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Relationship between the antinociceptive response to desipramine and changes in $GABA_B$ receptor function and subunit expression in the dorsal horn of the rat spinal cord

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Abstract

Although tricyclic antidepressants are among the drugs of choice for the treatment of neuropathic pain, their mechanism of action in this regard remains unknown. Because previous reports suggest these agents may influence γ-aminobutyric acid (GABA) neurotransmission, and $GABA_B$ receptors are known to participate in the transmission of pain impulses, the present experiments were undertaken to examine whether the administration of desipramine alters $GABA_B$ receptor subunit expression and function in the dorsal horn of the rat spinal cord. For the study, rats were injected (i.p.) once daily with desipramine (15 mg/kg) for 7 consecutive days, during which their thermal withdrawal threshold was monitored, and after which $GABA_B$ receptor function, and the levels of $GABA_B$ receptor subunit mRNA, were quantified in the spinal cord dorsal horn. The results indicate that 4–7 days of continuous administration of desipramine are necessary to observe a significant increase in the thermal pain threshold. Moreover, it was found that 7 days of treatment with desipramine enhances $GABA_B$ receptor function, as measured by baclofen-stimulated [^{35}S]GTPγS binding, and increases mRNA expression for the $GABA_{B(1a)}$ and $GABA_{B(2)}$, but not $GABA_{B(1b)}$, subunits. These findings suggest the antinociceptive effect of desipramine is accompanied by a change in spinal cord $GABA_B$ receptor sensitivity that could be an important component in the analgesic response to this agent.

Keywords: $GABA_B$ receptors; Tricyclic antidepressants; Desipramine; Nociception; Neuropathic pain; [^{35}S]GTPγS binding

1. Introduction

Neuropathic pain, which results from injury to a peripheral nerve or the central nervous system, is associated with a number of clinical conditions, including diabetic neuropathy, postherpetic neuralgia, trigeminal neuralgia, and stroke [1,2]. Drug classes of some benefit in the treatment of this symptom include the tricyclic antidepressants, such as amitriptyline and desipramine [1–3].

Efforts to identify molecular mechanisms responsible for the beneficial response to antidepressants in the treatment of neuropathic pain are hindered, in part, by the numerous sites of actions of these drugs and the number of neural systems associated with the propagation and perception of pain. Among the pathways that might possibly mediate the analgesic response to these agents is that which utilizes GABA as a transmitter [4,5]. Evidence to support this hypothesis includes the fact that $GABA_B$ receptor agonists display antinociceptive activity in a variety of animal models of pain, and in certain clinical conditions [6–14]. Moreover, chronic administration of some antidepressants to laboratory animals variably increases $GABA_B$ receptor binding and function in brain [15–20], and formalin-induced inflammatory pain increases the expression of the heterodimeric $GABA_B$ receptor subunit mRNAs and protein in the dorsal horn of the rat spinal cord [12,21]. It has also been reported that administration of desipramine or imipramine increases pain thresholds in mice, and that this action is attenuated by CGP 35348, a $GABA_B$ receptor antagonist [22]. Given these findings, it is possible the $GABA_B$ receptor may be a critical component in mediating the beneficial effect of antidepressants in the treatment of neuropathic pain.

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The present study was undertaken to define more precisely the effect of a tricyclic antidepressant on $GABA_B$ receptors involved in pain perception. The results indicate that continuous (7 consecutive days) administration of desipramine enhances, over time, the pain threshold to a thermal stimulus. In addition, this treatment increases $GABA_B$ receptor function and the expression of select $GABA_B$ receptor subunits in the dorsal horn of the spinal cord. These findings suggest that desipramine-induced changes in the expression of some $GABA_B$ receptor subunits, and the consequent effects on $GABA_B$ receptor function, may be important elements in mediating the analgesic response to this drug.

2. Materials and methods

2.1. Animals and drug administration

Harlan Sprague–Dawley male rats (150–200 g) were used throughout the study. The animals were housed three to a cage under a 12-hr light/dark cycle with food and water *ad lib*. In all cases, the rats were allowed to adapt to their home cage for at least 24 hr prior to drug treatment and behavioral testing. The animals were administered either vehicle (water) or desipramine (15 mg/kg, i.p.) once daily for 7 consecutive days. The dose and duration of treatment were selected on the basis of earlier work demonstrating that this regimen caused neurochemical changes in rat brain [23]. On the 7th day, 1 hr following injection of vehicle or desipramine, the animals were placed on a Hargreaves analgesiometer and withdrawal latencies to thermal stimuli measured. Following this test, the animals were sacrificed by decapitation and their spinal cords rapidly removed by a forceful injection (60 mL syringe and 16 gauge needle) of ice-cold isotonic saline into the caudal end of the vertebral canal. Immediately after removal, the spinal cords were snap-frozen on dry ice.

