and inhibitory receptor activities is crucial to maintain normal neuronal activities in the central nervous system (CNS). In the spinal dorsal horn, attenuation in glycinergic and/or γ -aminobutyric acid (GABA) inhibitory system contributes to the genesis of pathological pain. For example, reduction of glycinergic receptor activities in the spinal superficial dorsal horn is associated with inflammatory pain (Harvey *et al.* 2004, Bonin & De Koninck 2013) while impairment in GABAergic inhibitory synaptic activities in the spinal dorsal horn is an important mechanism contributing to the genesis of neuropathic pain induced by nerve injury (Coull *et al.* 2003, Coull *et al.* 2005, Bonin & De Koninck 2013, Moore *et al.* 2002). Currently, whether and how the spinal inhibitory system is altered in paclitaxel-induced neuropathic pain remains inadequately understood.

GABA is the major inhibitory neurotransmitter released from GABAergic interneurons in the spinal dorsal horn (Bardoni *et al.* 2013, Bonin & De Koninck 2013). GABA exerts its inhibitory effects through acting on ionotropic GABA_A receptors and metabotropic GABA_B receptors at presynaptic terminals to reduce presynaptic glutamate release (Bardoni *et al.* 2013). Activation of presynaptic GABA_B receptors with baclofen can ameliorate pathological pain (Gaillard *et al.* 2014, Fukuhara *et al.* 2013). GABA also acts on GABA_A receptors at postsynaptic neurons to cause influx of Cl⁻ and membrane hyperpolarization in postsynaptic neurons (Bardoni *et al.* 2013). Activation of synaptic GABA_A receptors by GABA released presynaptically produces phasic inhibition, while activation of extrasynaptic GABA_A receptors by ambient GABA is related to tonic inhibition of neurons (Belelli *et al.* 2009, Lee & Maguire 2014). Studies of GABAergic receptor activities in the spinal dorsal horn have mainly concentrated on understanding the fast synaptic (phasic) inhibition. Little is known about the regulation of GABAergic tonic inhibition in the spinal dorsal horn in normal and pathological pain conditions.

One important factor that regulates the clearance and maintenance of the homeostasis of extracellular inhibitory transmitters is the GABA transporter system (Zhou & Danbolt 2013). GABA transporters are located on the plasma membrane in neurons and astrocytes, which transport extracellular GABA into the cell as it is not metabolized extracellularly (Zhou & Danbolt 2013). In the CNS, there are mainly two types of GABA transporters, GABA transporter-1 (GAT-1) and GABA transporter-3 (GAT-3). Studies in the forebrain show that GABAergic tonic inhibition is controlled by GABA transporters. The regulation of GABA receptor activities by GAT-1 and GAT-3 and cellular types expressing GAT-1 and GAT-3 are region-specific (Park *et al.* 2009, Kersante *et al.* 2013, Belelli *et al.* 2009, Lee & Maguire 2014). Previous studies have suggested that the protein expression of GABA transporters is altered in animals with pathological pain induced by inflammation or nerve injury (Ng & Ong 2001, Ng & Ong 2002, Daemen *et al.* 2008). Currently, it is unclear whether changes of tonic GABAergic inhibition and GABA transporters contribute to the genesis of paclitaxel-induced neuropathic pain.

In this study, we revealed, for the first time, that GABAergic tonic inhibition in the spinal dorsal horn of rats with P-INP is reduced. We defined the cellular location of GAT-1 and

GAT-3 and the role of these transporters in GABAergic tonic inhibition in normal and P-INP. We demonstrated that blocking GAT-1 in the spinal dorsal horn is a powerful approach to ameliorating P-INP.

Methods and materials

Animals

Adult male Sprague-Dawley rats (body weight: 170-220 g, Harlan Laboratories) were used. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Georgia and were fully compliant with the National Institutes of Health Guidelines for the Use and Care of Laboratory Animals.

Paclitaxel-induced neuropathic pain model in rats

P-INP was induced in rats by intraperitoneal (i.p.) injection of paclitaxel (Taxol, Bristol-Myers Squibb) at a dose of 2 mg/kg on four alternate days (days 1, 3, 5, and 7) with a cumulative dose of 8 mg/kg. Paclitaxel and vehicle were prepared and injected as previously described (Gao *et al.* 2013).

Behavioral tests

All the behavioral testing were conducted in a quiet room with room temperature at 22 °C (Weng *et al.* 2003). To test mechanical sensitivity, rats were placed on a wire mesh, loosely restrained under a plexiglass cage (12 × 20 × 15 cm³) and allowed to acclimate for at least 30 min. Hind paw withdrawal response thresholds to mechanical stimuli were defined with a set of von Frey monofilaments (bending force from 0.58 to 14.68 g), which were applied in an ascending order to evoked a 50% or greater withdrawal responses (Yan *et al.* 2014). This value was later averaged across all animals in each group to yield the group response threshold. To determine thermal sensitivity, rats were placed on a smooth glass plate preheated at 30 °C. A radiant heat beam (diameter 5 mm²) was directed onto the mid-plantar surface of the hind paw from beneath (Hargreaves *et al.* 1988). The withdrawal latency was recorded as previously described (Maixner *et al.* 2015). When the behavioral tests and i.p. injection took place on the same day, the i.p. injection was made after the behavioral tests. The experimenters conducting the behavior tests were blinded to the type of treatments given to the animals.

To determine whether the tested agents cause impairment in motor functions or sedation, the rotarod test was conducted as described previously (Stone *et al.* 2014, Hara *et al.* 2014). Animals were placed on a rotating drum and the drum was set to rotate from 4 to 40 rpm over a period of 5 min. The period of time in seconds at which the animal fell from the drum was recorded. The mean time for each treatment group of animals was taken for statistical analysis.

Intrathecal catheter implantation

A polyethylene (PE-10) catheter that ended at the spinal L4 segment was intrathecally placed as previously described (Yaksh & Rudy 1976). Briefly, rats were anesthetized with 2-3% isoflurane. A PE-10 catheter was carefully inserted into an opening at the atlanto-

occipital membrane and advanced to the lumbar enlargement. Behavior tests and intrathecal drug administration were conducted 7 days after the intrathecal implantation of the catheter. At the end of the behavior experiments, 50 μ L of 2% lidocaine was injected into the catheter. If hind paw paralysis did not occur after the lidocaine injection, rats were omitted from the data analysis.

Western blotting

Animals were deeply anesthetized with urethane (1.3–1.5 g/kg, i.p.). The L4-L5 spinal segment was exposed and removed from the rats. The dorsal half of the spinal segment was isolated and quickly frozen in liquid nitrogen and stored at -80 °C for later use. Frozen tissues were homogenized and protein was isolated and quantitated as previously described (Gao *et al.* 2013, Maixner *et al.* 2015). Protein samples (40 μ g) were electrophoresed in 10 % SDS polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were blocked with 5% milk or 5% BSA, and then incubated respectively overnight at 4 °C with polyclonal rabbit anti- GAT-1 (1:1000, Abcam) polyclonal rabbit anti-GAT-3 (1:1000, Abcam) primary antibodies, or a monoclonal mouse anti- β -actin (1:2000, Sigma-Aldrich, St. Louis, USA) primary antibody as a loading control. Then the blots were incubated for 1 hr at room temperature with corresponding HRP-conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology, CA, USA), visualized in ECL solution (Super Signal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA) for 1 min, and exposed onto FluorChem HD2 System. The intensity of immunoreactive bands was quantified as previously described (Gao *et al.* 2013). Levels of each biomarker were expressed as the ratio to the loading control protein (β -actin).

