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Amitriptyline prevents thermal hyperalgesia and modifications in rat spinal cord $GABA_B$ receptor expression and function in an animal model of neuropathic pain

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GABA, γ-aminobutyric acid
$GABA_B$, γ-aminobutyric acid-B receptors
$GABA_{B(1a)}$, $GABA_{B(1b)}$, $GABA_{B(2)}$, γ-aminobutyric acid-B receptor subunits
GTP, guanosine triphosphate

ABSTRACT

Using an animal model of neuropathic pain, behavioral and biochemical experiments were performed to assess the effects of this condition on pain threshold and $GABA_B$ receptor sensitivity and subunit gene expression in the rat lumbar spinal cord. The results indicate that partial sciatic nerve ligation decreases thermal and mechanical pain withdrawal latencies, and increases baclofen-stimulated [^{35}S]GTPγS binding and $GABA_B$ receptor subunit gene expression in the rat lumbar spinal cord, suggesting that neuropathic pain may be due, in part, to a deficiency in GABAergic transmission. The experiments also demonstrate that daily administration (10 mg/kg, i.p.) of amitriptyline, a tricyclic antidepressant used for the treatment of neuropathic pain, for 1 week after surgery prevents the decline in thermal pain threshold, the increase in $GABA_{B(2)}$ gene expression, and development of increased $GABA_B$ receptor function in spinal cord resulting from nerve damage. These findings indicate that the efficacy of amitriptyline as a treatment for neuropathic pain may be related to an ability to maintain spinal cord $GABA_B$ receptor activity.

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1. Introduction

Neuropathic pain is characterized by neuronal hyperexcitability secondary to sensory nerve damage [1–3]. Symptoms include numbing, tingling and burning pain, hyperalgesia or hypoalgesia, and allodynia. The sensitization of central and peripheral nerve pathways resulting from neuropathies is due to the release of various neurotransmitters, neuromodulators, cytokines, and other substances [2]. Prolonged exposure to these endogenous agents modifies expression of cellular elements, such as transmitter receptors and ion channels, which regulate neuronal activity [1]. Included are changes in the number or function of $Ca_v\alpha_2\delta_{-1}$ channel subunits, the $Na_v1.3$ and 1.8 channels, bradykinin-1 and -2, capsaicin TRPV1, substance P, μ-opioid and glutamic acid NMDA receptors [1,4,5]. In addition, spinal cord inhibitory systems are compromised, with a loss of γ-aminobutyric acid (GABA) transporters and neurons, and a decrease in GABA levels associated with this condition [6–9]. While several drug classes, including antidepressants, such as amitriptyline, as well as antiepileptics, opioids, and topical anesthetics are used to treat this syndrome, none, either alone or in combination, is universally effective [2,3,10–14]. Thus, the quest continues to fully characterize the cellular changes responsible for generating and maintaining the sensory dysfunction associated with neuropathic pain in an attempt to design new therapeutic approaches for treating this disorder.

Chronic, but not acute, administration of antidepressants increases thermal withdrawal thresholds, modifies the $GABA_B$ receptor response to agonist, and alters subunit gene expression in the rat spinal cord and hippocampus [15–20]. These findings led to the suggestion that one or more of these effects may be an important component in the mechanism of action of this drug class as treatments for neuropathic pain [15]. Indeed, a role for GABA in pain transmission and perception has been apparent for some time, with both $GABA_A$ and $GABA_B$ receptor agonists being antinociceptive in a variety of animal models. Moreover, alterations in spinal cord $GABA_B$ receptor function and subunit expression are associated with persistent inflammatory pain, and baclofen, a $GABA_B$ receptor agonist, displays some efficacy in animal models of neuropathic pain and in the clinical management of this syndrome [21–28]. Furthermore, hyperalgesia is a phenotype associated with deletion of the $GABA_{B(1)}$ subunit, suggesting an important role for $GABA_B$ receptors in regulating pain threshold [29].