2.2. Behavioral testing

The rat hind paw withdrawal response to thermal stimuli was quantified using an analgesiometer [24] manufactured by George Ozaki. Freely moving rats were placed in a Plexiglas chamber and a noxious, thermal stimulus generated by a high-intensity light beam was focused on the skin of the right or left hind paw. The intensity of the light beam necessary to provide a withdrawal latency of approximately 10 s in control animals was predetermined empirically.

2.3. In situ hybridization

The $GABA_{B(1a)}$ probe, a 400-base pair *Sma*I cDNA fragment, the $GABA_{B(1b)}$ probe, a 310-base pair *Ksp*I cDNA fragment, and the $GABA_{B(2)}$ probe, a 724-base pair *Nhe*I/*Sac*I cDNA fragment, were all sub-cloned into Bluescript (Stratagene). The plasmids were donated by K. Kaupmann (Novartis Pharma). Riboprobes were synthesized with digoxigenin-11-UTP (Roche Molecular Biochemicals) using the 'MAXIscript' *in vitro* transcription kit (Ambion).

The spinal cords were cryostat-sectioned at 12 μm thickness and thaw-mounted in silanized slides. The sections were post-fixed in 4% paraformaldehyde for 15 min, followed by three washes in sterile PBS for 5 min each, and then covered with pre-hybridization buffer (50 mL deionized formamide; 20 mL 20× SSC; 0.2 mL 50× Denhardt's solution [Sigma Chemical Co]; 25 mL yeast tRNA [10 mg/mL, Gibco BRL]; 1.6 mL 50% dextran sulfate [Oncor]) and incubated for 1 hr at 60°. The sections were hybridized overnight at 60° in hybridization buffer that consisted of 1 μg/mL digoxigenin labeled probes in the pre-hybridization buffer. Nonspecifically bound probes were removed with post-hybridization washing as follows: 2 × 15 min in 2× SSC at 37°; 2 × 15 min in 1× SSC at 37°; and 2 × 30 min in 0.1× SSC at 37°. Following equilibration for 1 min in Buffer 1 (100 mM Tris–HCl, 150 mM NaCl, pH 7.5) the tissues were pre-incubated at room temperature for 1 hr with blocking reagent that consisted of Buffer 1 with 2% normal sheep serum and 0.1% Triton X-100. Immunohistochemical detection of digoxigenin labeled hybrids was accomplished using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Molecular Biochemicals), diluted 1:1000 with Buffer 1 containing 1% normal sheep serum and 0.1% Triton X-100. After 1 hr at 37°, the antibody-containing solution was removed and sections washed for 10 min in Buffer 1 and 10 min in Buffer 2 (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). A chromogen solution (45 μL of 75 mg/mL nitroblue tetrazolium in 70% dimethylformamide, 35 μL of 50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide in 10 mL Buffer 2) was used as a substrate for detecting the alkaline phosphatase marker. The reaction, resulting in a purple precipitate, was terminated with Buffer 3 (10 mM Tris–HCl containing 1 mM EDTA, pH 8.0). Sections from different groups of animals were processed simultaneously to allow for visual comparison of precipitate densities. The tissue sections were dehydrated through graded ethanol, cleared in xylene, and mounted with Permount. Controls included hybridization with sense probes or omission of either the labeled probe or the anti-digoxigenin alkaline phosphatase-conjugated antibody. No staining was observed in either case. Rat brain cerebral cortex sections were used as positive controls. Tissues originating from at least three different animals, with at least five sections per animal, were evaluated by image analysis.

For *in situ* analyses, grayscale values (0–255) of the nitroblue tetrazolium salt precipitate were used to com-

pare the levels of $GABA_B$ receptor subunits between groups. The image analysis system consisted of a Dage/MTI 72 CCD camera mounted on the trinocular port of Zeiss Axioplan microscope (Carl Zeiss, Inc.). The camera is connected to a Matrox MVP-AT array processor installed in a 486-based PC running IM3000B image processing and analysis software (Belvoir Consulting).