GABA uptake assay

GABA uptake activity in the lumbar spinal dorsal horn of rats was measured using synaptosome preparations according to previously established protocols with modifications (Wonnemann *et al.* 2000, Hu *et al.* 2003, Ozkan *et al.* 1997, Yan *et al.* 2014). The L4-L5 spinal segment was removed from the rat anesthetized with urethane (1.3–1.5 g/kg, i.p) and the dorsal half of the spinal cord was isolated. Synaptosome preparations were prepared immediately in Syn Per TM synaptic protein extraction reagent (Thermo scientific) and quantified by the BCA assay (Thermo scientific). Briefly the homogenates were centrifuged at $10,500 \times g$ for 10 min at 4 °C, and the supernatant was collected. The remaining pellets were re-suspended in the same solution and re-centrifuged at $9,200 \times g$ for 10 min at 4°C. The two supernatant were combined and centrifuged again at $9,200 \times g$ for 10 min at 4°C to obtain the synaptosomal pellets, which contained both neuronal and glial GABA transporters. The synaptosome protein (50 μ g) was incubated in Locke's buffer solution containing: 0.5 mM EDTA, 0.5 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.32 M sucrose, 5 μ g/ml pepstatin, 5 μ g/ml aprotinin, 20 μ g/ml trypsin inhibitor, 4 μ g/ml leupeptin, and 0.01 M phosphate-buffered saline. GABA uptake activity was determined by incubating the synaptosome preparation with a solution containing 0.4 μ Ci of γ -Aminobutyric Acid (GABA), γ -[2,3-³H(N)] (Perkin Elmer Life Sciences, Boston, MA) at room temperature for 10 min. The reaction was terminated by filtering the synaptosomes through a Whatman GF/B filter presoaked with the same buffer solution. The filter was washed two times with ice-cold Locke's buffer (2ml) and was then transferred to a vial containing a scintillation

cocktail. The radioactivity of the final samples was measured by a liquid scintillation counter (Beckman, LS6500).

Immunohistochemical Analysis

Male Sprague-Dawley rats were deeply anesthetized with urethane (1.3-1.5 g/kg, i.p.) and the L4 and L5 spinal cord was removed, fixed, and cyrosectioned as previously described (Weng *et al.* 2014). Sections were incubated overnight at 4 °C in 2% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS containing primary antibodies against the following targets: rabbit anti- GAT-1 (1:200, Abcam), rabbit anti-GAT-3 (1:200, Abcam), mouse anti-GFAP (a marker for astrocytes, 1:500, Cell Signaling), rat anti-OX-42 (a marker for microglia, 1:500, AbD Serotec), and mouse anti MAP2 (a marker for neuronal cytoskeleton 1:500, Cell Signaling) antibodies. After washing three times with 0.1 M PBS, the sections were incubated for 2 hr at room temperature with the corresponding Texas Red antibody (1:500 Vector Laboratories), Alexa Fluor 488 antibody (1:500 Life Technologies), or the Mouse Anti-NeuN Alexa Fluor 488 conjugated antibody (the neuronal cell body marker, 1:200, Millipore). After rinsing three times with 0.1M PBS, the sections were mounted onto gelatin-coated slides, air-dried, and cover-slipped with Vectashield mounting medium (Vector Laboratories). Non-adjacent sections were selected randomly, and the immunostaining for each antibody were viewed under an Olympus BX43 microscope with an Olympus U-CMAD3 camera. Images were processed using Olympus-cellSens Dimensions.

Recording of GABAergic currents

Spinal slices were obtained from rats as previously described (Weng *et al.* 2007). Briefly, rats were deeply anesthetized under isoflurane, and a laminectomy was then performed to remove the lumbar spinal cord. The L4 to L5 spinal segment was placed in ice-cold sucrose-based artificial cerebrospinal fluid (aCSF) pre-saturated with 95% O₂ and 5% CO₂. The sucrose-based aCSF contained 234 mM sucrose, 3.6 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 1.2 mM NaH₂PO₄, 12.0 mM glucose, and 25.0 mM NaHCO₃. Transverse spinal cord slices (400 μm thick) were cut in the ice-cold sucrose aCSF and then pre-incubated for at least 2 hours in Krebs solution oxygenated with 95% O₂ and 5% CO₂ at 35 °C before they were transferred to the recording chamber. The Krebs solution contained 117.0 mM NaCl, 3.6 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 1.2 mM NaH₂PO₄, 11.0 mM glucose, and 25.0 mM NaHCO₃. Following pre-incubation, a rat spinal slice was placed in the recording chamber (1.5 ml in volume), perfused with Krebs solution at 35 °C, and saturated with 95% O₂ and 5% CO₂. Borosilicate glass recording electrodes (resistance, 3–5 MΩ) were made and filled with an internal solution containing 110 mM Cs₂SO₄, 5 mM KCl, 2.0 mM MgCl₂, 0.5 mM CaCl₂, 5.0 mM HEPES, 5.0 mM EGTA, 5.0 mM ATP-Mg, 0.5mM Na-GTP, and 10 mM lidocaine *N*-ethyl bromide (QX314), adjusted to pH 7.2–7.4 with 1 M CsOH (290–300 mOsm) (Jiang *et al.* 2012). The recording electrodes were directed to the out layer of spinal dorsal horn lamina II. Whole-cell configurations were established by applying moderate negative pressure after electrode contact (Weng *et al.* 2007). A seal resistance of 2 GΩ and an access resistance of about 20 MΩ were considered acceptable. GABAergic currents were recorded in the presence of 0.5 μM strychnine (a glycine receptor inhibitor), 10 μM DNQX (an AMPA/kainate receptor inhibitor) and 25 μM D-AP5 (NMDA receptor

inhibitor) at a holding potential of 0 mV (Moore *et al.* 2002). The depth of the recorded cell in the slice (about 50 μm below the surface) was kept constant across all experiments.

Materials

NO-711 and SNAP5114 were obtained from Sigma-Aldrich (St. Louis, MO).

Data analysis

All data are presented as the mean \pm SEM. One way or two way ANOVA with repeated measures over time or different treatments was used to detect differences on paw-withdrawal response thresholds and latencies, followed by the Bonferroni post hoc test to determine sources of differences. Student's t-tests were used to make comparison between groups (non-paired t) and within the same group (paired t). A p value less than 0.05 was considered statistically significant.

Results

In this study, rats used for the mass spectrometry, electrophysiology, and Western blot experiments were assigned into two groups, paclitaxel treated group and vehicle treated group. Paclitaxel (2 mg/kg, i.p.) or vehicle was respectively injected into animals in the paclitaxel group or vehicle group on days 1, 3, 5, and 7. Hind paw withdrawal response thresholds to mechanical and thermal stimuli were measured on days 1, 5 and 10. Consistent with previous findings by us (Gao *et al.* 2013) and others (Polomano *et al.* 2001, Okubo *et al.* 2011), rats receiving paclitaxel in this scheme developed mechanical allodynia and thermal hyperalgesia. These were evident by a significant reduction (51 vehicle-treated rats versus 48 paclitaxel-treated rats) of mechanical thresholds ($F_{(2, 46)} = 196, P < 0.001$) and latencies ($F_{(2, 46)} = 98.65, P < 0.001$) of hind paw withdrawal responses on day 5 and day 10 after the first injection (Fig.1). Electrophysiology and Western blot experiments were performed on rats 10 days after the first i.p. injection, when all rats treated with paclitaxel developed mechanical allodynia and thermal hyperalgesia.