Although various aspects of GABA transmission have been examined in animal models of neuropathic pain [6–9], little is known about the effects of peripheral nerve damage on $GABA_B$ receptor function and subunit gene expression. The present study was undertaken to examine this issue. The results indicate that amitriptyline administration prevents the thermal hyperalgesia, but not the mechanical allodynia, that develops following partial sciatic nerve ligation in rat, and blocks the enhancement of dorsal horn $GABA_B$ receptor function that develops in this animal model of persistent pain. The ability to normalize alterations in spinal cord $GABA_B$ receptor activity resulting from peripheral nerve damage may be an important component in the mechanism of action of amitriptyline, and perhaps other agents, as treatments for neuropathic pain.

2. Materials and methods

2.1. Animals, surgical procedure, and drug administration

All animal protocols were approved by the University of Kansas Medical Center Animal Care and Use Committee. Male, Harlan Sprague-Dawley rats (200–250 g) (Indianapolis, IN) were divided randomly into three groups. Control animals were not subjected to anesthesia or surgery. Animals in both the ligated and sham groups were anesthetized by i.p. administration of 65 mg/kg ketamine HCl and 5.5 mg/kg xylazine HCl. The left sciatic nerve was exposed and dissected from surrounding connective tissue near the trochanter, just distal to the branching point of the posterior biceps semitendinosus nerve. Povidone iodine was applied externally to the area of incision before and after surgery, and directly on the wound before closure. In the sham-operated group, the sciatic nerve was isolated but not ligated, whereas in the neuropathic pain group 1/3 to 1/2 the diameter of the nerve was tightly ligated with 8–0 silk suture [30]. After closure an antibiotic ointment containing bacitracin, neomycin, and polymyxin B, was applied to the wound, and the animals placed in their home cages. Beginning 24 h after surgery, amitriptyline (10 mg/kg, i.p.), or an equivalent volume of vehicle (water), was administered to members of the sham and ligated groups, and vehicle only to the control animals, once daily for 7 consecutive days. This amitriptyline dosing regimen was selected on the basis of previous work showing it induces behavioral and neurochemical changes in rat [15].

Thermal and mechanical pain thresholds were quantified in all three groups on the seventh day after surgery, 1 h after administration of the last dose of amitriptyline or vehicle. Approximately 1 h after the behavioral tests, the animals were sacrificed by decapitation and their spinal cords rapidly removed by forceful injection (60 ml syringe and 16 gauge needle) of ice-cold isotonic saline into the caudal end of the vertebral canal. The isolated cords were immediately snap-frozen for subsequent mRNA or [^{35}S]GTPγS binding analyses.

2.2. Behavioral testing

The rat hind paw withdrawal response to noxious thermal stimulation was quantified using a radiant thermal analgesiometer [31]. For the test, animals were placed in a Plexiglas chamber and, after a 10–15-min period of acclimation to ensure all four paws were resting comfortably on the floor of the apparatus, were exposed to a thermal stimulus generated by a high-intensity light beam focused on the skin of the right or left hind paw. The results are expressed as the amount of time in seconds that elapsed between activation of the light beam and removal of the paw from the heat source by the animal (withdrawal latency).

For testing mechanical allodynia, von Frey monofilaments (Stoelting Inc., Wood Dale, IL) of graded bending forces (2.6–522 mN) were applied to the plantar aspect of the hind paws of unrestrained rats placed in a Plexiglas chamber with a wire mesh grid bottom. Monofilaments were applied perpendicular to the hind paw surface with sufficient force to cause a slight bending of the filament in increasing order of intensity until the rat responded by vocalization or brisk withdrawal of the

paw. Mechanical stimulation was repeated three times at 5–10 min intervals, with the order of testing randomized for each paw [32]. Monofilament thresholds were converted to grams of force using the manufacturer's table.