All preparations were measured using a single illumination setting. The lamp was warmed and a 10× magnification selected. Settings were manipulated to produce maximum contrast (spread of grayscale values) in the images. Five different regions in the superficial dorsal horn containing immunoreactive product were outlined using a circle drawing command. The area was then measured and the density of immunoreactivity analyzed within the circular region. The five circular regions were pooled to obtain a final mean value. The results were compared by SuperAnova using a one-way ANOVA with Fisher's PLSD post hoc. Differences between means were considered significant when $P < 0.05$.

2.4. GTPγS binding assay

The rat spinal cords were cryostat-sectioned at 20 μm, thaw-mounted onto gelatin-coated slides, air-dried, and then stored at −70° until use. The slides were brought to room temperature and equilibrated to assay conditions by a 10-min incubation in assay buffer (4 mM $MgCl_2$, 160 mM NaCl, 0.267 mM EGTA, 67 mM Tris, pH 7.4) and then placed for 15 min into assay buffer containing 2 mM GDP. Incubation buffers (1.25 mL assay buffer, 1.25 mL 8 mM GDP, 1.25 mL [^{35}S]GTPγS at 27,500–30,000 dpm, and 1.25 mL 4 mM baclofen) were prepared in mailer vials along with nonspecific binding (40 μM unlabeled GTPγS replacing baclofen) and basal (water replacing baclofen) controls. The slides were incubated for 2 hr at room temperature, followed by two rinses in ice-cold 50 mM Tris–HCl, pH 7.4, and one rinse in distilled water at room temperature. The slides were air-dried overnight, apposed to autoradiography film for 2 days, and then developed in a Kodak X-ray developer. Quantification of autoradiographic grain densities on the tissue sections was accomplished using densitometric analysis (Scion Image). To this end, the dorsal horn was outlined using a drawing command and the grain density within this region measured and recorded. Background levels from the film and nonspecific binding over the superficial dorsal horn were subtracted from all tissue sections. The data are presented as percent stimulation of [^{35}S]GTPγS binding over basal [((total density of agonist-stimulated sections/basal density) × 100%) − 100]. The values reported are the means ± SEM from four to six different sections from three different rats in each age group.

For all experiments, the results were compared by SuperAnova using a one-way ANOVA with Fisher's PLSD post hoc. Differences between means were considered significant when $P < 0.05$.

2.5. Materials

Desipramine HCl and guanosine diphosphate was purchased from Sigma Chemical Co. Unlabeled GTPγS was purchased from CalBiochem, and [^{35}S]GTPγS (1250 Ci/mmol) from Amersham Pharmacia Biotech.

3. Results

3.1. Nociceptive response to desipramine

Administration to rats of a single dose (15 mg/kg, i.p.) of desipramine for 7 consecutive days increased, by approximately 30%, the latency to withdraw the hind paw in response to a noxious thermal stimulus (Fig. 1). A time course study comparing the duration of drug treatment with the effect on nociceptive threshold revealed no effect on withdrawal latency, as compared to vehicle-treated controls, after a single dose of desipramine or following 3 consecutive days of drug administration (Fig. 1). In all cases, nociceptive threshold was quantified 1 hr following administration of the preceding dose of desipramine or vehicle.

3.2. Spinal cord $GABA_B$ receptor subunit expression in response to desipramine

In situ hybridization analysis of $GABA_B$ receptor subunit expression in the dorsal horn of the rat spinal cord revealed that 7 days of continuous administration of desipramine significantly increased mRNA levels of $GABA_{B(1a)}$ and $GABA_{B(2)}$ subunits, but not of $GABA_{B(1b)}$ (Fig. 2). Thus, both $GABA_{B(1a)}$ and $GABA_{B(2)}$ mRNA increased approximately 30% relative to their basal levels in the dorsal horn of vehicle-treated controls. Hybridization with sense probes, or omission of either the labeled probe or the anti-digoxigenin alkaline phosphate-conjugated antibody, yielded no staining in the tissues, demonstrating the specificity of these probes (data not shown).

3.3. Spinal cord $GABA_B$ receptor function in response to desipramine

$GABA_B$ receptor function in the dorsal horn was measured by quantifying baclofen-stimulated [^{35}S]GTPγS binding in slices of rat spinal cord (Fig. 3). To this end, spinal cord slices were incubated with the radioligand in the presence or absence of 1 mM baclofen, a saturating concentration of this selective $GABA_B$ receptor agonist. Seven consecutive days of desipramine treatment doubled the response to this agonist as compared to [^{35}S]GTPγS binding in tissue obtained from vehicle-treated control subjects (Fig. 3).