GABAergic tonic inhibition in the spinal dorsal horn is weakened in paclitaxel-treated rats

To determine whether altered GABAergic receptor activities in the spinal cord contribute to the genesis of P-INP, we compared GABAergic tonic inhibition in spinal dorsal horn neurons in rats treated with paclitaxel and rats treated with vehicle. Following previously published protocols (Bai *et al.* 2001, Jia *et al.* 2005, Clarkson *et al.* 2010), we analyzed GABAergic tonic inhibition by bath-perfusing the GABA_A receptor inhibitor bicuculline (25 μM) and measuring changes of holding currents when GABAergic currents in the spinal dorsal horn neurons were recorded. We found that perfusion of bicuculline into the recording chamber blocked spontaneous GABAergic IPSCs and revealed a shift in the holding currents in slices taken from vehicle control rats (tested in 11 neurons) (Fig. 2A) and paclitaxel-induced neuropathic rats (tested in 11 neurons) (Fig. 2B). The changes of holding currents induced by bicuculline in rats with P-INP (23.93 ± 2.76 pA) were significantly less (Fig. 2C, $P < 0.001$) than those in vehicle control rats (63.93 ± 3.53 pA) (Fig. 2C). These data indicated that GABAergic tonic inhibition is reduced in the spinal dorsal horn of rats

with P-INP. Thus, enhancing GABAergic tonic inhibition may be an effective approach to alleviate P-INP.

Tonic GABA receptor activities in the spinal dorsal horn are mainly regulated by GAT-1 under normal conditions

Studies in the forebrain have shown that tonic GABAergic inhibition is regulated both by GABA synthesis (Nishikawa *et al.* 2011, Fatemi *et al.* 2005) and by GABA transporters (Semyanov *et al.* 2004, Wu *et al.* 2006, Smith *et al.* 2007, Kirmse *et al.* 2008, Nishikawa *et al.* 2011). To determine the role of GABA transporters in the regulation of GABAergic receptor activities in the spinal dorsal horn, we first determined the cellular location of the GABA transporters GAT-1 and GAT-3 in the spinal dorsal horn of normal control animals using immunohistochemistry technique. As shown in Figure 3A and B, GAT-1 and GAT-3 were widely expressed in the entire spinal dorsal horn with more dense expression in the superficial dorsal horn (laminae I and II). Furthermore, expression of GAT-1 was co-localized with a marker for neuronal cytoskeleton (MAP2) and the astrocyte marker (GFAP), but not with the neuronal cell body marker (NeuN) or microglia marker (OX42) (Fig. 3C). In contrast, GAT-3 was predominantly co-localized with GFAP, but not OX42, MAP2, or NeuN (Fig. 3D). These data indicate that: a) GAT-1 is expressed in neuronal presynaptic terminals and astrocytes; b) GAT-3 is only present in astrocytes; c) neither GAT-1 nor GAT-3 is expressed in microglia.

We next determined whether GABAergic tonic inhibition in the spinal dorsal horn is regulated by GAT-1 and GAT-3 in normal controls. NO-711 and SNAP5114 are widely used to selectively block GAT-1 and GAT-3 respectively (Nusser & Mody 2002, Rossi *et al.* 2003, Keros & Hablitz 2005). We recorded GABAergic tonic currents before and during bath-perfusion of the GAT-1 inhibitor (NO-711, 10 μ M). As shown in Figure 4A and C, the GAT-1 inhibitor significantly ($P < 0.001$) increased the holding currents (which reflects tonically active GABA conductance) by -37.05 ± 2.36 pA ($n = 8$). Under the condition when GAT-1 was inhibited by NO-711 (10 μ M), the addition of the GAT-3 inhibitor SNAP5114 (100 μ M) resulted in a further increase of holding currents ($n = 8$, $P < 0.001$) (Fig. 4A and C). Interestingly, when GAT-3 was blocked alone by bath-perfusion containing SNAP5114 (100 μ M), we did not observe a significant change in the holding currents ($n = 9$, $P = 0.58$) (Fig. 4B). These data indicated that GAT-1 is critical in maintaining tonic GABA inhibition in the spinal dorsal horn.

Expression of GAT-1 is increased while expression of GAT-3 is reduced in the spinal dorsal horn in rats with P-INP, which is concurrently associated with increased GABA uptake activities

We then determined whether altered functions of GAT-1 and GAT-3 are implicated in the genesis of P-INP. We examined protein expressions of GAT-1 and GAT-3 in the dorsal half of the L4 to L5 spinal segment in the paclitaxel group or vehicle group. As shown in Figure 5A, in comparison with rats treated with vehicles ($n = 10$), the expression of GAT-1 in the spinal dorsal horn was significantly increased ($n = 11$, $P < 0.001$) while the expression of GAT-3 in the spinal dorsal horn was significantly reduced in rats with P-INP. We next determined the global GABA uptake activities carried by both GAT-1 and GAT-3 in the

spinal dorsal horn of rats with P-INP and rats receiving vehicle treatment using synaptosome preparations (Mitrovic *et al.* 1999, Sung *et al.* 2003). We found that GABA uptake activities were significantly increased in rats with P-INP ($n = 4$, $P < 0.001$) in comparison with rats ($n = 3$) in the vehicle group (Fig. 5B). These data indicate that in the spinal dorsal horn of rats with P-INP GABA uptake activities are increased, which is ascribed to the increased GAT-1 function.

The paclitaxel-induced GABAergic disinhibition is ameliorated by blocking GAT-1 but not GAT-3

We then investigated whether the paclitaxel-induced suppression of GABAergic tonic inhibition in the spinal dorsal horn can be ameliorated by blocking GAT-1 and GAT-3. As shown in Figure 6A and C, the GAT-1 inhibitor (NO-711, 10 μM) significantly increased the holding currents. The degree of increases induced by the GAT-1 inhibitor on the holding currents in the paclitaxel-treated rats (-66.27 ± 3.80 pA, $n = 7$) (Fig. 6C) was significantly ($P < 0.001$) higher than those in vehicle treated rats (-37.05 ± 2.36 pA, $n = 8$) (Fig. 4C). Furthermore, we did not observe significant ($P < 0.05$) alterations in the holding current in paclitaxel treated rats when the GAT-3 inhibitor (SNAP5114, 100 μM) was perfused into the bath in the presence of the GAT-1 inhibitor (NO-711, 10 μM) (Fig. 6A and C). The holding current was not significantly altered by the GAT-3 inhibitor alone (Fig. 6B) ($n = 9$, $P = 0.39$). These data indicate that inhibition of GAT-1 is a potent approach to restore the paclitaxel-induced suppression of GABA receptor tonic activities in the spinal dorsal horn.