For the response to the thermal stimulus the results are displayed as withdrawal latency in seconds, whereas data from the mechanical test are shown as withdrawal threshold in grams of force. Thermal test results were compared by SuperAnova (Abacus Concepts, Berkeley, CA) using a one-way ANOVA with Fisher's PLSD post hoc, whereas mechanical threshold data were analyzed using ANOVA with Wilcoxson Signed-rank tests (for non-continuous data) employing Systat 10.2 software (Point Richmond, CA). Differences between means were considered significant with $P < 0.05$.

2.3. Measurement of subunit gene expression

Total RNA, which was isolated from spinal cord tissue taken from the dorsal half of the lumbar enlargement (L_I-L_{VI}) ipsilateral to the ligated nerve, was analyzed for $GABA_B$ receptor subunit and β-actin mRNAs using solution hybridization–nuclease protection assays [19,22]. Briefly, 25–50 μg samples of total RNAs were assayed for $GABA_B$ receptor subunits and 5 μg total RNA assayed for β-actin mRNA expression. Twenty to one hundred picograms of cRNA quantification samples were used to generate a standard curve, with *E. coli* tRNA as a negative control. Digested samples were electrophoresed on denaturing gels containing 7 M urea. The gels were fixed, dried, and exposed to phosphor plates for 16–24 h. The resulting densitometric images were analyzed using a Molecular Dynamics PhosphorImager SF (Sunnyvale, CA). Specific mRNA amounts were determined by comparison to cRNA standards and analyzed using IP Lab Gel (Signal Analytics, Vienna, VA). The results, which are displayed as pg-specific mRNA/ng β-actin mRNA, are compared among groups by SuperAnova using a one-way ANOVA with Fisher's PLSD post hoc.

2.4. Measurement of $GABA_B$ receptor function

Baclofen-stimulated [^{35}S]GTPγS binding in the dorsal horn of the rat spinal cord ipsilateral to the partially ligated nerve was used as a measure of $GABA_B$ receptor function [15,18]. For the assay, 20 μm transverse sections from between L_I and L_{VI} of the snap-frozen spinal cords were thaw-mounted onto gelatin-coated slides, air-dried, then stored at −70 °C until use. On the day of testing, the slides were brought to room temperature and placed for 10 min into the assay buffer, which consisted of 3 mM $MgCl_2$, 120 mM NaCl, 0.2 mM EGTA, and 50 mM Tris. Following this incubation, the tissues were transferred for 15 min into assay buffer containing 2 mM GDP. The tissue slices were then incubated at room temperature for 2 h in one of three types of buffer. The buffer used to quantify total baclofen-stimulated [^{35}S]GTPγS binding consisted of 2 mM GDP, 0.1 nM [^{35}S]GTPγS, and 100 μM baclofen, a saturating concentration of this $GABA_B$ receptor agonist. Nonspecific and basal binding of the radiolabeled material were assessed by placing tissues in buffer devoid of baclofen but containing 10 μM unlabeled GTPγS or water, respectively. Following 2 h of incubation the tissue slices were rinsed twice with ice-cold 50 mM Tris–HCl, pH 7.4, and once with distilled water. After air-drying overnight, the slides were apposed to Kodak MR autoradiography film for 2 days and then placed in a Kodak film developer. Densitometric analysis (Scion Image) was used to quantify radioligand binding. Background levels from the film and nonspecific binding to the tissue were subtracted from all sections. The data are displayed as percent stimulation of [^{35}S]GTPγS binding over basal, and compared by SuperAnova using a one-way ANOVA with Fisher's PLSD post hoc. Differences between means were considered statistically significant when $P < 0.05$.

2.5. Materials and supplies

Amitriptyline HCl, unlabeled GTPγS, and guanosine diphosphate were purchased from Sigma Chemical Co., St. Louis, MO, and [^{35}S]GTPγS (1250 Ci/mmol) from Amersham Pharmacia Biotech, Piscataway, NJ.