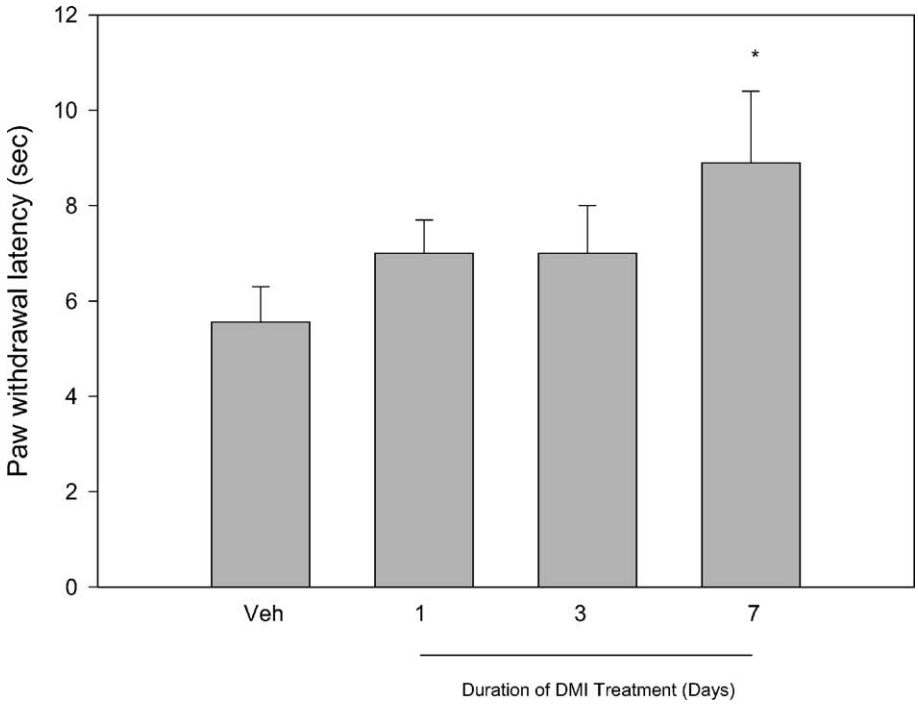

Fig. 1. Rat hind paw withdrawal latency in response to a noxious thermal stimulus following treatment with desipramine (DMI) (15 mg/kg, i.p., once daily) for 7 consecutive days. Vehicle-treated animals (Veh) received an equivalent volume of water once daily. In all cases, pain thresholds were quantified 1 hr following the preceding dose of desipramine or saline. The height of each bar represents the mean ± SEM of results from six animals. $^{*}P \leq 0.05$ compared to vehicle-treated controls.

4. Discussion

The tricyclic antidepressants, in particular amitriptyline, desipramine, imipramine, and nortriptyline, have for decades been among the drugs of choice, either alone or in combination with other agents, for the treatment of neuropathic pain [1–3]. While it is established that these agents provide some relief from this symptom, their precise mechanism of action in this regard remains unknown. It seems unlikely that their benefit is solely related to their ability to attenuate an affective component of chronic pain since their onset of action is more rapid, and the effective