Mechanical allodynia and thermal hyperalgesia in rats with P-INP are attenuated by intrathecal injection of GAT-1 inhibitors

Finally, we determined whether inhibition of GAT-1 can ameliorate mechanical allodynia and thermal hyperalgesia induced by paclitaxel. Rats pre-implanted with intrathecal catheters were assigned into 4 groups: paclitaxel+saline group, paclitaxel+GAT-1 inhibitor group, vehicle+saline group, and vehicle+GAT-1 inhibitor group. After taking baseline withdrawal response thresholds to radiant heat and mechanical stimuli, rats received either 4 injection of paclitaxel (2 mg/kg, i.p) or vehicle on alternative days. Ten days following the first injection, mechanical allodynia and thermal hyperalgesia were confirmed by behavioral tests using von Frey monofilament (Fig. 7A) and radiant heat stimuli (Fig. 7B). Meanwhile mechanical and thermal thresholds in the vehicle+saline group and naïve+GAT-1 inhibitor group remained stable. We then topically applied the GAT-1 inhibitor NO-711 (10 μg in a volume of 10 μl) onto the spinal lumbar enlargement in the paclitaxel+GAT-1 inhibitor group and naïve+GAT-1 inhibitor group through the pre-implanted intrathecal (i.t.) catheter. Rats in the paclitaxel+saline group and vehicle+saline group received 10 μl of saline via intrathecal injection. Changes in thermal and mechanical sensitivities were determined at 0.5, 1, 3, and 24 hours after the i.t. injection. The paclitaxel-induced mechanical allodynia and thermal hyperalgesia were significantly attenuated by a single intrathecal injection of the GAT-1 inhibitor NO-711 (10 μg). As shown in Fig. 7A, NO-711 significantly increased the mechanical thresholds in the paclitaxel+GAT-1 group at 30 min (10.00 ± 0.01 g) and 60 min (8.00 ± 0.89 g) in comparison with their own values (4.00 ± 0.89 g) before the NO-711 injection ($F_{(4,20)} = 30.18$, $P < 0.001$), or in comparison with their counterparts in the paclitaxel+saline group at 30 min (5.2 ± 0.87 g) and 60 min (3.6 ± 0.97 g) (main effects of

drug: $F_{(1,45)} = 12.39, P < 0.01$; main effect of time: $F_{(4,45)} = 8.90, P < 0.001$; interaction: $F_{(4,45)} = 4.30, P < 0.005$; 5 to 6 rats/group). Similarly, latencies of withdrawal responses to radiant heat stimuli in the paclitaxel+GAT-1 group were also significantly increased at 30 min (13.50 ± 1.07 s) and 60 min (11.51 ± 0.87 s) after the injection of NO-711 (10 μ g) in comparison with the values (7.45 ± 0.50 s) prior to the NO-711 injection ($F_{(4,20)} = 14.33, P < 0.001$), and their counterparts in the paclitaxel+saline group at 30 min (8.13 ± 0.38 s) and 60 min (7.97 ± 0.77 s, Fig. 7B) (main effects of drug: $F_{(1,45)} = 19.97, P < 0.001$; main effect of time: $F_{(4,45)} = 5.52, P < 0.01$; interaction: $F_{(4,45)} = 6.46, P < 0.003$; 5 to 6 rats/group). The effects of GAT-1 inhibitors on mechanical and thermal thresholds disappeared by 3 hours after drug administration. These results indicated that the blockade of GAT-1 activities is a powerful approach to reverse P-INP. In contrast, administration of saline in the vehicle+saline group or the GAT-1 inhibitor (10 μ g in a volume of 10 μ l) in the naive +GAT-1 inhibitor group did not significantly alter mechanical or thermal thresholds in these two groups (Fig. 7A and B). To further determine whether the GAT-1 inhibitor at the tested dose (10 μ g) cause impairment on motor functions and/or sedation, the rotarod test was performed. Retention time on an accelerating rotarod is a widely used index to monitor motor functions and/or sedation in animals, since sedation also causes the animals to fall (Stone *et al.* 2014, Hara *et al.* 2014). We found that in comparison with naïve rats ($n = 8$) receiving intrathecal saline injection (10 μ l), intrathecal administration of the GAT-1 inhibitor NO-711 (10 μ g in a volume of 10 μ l) to naïve rats ($n = 8$) did not significantly ($P = 0.22$ to 0.77) alter the retention time on the rotarod over the observation period (0.5 to 24 hours after the intrathecal injection) (data not shown). These results indicate that NO-711 at a dose of 10 μ g does not cause motor impairment or sedation.

Discussion

Mechanisms underlying paclitaxel-induced neuropathic pain

Pathological changes in both peripheral nerves and the spinal dorsal horn have been reported in animals with P-INP. For example, P-INP in rats is associated with the degeneration of intraepidermal terminal arbors of sensory neurons and activation of Langerhans cells in the skin (Siau *et al.* 2006, Boyette-Davis *et al.* 2011). Furthermore, the incidence of swollen and vacuolated axonal mitochondria is increased in myelinated and unmyelinated sensory axons (Xiao & Bennett 2012, Zheng *et al.* 2011). Agents that enhance mitochondrial function have been shown to be effective in the prevention or attenuation of P-INP (Jin *et al.* 2008). The protein and mRNA expressions of TRPV1 in dorsal root ganglion sensory neurons are increased in rats receiving repeated paclitaxel treatments (Hara *et al.* 2013). More recently, it was reported that chronic paclitaxel significantly increases the protein expression of the chemokine CX3CL1 in A-fiber primary sensory neurons and infiltration of macrophages into the dorsal root ganglion (DRG) in rats, which contributes to the paclitaxel-induced DRG neuronal apoptosis of A fibers and pathological pain (Huang *et al.* 2014). At the spinal cord level, we have previously shown that wide-dynamic range neurons in the spinal dorsal horn in rats with paclitaxel-induced neuropathic pain display a significant increase in both spontaneous activities (action potentials) and responses to noxious mechanical, heating, and cooling stimuli, as well as an abnormal wind-up to transcutaneous electrical stimuli (Cata *et al.* 2006). Loss of homeostasis between excitatory and inhibitory inputs to neurons is a

major factor leading to exaggerated neuronal activation. In this regard, the down-regulation of glial glutamate transporters in the spinal dorsal horn has been demonstrated to be causal in the genesis of P-INP (Weng *et al.* 2005, Doyle *et al.* 2012, Gao *et al.* 2013). Glial glutamate transporters control neuronal activation by promptly clearing the excitatory neuronal transmitter glutamate released from presynaptic terminals in the spinal dorsal horn (Nie & Weng 2010, Weng *et al.* 2007, Nie & Weng 2009). Improving glial glutamate transporter functions by reducing levels of peroxynitrite (Doyle *et al.* 2012) or suppression of GSK3 β activities (Gao *et al.* 2013) in the spinal dorsal horn effectively prevents and attenuates P-INP. It was recently reported that up-regulation of NKCC1 in the spinal dorsal horn causes a depolarizing shift and reduces GABA-induced membrane hyperpolarization in GABAergic neurons following P-INP (Chen *et al.* 2014). Whether the tonic GABAergic inhibition is altered in P-INP has not been explored. Our current study provided direct evidence that GABAergic tonic disinhibition in the spinal dorsal horn is implicated in the genesis of P-INP.