3. Results

3.1. Partial sciatic nerve ligation and pain threshold

A significant reduction in rat hind paw withdrawal latency in response to a thermal stimulus was noted 7 days following either partial sciatic nerve ligation or an identical surgery without nerve ligation (sham) as compared to controls (Fig. 1). In contrast, no difference in thermal stimulus withdrawal latency was noted between these subjects and controls when

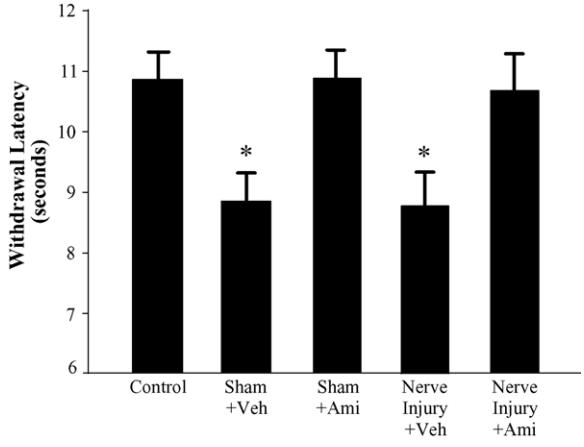

Fig. 1 – Thermal withdrawal latencies in the rat hindpaw 7 days after sham surgery or partial nerve ligation. Some animals received daily i.p. injections of 10 mg/kg amitriptyline (Ami) or an equivalent volume of water (Veh) beginning 24 h after surgery and continuing for 7 consecutive days. Thermal withdrawal latencies were assessed 1 h after the final injection of drug or vehicle. Unlike the sham and nerve injured groups, control animals did not undergo general anesthesia or surgery to isolate the sciatic nerve, although they did receive daily injections of vehicle. The height of each bar represents the mean ± S.E.M. of results obtained from 10 animals. *$P < 0.05$ compared to control values.

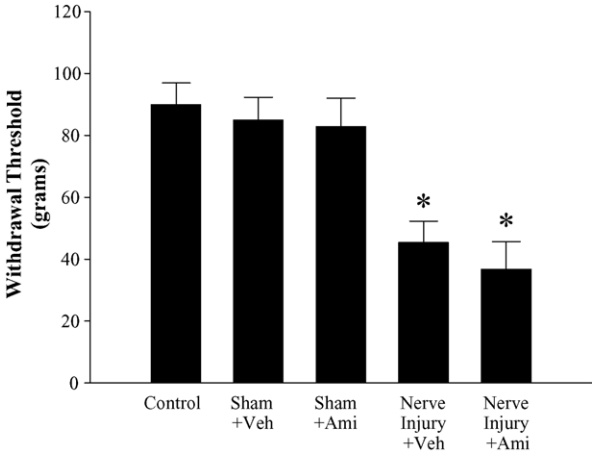

Fig. 2 – Mechanical withdrawal threshold in the rat hind paw 7 days after sham or partial nerve ligation (nerve injured) surgery. Some animals received daily i.p. injections of 10 mg/kg amitriptyline (Ami) or an equivalent volume of water (Veh) beginning 24 h after surgery and continuing for 7 consecutive days. Mechanical withdrawal thresholds were assessed 1 h after the final injection of drug or vehicle. Unlike the sham and nerve injured groups, control animals did not undergo general anesthesia or surgery to isolate the sciatic nerve, although they did receive daily injections of vehicle. The height of each bar represents the mean ± S.E.M. of results obtained from 10 animals. $^{*}P < 0.05$ compared to control values.