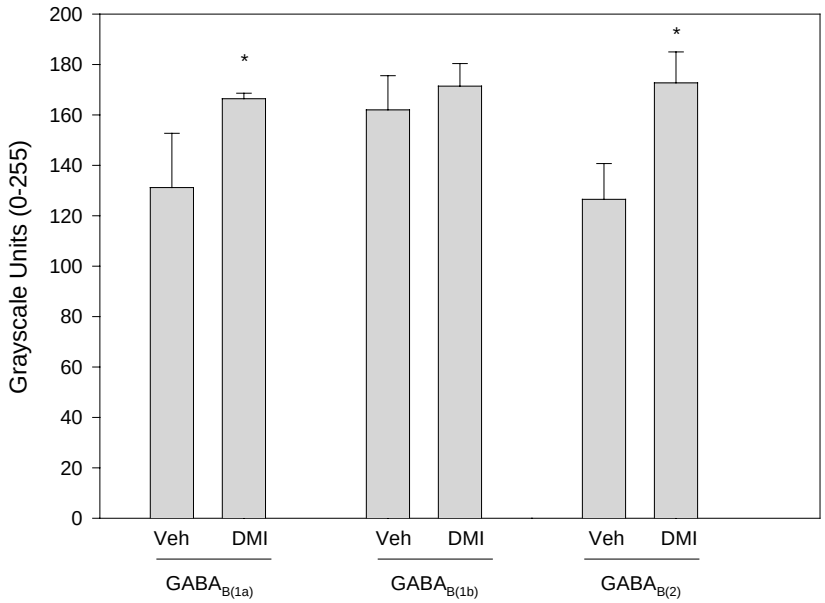

Fig. 2. Expression levels of $GABA_{B(1a)}$, $GABA_{B(1b)}$, and $GABA_{B(2)}$ subunits in the dorsal horn of the rat spinal cord as measured by *in situ* hybridization. Desipramine (DMI) (15 mg/kg), or an equivalent volume of water (Veh), was administered (i.p.) once daily for 7 consecutive days. The height of each bar represents the mean ± SEM of results obtained from three different animals. $^{*}P \leq 0.05$ compared to corresponding vehicle-treated controls.

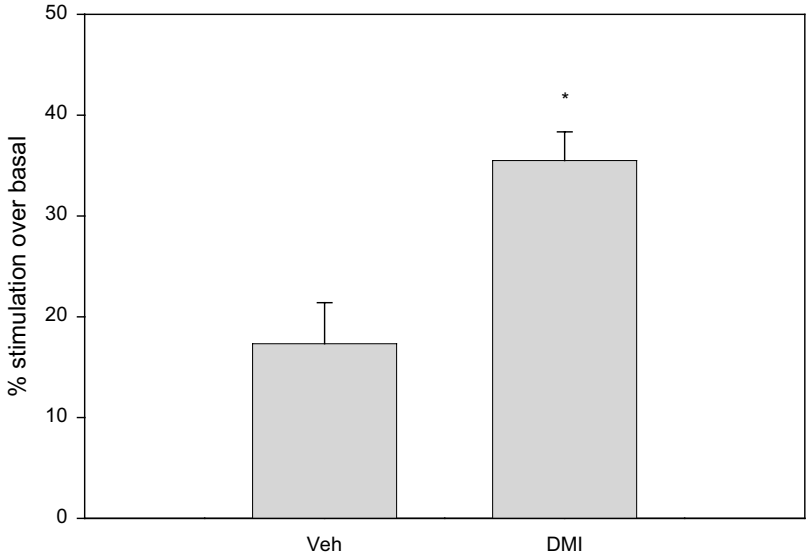

Fig. 3. Baclofen (1 mM)-stimulated [^{35}S]GTPγS binding in the dorsal horn of rat spinal cord slices, following 7 consecutive days of desipramine (DMI) administration (15 mg/kg, i.p., once daily), or an equivalent volume of water (Veh). The animals were sacrificed 2 hr following the final injection of desipramine or vehicle. The height of each bar represents the mean ± SEM of data obtained from six animals. $^{*}P \leq 0.05$ vs. vehicle-treated subjects.

doses somewhat less, as analgesics than as antidepressants [25]. Moreover, if relief from depression was primarily responsible for the analgesic response, antidepressants as a class should be effective therapies for neuropathic pain, which is not the case [1]. Rather, it seems the analgesic response to this select group of agents may be due to some action separate from that responsible for their antidepressant action. Numerous possibilities exist given the broad range of actions of the tricyclic pharmacophore, including blockade of cholinergic muscarinic, alpha-adrenergic, and histamine H1 receptors, along with inhibition of monoamine reuptake, and neuronal Na^+, Ca^{2+}, or K^+ channel activity [26]. Thus, it is conceivable that one, or a combination, of these effects may directly or indirectly influence the transmission of a pain impulse.

Previous reports suggest that subchronic, but not acute, administration of antidepressants variably increases $GABA_B$ receptor binding and function in rat brain [15–20]. It has also been demonstrated that $GABA_B$ receptors located in the spinal cord dorsal horn are involved in modulating the perception of pain [12,21,27–29], and that the analgesic response to desipramine and imipramine is inhibited by a $GABA_B$ receptor antagonist [22]. These findings, coupled with the knowledge that baclofen, a $GABA_B$ receptor agonist, displays analgesic activity in laboratory animals and humans [4–14], suggest this neurotransmitter receptor system may play a role in the clinical response to tricyclic antidepressants in the treatment of neuropathic pain. The present series of experiments was undertaken to test this hypothesis by examining the effect of desipramine on pain threshold in the rat, as well as on the expression and function of $GABA_B$ receptor subunits in the dorsal horn of the spinal cord, a region known to be an important relay for pathways transmitting pain impulses.