Regulation of GABAergic receptor activities in the spinal dorsal horn in pathological pain conditions

Disinhibition in the spinal dorsal horn is a critical factor leading to excessive neuronal activation in animals with neuropathic pain induced by mechanical injury. Extracellular GABA level in the lumbar dorsal horn is reduced by nerve injury (Stiller *et al.* 1996, Castro-Lopes *et al.* 1993). In neuropathic rats, amplitudes and frequencies of GABA_A receptor-mediated IPSCs in neurons in the superficial dorsal horn are attenuated (Moore *et al.* 2002). Additionally, the spinal application of GABA agonists attenuate mechanical allodynia and thermal hyperalgesia induced by nerve injury (Malan *et al.* 2002). It is known that GABAergic receptor activities are governed by at least the following factors: the amount of GABA released from presynaptic terminals, the function of GABA transporters, and the function of GABA receptors at the postsynaptic neuron (Zhou & Danbolt 2013). GABA produces phasic inhibition by acting on GABA_A receptors inside the synapse, and tonic inhibition by acting on extrasynaptic GABA_A receptors (Belelli *et al.* 2009). Studies of GABA receptor activities in the spinal dorsal horn have been mainly focused on the phasic GABA inhibition. For example, synaptic GABAergic activities (phasic inhibition) are reduced when the release probability of GABA neurotransmitters is decreased by activation of presynaptic A1 adenosine-receptors or GABA_B receptors (Hugel & Schlichter 2003, Yang *et al.* 2004). Activation of neuronal acetylcholine receptors (Takeda *et al.* 2003) or M3 muscarinic acetylcholine receptors (Zhang *et al.* 2006) at presynaptic terminals increases the GABA release from the presynaptic terminals and GABAergic phasic inhibition. Reduction of GABA synthesis through the glutamate-glutamine cycle at the presynaptic terminals also decreases GABAergic phasic inhibition (Jiang *et al.* 2012). At the post-synaptic site, phasic inhibition induced by the activation of GABA receptors are reduced by the down-regulation of the K⁺-Cl⁻ cotransporter KCC2, which disrupts Cl⁻ homeostasis in neurons (Coull *et al.* 2003, Coull *et al.* 2005).

Previous studies have shown that GABAergic tonic inhibition is present in the spinal dorsal horn (Ataka & Gu 2006, Takahashi *et al.* 2006, Maeda *et al.* 2010). It is generally believed that extrasynaptic GABA_A receptors (which contain the δ subunit) are responsible for the

generation of GABAergic tonic inhibition. It has been demonstrated that selective activation of the extrasynaptic GABA_A receptors with the δ GABA_A receptor-preferring agonist 4,5,6,7-tetrahydroisoxazolo [5,4-c]pyridine-3-ol (THIP) increases the tonic GABA inhibition, suppresses neuronal excitability in the spinal dorsal horn, and acute nociception in mice (Bonin *et al.* 2011). Currently, whether GABAergic tonic inhibition in the spinal dorsal horn is regulated by GABA transporters remains unexplored. Our study filled this gap by demonstrating that tonic GABA receptor activities in the spinal dorsal horn are regulated by GABA transporters both in normal and neuropathic pain induced by paclitaxel treatment.

Role of GABA transporters in the regulation of GABA receptor activities in the CNS

GABA transporters play an important role in the clearance and homeostasis of extracellular GABA in the CNS because no enzymes are available in the extracellular space to convert GABA into a biologically inert molecule (Zhou & Danbolt 2013). Among the four GABA transporters, GAT-1 and GAT-3 are widely expressed in the CNS (Melone *et al.* 2013, Guastella *et al.* 1990). Expression of GAT-1 and GAT-3 transporters is region-specific. In the forebrain area, GAT-1 is abundantly expressed in terminals of cortical neurons but not in thalamic, Purkinje and striatonigral synapses where GAT-3 is expressed mainly in astrocytes (Zhou & Danbolt 2013). Studies on the cellular location of GAT-1 and GAT-3 in the spinal dorsal horn are limited. In one study on the rat spinal trigeminal nucleus, GAT-1 and GAT-3 are expressed in astrocytes (Ng & Ong 2001). Expressions of GAT-1 in presynaptic terminals and GAT-3 in astrocytes in the mouse spinal dorsal horn were recently reported (Kim *et al.* 2014). Our current study on rats further confirmed that: a) GAT-1 is positioned at the presynaptic terminals but not at the neuronal cell body; b) GAT-1 is also expressed in astrocytes; c) GAT-3 is only expressed in astrocytes; d) neither GAT-1 nor GAT-3 is expressed in microglia.

Regulation of GABAergic tonic inhibition by GAT-1 and GAT-3 varies depending on different regions. For example, pharmacological inhibition of GAT-1 increases GABAergic tonic currents in the hippocampus (Nusser & Mody 2002) and cerebellum (Rossi *et al.* 2003) but not in the sensorimotor cortex (Keros & Hablitz 2005). When GAT-3 is inhibited, GABAergic tonic currents are increased in the supraoptic nucleus (Park *et al.* 2006) but not in the sensorimotor cortex (Keros & Hablitz 2005). A combined inhibition of GAT-1 and GAT-3 is required to significantly enhance tonic inhibition in the sensorimotor cortex (Keros & Hablitz 2005). The regulation of tonic inhibition in the spinal dorsal horn is unknown. Our study showed that under normal conditions, GABAergic tonic inhibition in the spinal dorsal horn was increased when GAT-1 but not GAT-3 was inhibited. Only under the condition when GAT-1 had been inhibited, further inhibition of GAT-3 increased GABAergic tonic inhibition. These data suggest that in comparison with GAT-3, GAT-1 is positioned in proximity to the presynaptic terminals, and the GABA uptake by GAT-1 is more powerful, which may result from the higher efficacy or number of GAT-1 transporters. Consistent with this notion, GAT-1 was found to be expressed in presynaptic terminals by us (Fig. 3) and others (Kim *et al.* 2014), and the global GABA uptake is increased in rats with the up-regulation of GAT-1 protein expression and down-regulation of GAT-3 expression (Fig. 5).

Protein expressions of GAT-1 and GAT-3 in the spinal dorsal horn are altered under different pathological pain conditions but controversy remains. Animals with neuropathic pain induced by chronic constriction of the sciatic nerve or spared nerve injury have an increased protein expression of GAT-1 in the spinal dorsal horn (Daemen *et al.* 2008) or the gracile nucleus (Gosselin *et al.* 2010). Using the same animal model, others have reported that the protein expression of GAT-1 in the same area is reduced (Shih *et al.* 2008, Miletic *et al.* 2003). Expressions of GAT-1 and GAT-3 transporters are increased in the spinal trigeminal nucleus in rats with inflammatory pain induced by carrageenan injection (Ng & Ong 2001). In another inflammatory pain model induced by formalin injection, global GABA uptake in the mouse spinal cord is increased at 20 min and 120 min after formalin injection (Hu *et al.* 2003). Our present study demonstrates that GAT-1 protein expression is increased while GAT-3 protein expression is reduced in the spinal dorsal horn in rats with P-INP, and these changes are concurrently associated with an increase of global GABA uptake at the same region. These data suggest that enhanced GABA uptake may in part contribute to the reduced GABAergic tonic inhibition in rats treated with paclitaxel. Targeting GABA transporters to produce analgesic effects has been reported. It was reported that latencies in the tail flick reflex (Hu *et al.* 2003) or the hot plate test (Kubo *et al.* 2009) were prolonged in mice receiving intraperitoneal injection of NO-711 (3 to 10 mg/kg). However, others showed that intrathecal application of GAT-1 inhibitor NO-711 (up to 100 µg) did not produce analgesic effects in the sham-operated control rats (Li *et al.* 2011). In agreement with this study, our findings show that intrathecal application of NO-711 (10 µg) does not alter mechanical and thermal thresholds of paw withdrawal responses, or motor functions in rats. Systemic or intrathecal administration of GAT-1 inhibitors ameliorates neuropathic pain induced by chronic constriction of the sciatic nerve (Daemen *et al.* 2008, Li *et al.* 2011), and the second phase nociceptive behaviors in the formalin pain model (Hu *et al.* 2003). Similarly, spinal inhibition of GAT-3 suppresses the second-phase response in the formalin pain model and attenuates mechanical allodynia induced by chronic constriction injury in rats (Kataoka *et al.* 2013). Currently, the regulation of GABAergic tonic inhibition by GABA transporters in pathological pain conditions has not been investigated. In this study, we found that the attenuation of GABAergic tonic inhibition in the spinal dorsal horn of rats with P-INP is significantly reversed by the GAT-1 inhibitor but not GAT-3 inhibitor. In intact animals, we demonstrated that inhibition of spinal GAT-1 ameliorates mechanical allodynia and thermal hyperalgesia induced by paclitaxel treatment.