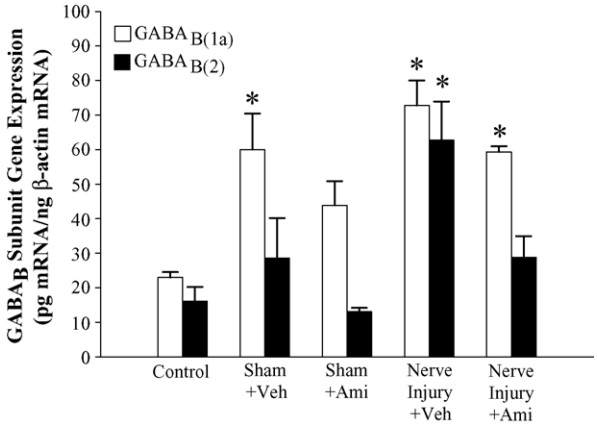

Fig. 3 – Effect of amitriptyline (Ami) administration, partial sciatic nerve ligation (nerve injury) and sham surgery (Sham) on $GABA_B$ receptor subunit gene expression in the rat lumbar dorsal spinal cord ipsilateral to the nerve injury. Some animals received daily i.p. injections of 10 mg/kg amitriptyline or an equivalent volume of water (Veh) beginning 24 h after surgery and continuing for 7 consecutive days. Unlike the sham and nerve-injured groups, control animals did not undergo general anesthesia or surgery to isolate the sciatic nerve, although they did receive daily injections of vehicle. The animals were sacrificed 7 days after sham surgery or partial nerve ligation, within approximately 1 h after measurement of thermal and mechanical pain thresholds. The height of each bar represents the mean ± S.E.M. of results obtained from 3 to 10 animals. $^{*}P < 0.05$ compared to control values.

the nerve injured or sham animals were administered 10 mg/kg amitriptyline i.p. once daily for 7 consecutive days (Fig. 1). In all cases, the withdrawal threshold was measured 1 h after administration of the final dose of amitriptyline or vehicle.

Although mechanical withdrawal threshold was lowered significantly 7 days following partial sciatic nerve ligation, it was not modified in the sham animals as compared to controls (Fig. 2). The i.p. administration of 10 mg/kg amitriptyline for 7 consecutive days had no significant effect on the nerve injury-induced reduction in mechanical withdrawal threshold, which was measured 1 h after administration of the final dose of antidepressant or vehicle (Fig. 2).

3.2. *Effect of partial sciatic nerve ligation on $GABA_B$ receptor subunit expression*

The animals were sacrificed by decapitation within 1 h of the pain threshold measurement 7 days after surgery. Solution-hybridization–nuclease protection assays conducted on total RNAs isolated from lumbar spinal cord tissue ipsilateral to the nerve injury revealed an increase in $GABA_{B(1a)}$ subunit gene expression relative to controls in both the sham operated and partial nerve ligation subjects (Fig. 3). While $GABA_{B(2)}$ subunit gene expression is unchanged in the sham animals, it increased significantly following partial nerve ligation. Daily administration of amitriptyline during the 1-week period following sham surgery or nerve injury prevented the increase in $GABA_{B(1a)}$ subunit gene expression in the sham group, but not in the neuropathic animals, and blocked the increase in $GABA_{B(2)}$ subunit gene expression in those subjected to partial nerve ligation (Fig. 3).

3.3. *Effect of partial sciatic nerve ligation on $GABA_B$ receptor sensitivity*

Seven days following partial sciatic nerve ligation, there was a significant increase in baclofen-stimulated [^{35}S]GTPγS binding in the spinal cord dorsal horn ipsilateral to the nerve injury as compared to control animals (Fig. 4). This effect of nerve damage was prevented by the daily administration of 10 mg/kg amitriptyline for 7 consecutive days following surgery. Neither sham surgery alone, nor the daily injection of amitriptyline to sham-operated animals, had any effect on baclofen-stimulated [^{35}S]GTPγS binding in the spinal cord dorsal horn (Fig. 4).

4. Discussion

The development and persistence of neuropathic pain are thought to be due to changes in cellular elements that regulate neuronal sensitivity [1–5]. Using animal models of this condition it has been shown that the number and function of various central and peripheral nervous system ion channels and neurotransmitter receptors are modified as a consequence of nerve damage [1,4,5,6–9]. The results of the present