The results indicate that continuous administration of desipramine increases the thermal pain threshold in rats. A time course study revealed the change in threshold required 4–7 days of continuous treatment with this antidepressant. This is reminiscent of the fact that at least a week of continuous therapy with a tricyclic antidepressant is required to obtain a maximal response in the treatment of neuropathic pain [1].

It was also found that following 7 days of continuous administration, at the time when pain threshold is increased, desipramine increases the levels of mRNA for two $GABA_B$ receptor subunits, $GABA_{B(1a)}$ and $GABA_{B(2)}$, but not for a third, $GABA_{B(1b)}$, in the dorsal horn of the rat spinal cord. Moreover, the increase in the expression of these gene products apparently results in an increase in the production of heterodimeric $GABA_B$ receptors since there is also an increase in $GABA_B$ receptor function, as measured by baclofen-stimulated [^{35}S]GTPγS binding, in the dorsal horn. From the present data, it is impossible to determine whether the change in pain threshold is contemporaneous with the modifications in $GABA_B$ receptor subunit expression. Nonetheless, the results suggest that desipramine may enhance the sensitivity of $GABA_B$ receptors in an area of the spinal cord that is critical for nociceptive processing.

The present data are intriguing in light of previous reports concerning the regulation of $GABA_B$ receptors in spinal cord [12,21]. Thus, as opposed to desipramine treatment, formalin-induced inflammatory pain increases the levels of mRNA and protein for $GABA_{B(1b)}$ and $GABA_{B(2)}$, but not $GABA_{B(1a)}$, in the dorsal horn of the rat spinal cord. Curiously, these inflammatory pain-induced increases in subunit levels do not result in a change in $GABA_B$ receptor function [21]. Taken together with the

present data, this suggests that functional modifications in spinal cord dorsal horn $GABA_B$ receptors, at least as measured by [^{35}S]GTPγS binding, require an increase in the production of the $GABA_{B(1a)}$ subunit, but not the $GABA_{B(1b)}$, in combination with $GABA_{B(2)}$. This is perplexing since it appears that a functional $GABA_B$ receptor results only from the dimerization of $GABA_{B(2)}$ with either $GABA_{B(1a)}$ or $GABA_{B(1b)}$, with neither homodimers nor dimerization of the $GABA_{B(1)}$ splice variants yielding a functional site [30–35]. The different findings with formalin- and desipramine-induced changes in $GABA_B$ receptor subunits suggest receptor sensitivity in the spinal cord is more dependent on the expression of the $GABA_{B(1a)}$ than of the $GABA_{B(1b)}$ subunit, with the latter perhaps having some other cellular function independent of the $GABA_B$ receptor system [36].

The results of the present work do not reveal how desipramine administration modifies $GABA_B$ receptor subunit expression and function. It seems likely that this effect is secondary to its action on some other system(s) that, in turn, results in an increase in the transcription of $GABA_B$ receptor subunits. While it is possible that tricyclic antidepressants may indirectly enhance GABA release and thereby stimulate the $GABA_B$ receptor, this seems an unlikely explanation for the present results since it is reported that direct and prolonged stimulation of spinal cord $GABA_B$ receptors causes a decrease, rather than an increase, in receptor sensitivity through nongenomic mechanisms [21]. It is possible that these findings indicate that desipramine treatment causes receptor supersensitivity by reducing $GABA_B$ receptor activation. In this case, the increased production of $GABA_B$ receptor subunits and functional supersensitivity might reflect a compensatory mechanism required to fully elaborate the analgesic response to desipramine. Alternatively, if the increase in $GABA_B$ receptor expression and function is due to a drug-induced decrease in spinal cord GABAergic activity, the latter effect might be responsible for the limited efficacy of desipramine in the treatment of neuropathic pain. Because it is likely spinal cord $GABA_B$ receptor function and subunit production are modified by neuropathic pain, the effect of desipramine on these parameters may be different from those that occur in the absence of this pathology.

In conclusion, these findings indicate that desipramine administration increases the sensitivity of $GABA_B$ receptors in the dorsal horn of the spinal cord. Such an action could be an important component in mediating the analgesic response to this agent in the treatment of neuropathic pain.

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