Conclusions

In this study, we found that disinhibition in the spinal dorsal horn contributes to the genesis of P-INP. This is at least in part due to enhanced GABA uptake by GAT-1 in the spinal dorsal horn. Suppression of GAT-1 activities reverses disinhibition in the spinal dorsal horn and attenuates mechanical allodynia and thermal hyperalgesia induced by paclitaxel treatment. Together, these results suggest targeting GAT-1 activity may be useful for the development of therapeutics in P-INP.

Acknowledgments

This project was supported by the National Institute of Neurological Disorders and Stroke grant RO1NS064289 to H.R. Weng.

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Abbreviations

P-INP	Paclitaxel induced neuropathic pain
IPSCs	Inhibitory postsynaptic currents
GAT-1	GABA transporter-1
GAT-3	GABA transporter-3
DPI	Days post-intraperitoneal injection
i.p.	Intra-peritoneal
MAP2	Microtubule-associated protein 2
GFAP	glial fibrillary acidic protein
NeuN	Neuronal Nuclei
OX42	CD11b -cluster of differentiation molecule 11B
L4	Lumbar 4
L5	Lumbar 5
TRPV1	transient receptor potential cation channel, subfamily V, member 1
CX3CL1	chemokine (C-X3-C motif) ligand 1
DRG	Dorsal root ganglion
GSK3β	Glycogen synthase kinase 3 beta
KCC2	K-Cl cotransporter

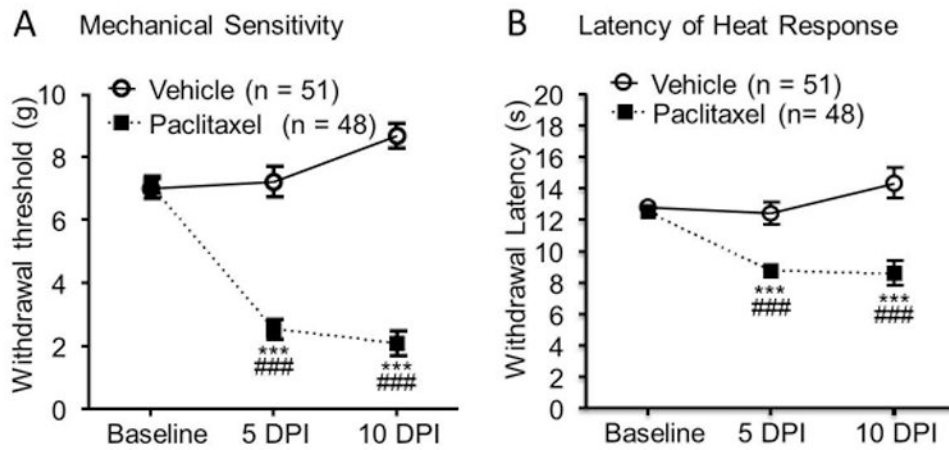


Figure 1. Rats treated with paclitaxel develop mechanical allodynia and thermal hyperalgesia
 Rats were injected (i.p.) with either vehicle or paclitaxel (2.0 mg/kg) on four alternate days (days 1, 3, 5 and 7). The mean (\pm SEM) mechanical thresholds of hind paw withdrawal responses (A) and latencies of the withdrawal response to heat stimuli (B) prior to the i.p. injection (Baseline), and on 5 days (5 DPI) and 10 days (10 DPI) post the first injection were plotted. Comparisons of data between baseline and each time point in the paclitaxel group are labeled with *. Comparisons of data between the vehicle group and the paclitaxel group at each time point are indicated with #. Three symbols, $P < 0.001$.

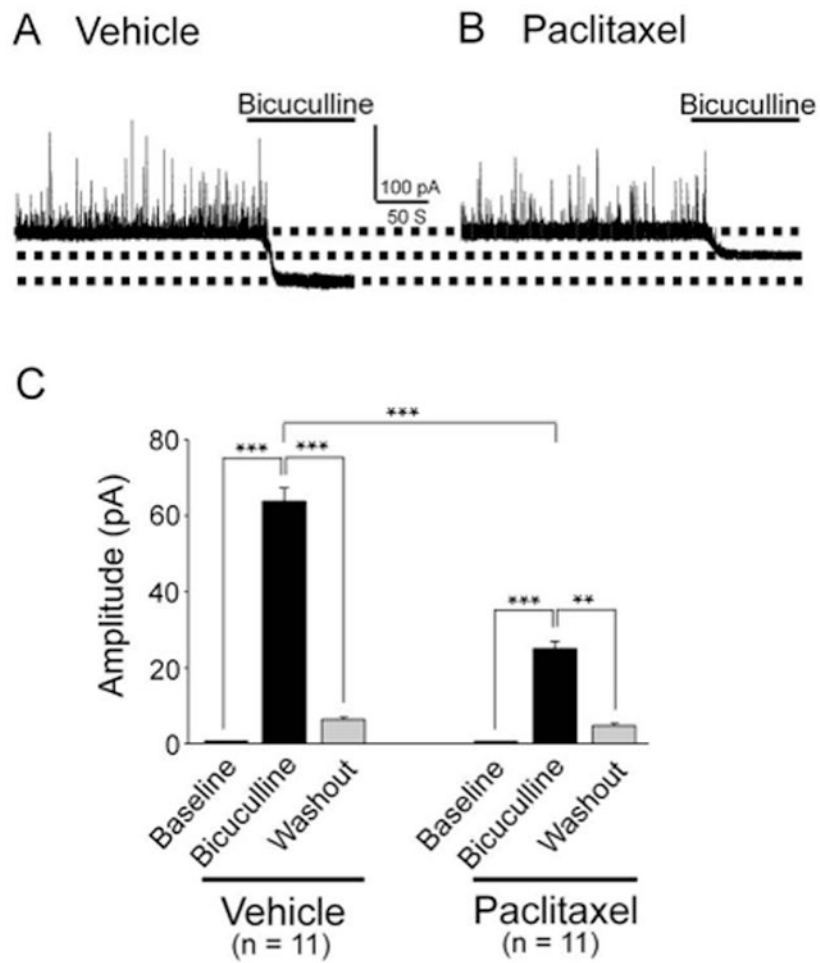


Figure 2. GABAergic tonic inhibition in the spinal dorsal horn is weakened in the paclitaxel-treated rats

Raw data show recordings of GABAergic currents before and after blocking of GABA_A receptors with bicuculline (25 μ M) obtained from vehicle (A) and paclitaxel (B) treated rats. (C) Shows the mean (\pm SEM) changes of holding currents induced by bicuculline in vehicle and paclitaxel treated rats. ** $P < 0.01$; *** $P < 0.001$.