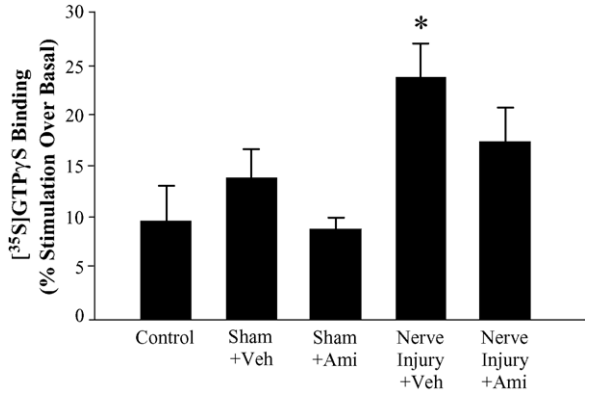

Fig. 4 – Effects of partial nerve ligation (nerve injury), sham surgery (Sham), and amitriptyline (Ami) administration on baclofen (100 μM)-stimulated [^{35}S]GTPγS binding in the dorsal horn of the rat spinal cord ipsilateral to the nerve injury. Some animals received daily i.p. injections of 10 mg/kg amitriptyline or an equivalent volume of water (Veh) beginning 24 h after surgery and continuing for 7 consecutive days. Unlike the sham and nerve injured groups, control animals did not undergo general anesthesia or surgery to isolate the sciatic nerve, although they did receive daily injections of vehicle. The animals were sacrificed 7 days after sham surgery or partial nerve ligation, approximately 1 h after measurement of the thermal and mechanical pain thresholds. The height of each bar represents the mean ± S.E.M. of results obtained from 4 to 5 animals. $^{*}P < 0.05$ compared to control values.

study indicate that alterations in $GABA_B$ receptor function and subunit expression can be added to this list.

The current work reveals that both thermal and mechanical withdrawal thresholds are lowered significantly in rats 7 days following a partial ligation of the sciatic nerve, while this nerve damage increases the gene expression of both the $GABA_{B(1a)}$ and $GABA_{B(2)}$ in the ipsilateral lumbar spinal cord and enhances baclofen-stimulated [^{35}S]GTPγS binding in the ipsilateral lumbar dorsal horn. Together, these findings suggest that nerve damage sufficient to lower the pain threshold increases the function of spinal cord $GABA_B$ receptors. As it has been shown previously that neuropathic pain is associated with a reduction in spinal cord GABAergic neurons and GABA content [6–9], and since $GABA_B$ receptor agonists display antinociceptive activity in humans and in animal models of neuropathic pain [21,23–28], the observed increase in the gene expression of $GABA_B$ receptor subunits and the apparent increased production of functional $GABA_B$ receptors may reflect a compensatory change in response to the loss of GABAergic tone. The likelihood that formation of new $GABA_B$ receptors is responsible for the enhanced function is supported by the finding that nerve injury increases gene expression of both $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits, a prerequisite for elaboration of this heterodimeric receptor [33–35]. This interpretation is supported by reports that pain-induced changes in $GABA_B$ subunit gene expression are associated with concomitant changes in subunit protein, and that prolonged administration of a $GABA_B$ receptor antagonist leads to an up-regulation of the $GABA_B$ receptor system [19,36]. However, since protein was not measured in the present study, it is impossible to know whether the increase in [^{35}S]GTPγS in response to a saturating concentration of baclofen reflects a change in receptor number, or efficiency in receptor–effector coupling, following nerve injury.

As with nerve injury, sham surgery causes a reduction in thermal withdrawal latency and an increase in $GABA_{B(1a)}$ subunit gene expression. However, the two groups differ in that the sham animals, unlike the ligated subjects, do not display a change in responsiveness to mechanical stimulation, an increase in the gene expression of the $GABA_{B(2)}$ subunit, or an enhancement in baclofen-stimulated [^{35}S]GTPγS binding in the dorsal horn. These results indicate that although the surgical procedure itself causes thermal hyperalgesia, presumably in association with inflammation resulting from the skin incision, the probing necessary to isolate the sciatic nerve, and the closing of the wound, there are qualitative differences between the partial ligation and sham animals in the surgical sequela. The finding that persistent inflammatory pain modifies spinal cord $GABA_B$ receptor subunit gene expression is in accord with earlier reports [16,19,22].

Tricyclic antidepressants, in particular amitriptyline, are among the drugs of choice for the management of neuropathic pain [10–14]. The present findings reveal that when amitriptyline is administered once daily beginning 24 h after partial sciatic nerve ligation and throughout the following week, it prevents some of the behavioral and biochemical changes induced by this trauma. With regard to pain threshold, amitriptyline injection blocks the nerve damage-induced decline in withdrawal latency to a thermal stimulus, but is without effect on the increased sensitivity to mechanical stimulation. This corresponds with earlier reports indicating this drug moderates nerve injury-induced changes in thermal hypersensitivity but not in mechanical allodynia [37,38]. The current data also indicate that amitriptyline administration prevents the increase in $GABA_B$ receptor function, as measured by [^{35}S]GTPγS binding, that develops following nerve damage. This may be explained by the finding that continuous daily injections of amitriptyline block the partial nerve ligation-induced increase in $GABA_{B(2)}$ gene expression, thereby preventing the increased formation of heterodimeric $GABA_B$ receptors. However, in the absence of information regarding the production of subunit protein, such an interpretation remains speculative. In contrast to the $GABA_{B(2)}$ subunit, amitriptyline administration did not affect the increase in $GABA_{B(1a)}$ gene expression that occurs following partial nerve ligation, although it did attenuate the increase in the expression of this subunit that occurs in conjunction with sham surgery. As $GABA_{B(1a)}$ gene expression in spinal cord is known to increase in response to a variety of stimuli, including pain, stress, and drug treatments, it is thought to be a marker for alterations in neuronal activity in addition to its role as a $GABA_B$ receptor subunit [15,17–19].

The present data could be interpreted as indicating that an amitriptyline-induced increase in GABAergic activity following nerve damage might account for the lack of change in $GABA_{B(2)}$ subunit gene expression and $GABA_B$ receptor function, since there would be no need for a compensatory increase in the production of these cellular components.

Support for this hypothesis is provided by the report that administration of a $GABA_B$ receptor antagonist inhibits tricyclic antidepressant-induced analgesia following nerve ligation [39]. As amitriptyline is not known to directly affect neuronal GABA uptake, synthesis, release, or metabolism, nor directly interact with $GABA_B$ receptors, the enhancement in GABAergic transmission is presumably secondary to one or more of the effects of amitriptyline on monoamine uptake, cholinergic, adrenergic, and histaminergic transmission, and on neuronal Na^+, Ca^{++}, and K^+ channel activity [40]. A relationship between GABAergic activity and central nervous system monoaminergic pathways has been reported [41].

Using a similar treatment protocol, earlier studies revealed that administration of amitriptyline, and other antidepressants, to rats not subjected to partial nerve ligation increases $GABA_{B(1a)}$ subunit gene expression, has a variable effect on the expression of other $GABA_B$ receptor subunits, and increases $GABA_B$ receptor function in rat lumbar spinal cord and hippocampus [15,17,18,20]. That amitriptyline is found in the current study to block the neuropathy-induced increase in $GABA_B$ receptor activity suggests that the neurochemical and cellular changes occurring as a result of nerve damage influence the effect of amitriptyline on spinal cord GABAergic tone. This demonstrates the importance of studying drug responses in animal models of the clinical condition, since they may differ substantially from those obtained in normal subjects.

The results of this study indicate that $GABA_B$ receptor function and subunit gene expression are increased in the rat lumbar spinal cord following partial sciatic nerve ligation, suggesting that neuropathic pain may be due, in part, to a deficiency in GABAergic transmission. Furthermore, the present work reveals that amitriptyline administration prevents enhancement of the $GABA_B$ receptor response to baclofen that occurs as a result of nerve damage, pointing to the possibility that it blocks the partial nerve ligation-induced decline in synaptic GABA activity. These findings indicate that maintenance of $GABA_B$ receptor function may be an important component in the clinical response to amitriptyline as a treatment for neuropathic pain.

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