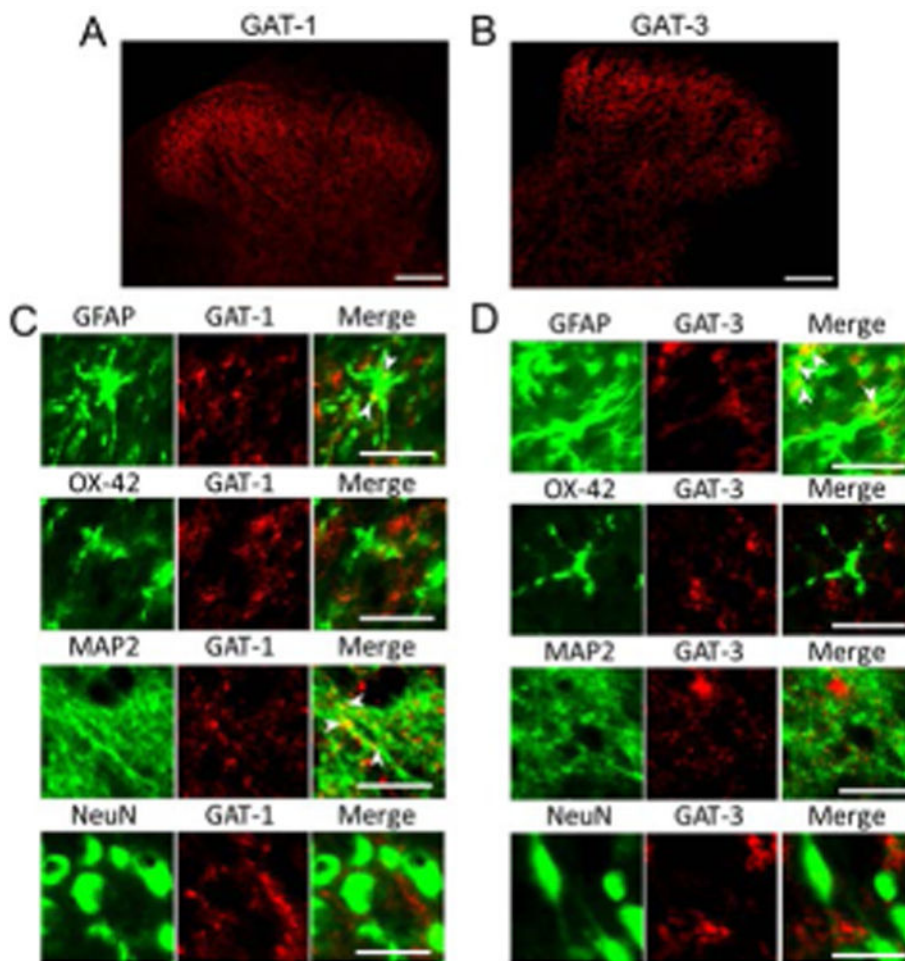


Figure 3. GAT-1 is expressed in neurons and astrocytes and GAT-3 only is present in astrocytes Samples were obtained from the spinal dorsal horn of normal control rats. (A) and (B) respectively show staining of GAT-1 and GAT-3 in the spinal dorsal horn, note higher expressions of GAT-1 and GAT-3 at the superficial dorsal horn (Scale bar = 100 μ M). (C) Shows that expression of GAT-1 is colocalized with MAP2 (a marker for neuronal cytoskeleton) and GFAP (an astrocyte marker), but not with NeuN (a neuronal cell body marker) or OX42 (a microglia marker) (Scale bar = 20 μ M). (D) Shows that GAT-3 is predominantly colocalized with GFAP, but not OX42, MAP2, or NeuN (Scale bar = 20 μ M).

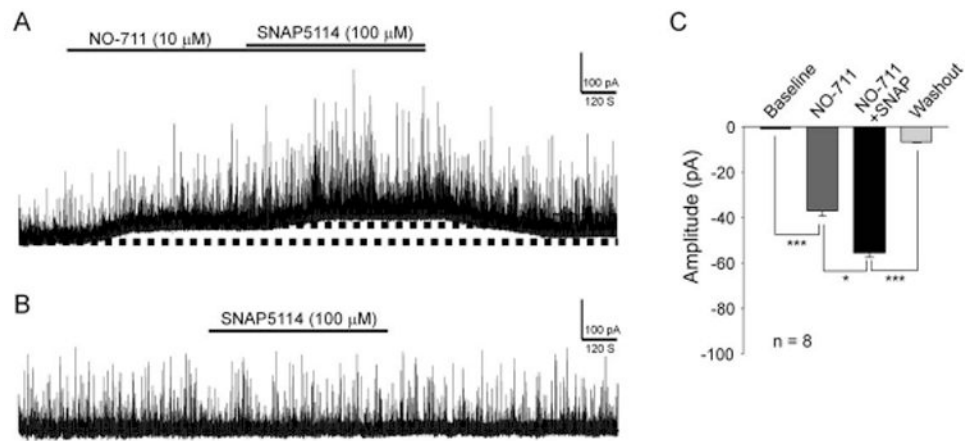


Figure 4. Tonic GABA receptor activities in the spinal dorsal horn are mainly regulated by GAT-1 under normal conditions

Data were obtained from normal control rats. (A) Shows recordings of GABAergic currents before and after blocking of GAT-1 with NO-711 (10 μM) and then further inhibition of GAT-3 with SNAP5114 (100 μM). (B) Shows recordings of GABAergic currents before and after blocking of GAT-3 alone with SNAP5114 (100 μM). (C) Shows the mean (± SEM) changes of holding currents induced by NO-711 and then NO-711 plus SNAP5114. ** $P < 0.01$; *** $P < 0.001$.

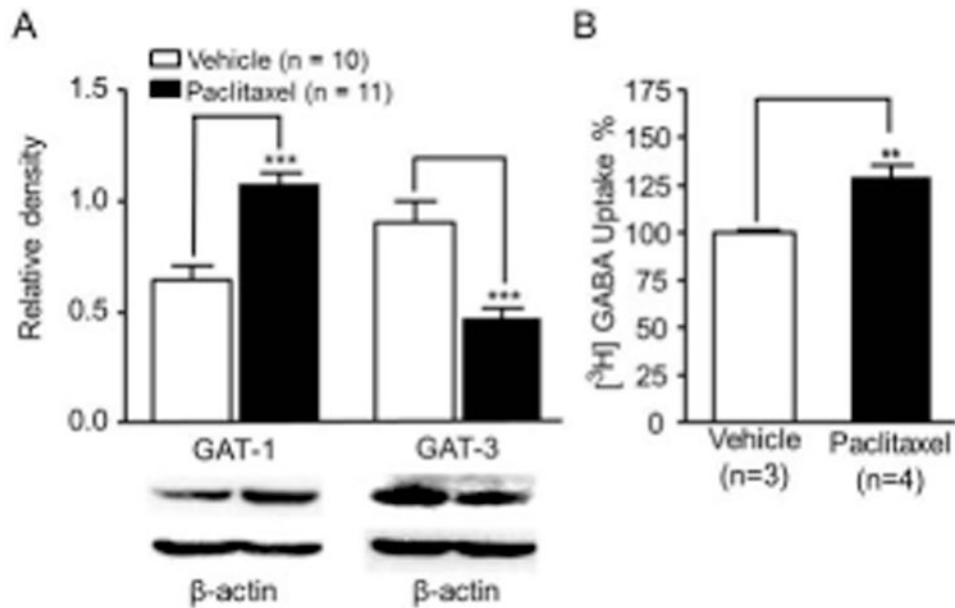


Figure 5. Rats with P-INP have an increased protein expression of GAT-1 and reduced protein expression of GAT-3, which was accompanied with increased GABA uptake activities
 (A) Shows the mean (\pm SEM) relative density of GAT-1 and GAT-3 to β -Actin in the spinal dorsal horn of rats treated with vehicle and paclitaxel. Samples of GAT-1 and GAT-3 expressions in the spinal dorsal horn at the L4 to L5 segment in paclitaxel and vehicle treated rats are shown. (B) Shows the mean (\pm SEM) GABA uptake activities obtained from the spinal dorsal horn of rats receiving vehicle and paclitaxel treatment. The GABA uptake activities in the synaptosome preparation from rats treated with paclitaxel treatment were normalized with those treated with vehicle measured at the same time. ** $P < 0.01$; *** $P < 0.001$.

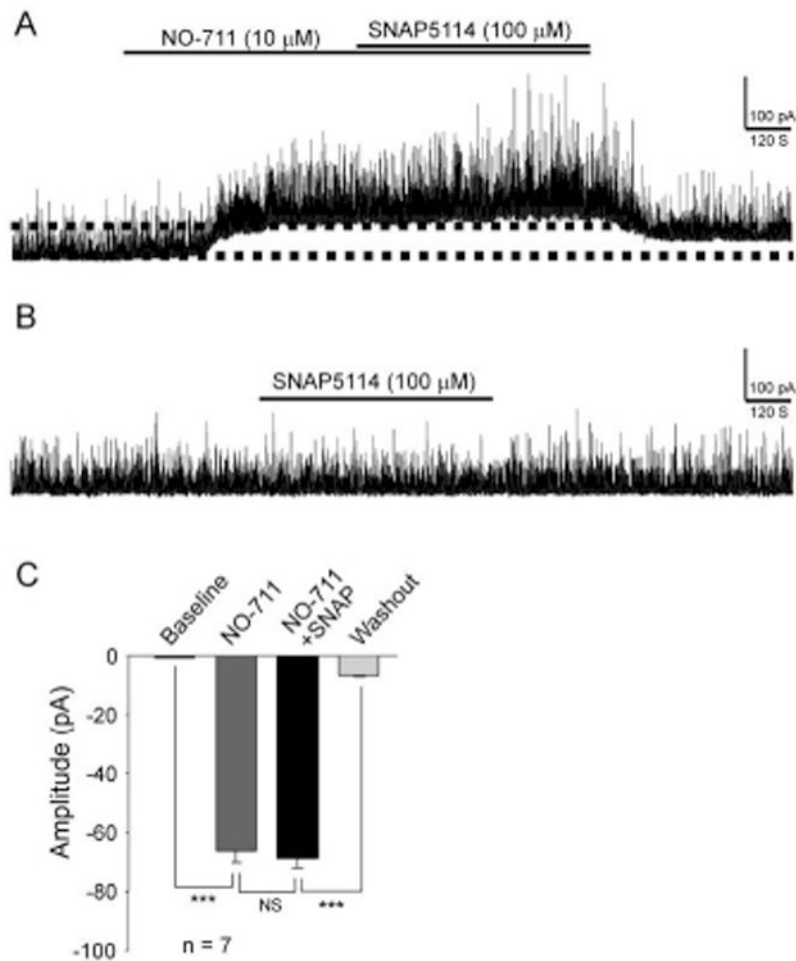


Figure 6. The paclitaxel-induced GABAergic disinhibition is ameliorated by blocking GAT-1 but not GAT-3

The original recordings in (A) and (B) were obtained from rats with P-INP. (A) Shows recordings of GABAergic currents before and after blocking of GAT-1 with NO711 (10 μM) and then further inhibition of GAT-3 with SNAP5114 (100 μM). (B) Shows recordings of GABAergic currents before and after blocking of GAT-3 alone with SNAP5114 (100 μM). (C) Shows mean (\pm SEM) changes of holding currents induced by NO-711 and then NO-711 plus SNAP5114 in rats treated with paclitaxel. ** $P < 0.01$; *** $P < 0.001$.

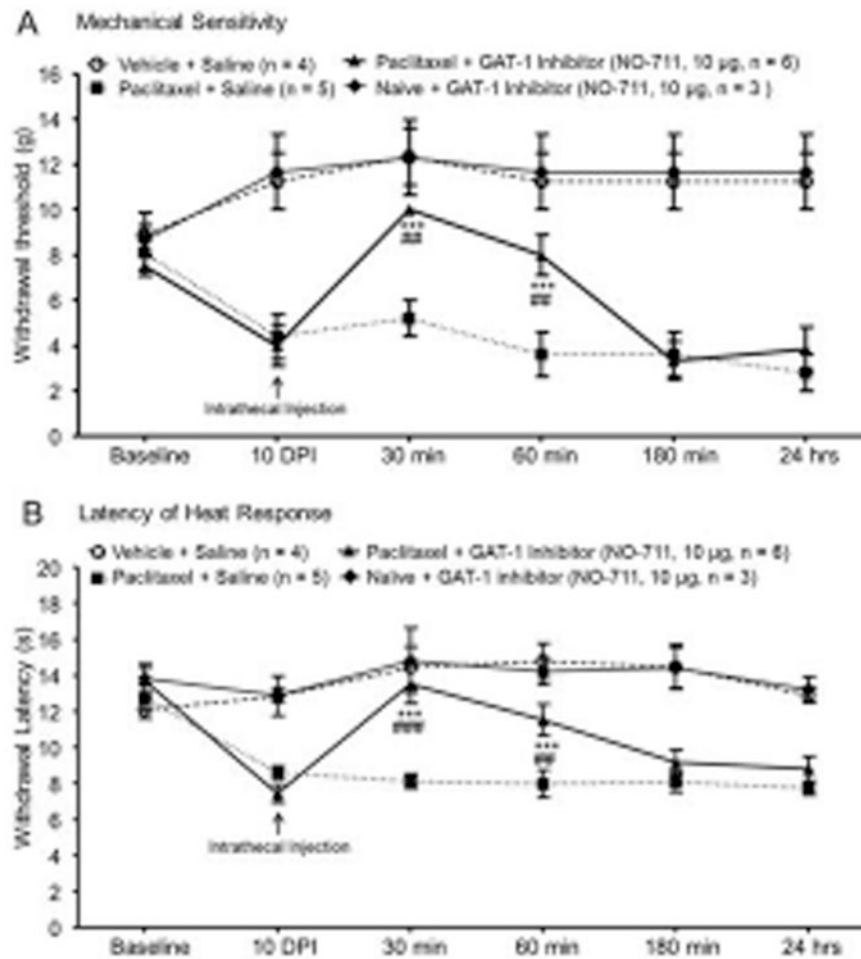


Figure 7. Mechanical allodynia and thermal hyperalgesia in rats with P-INP are attenuated by an intrathecal injection of a GAT-1 inhibitor

Line plots show measurements of mechanical thresholds of hind paw withdrawal responses (A) and latencies of the withdrawal response to heat stimuli (B) collected at baseline, 10 days post-i.p. vehicle or paclitaxel injection (10 DPI), and then 30, 60, 180 min and 24 hours after the intrathecal administration of the tested agent. Baseline indicates the measurement before animals received i.p. vehicle or paclitaxel injection. Comparisons between data collected on 10 DPI and at following each time point are indicated with * for the paclitaxel +GAT-1 inhibitor group. Comparisons between the paclitaxel+saline group and paclitaxel +GAT-1 inhibitor group are labeled with #. Two symbols: $P < 0.01$, three symbols: $P < 0.001$, data shows mean (\pm SEM).