



Muscimol prevents long-lasting potentiation of dorsal horn field potentials in rats with chronic constriction injury exhibiting decreased levels of the GABA transporter GAT-1

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Received 25 November 2002; received in revised form 30 April 2003; accepted 30 May 2003

Abstract

The inhibitory activity of gamma-aminobutyric acid (GABA) is considered critical in setting the conditions for synaptic plasticity, and many studies support an important role of GABA in the suppression of nociceptive transmission in the dorsal horn. Consequently, any injury-induced modification of the GABA action has the potential to critically modify spinal synaptic plasticity. We have previously reported that chronic constriction injury of the sciatic nerve was accompanied by long-lasting potentiation of superficial spinal dorsal horn field potentials following high-frequency tetanus. In this study we examined whether the GABA-A receptor agonist muscimol would modify post-tetanic responses in rats with chronic constriction injury. In animals exhibiting maximal thermal hyperalgesia as one sign of neuropathic pain 7 days after loose ligation of the sciatic nerve, spinal application of muscimol (5, 10 or 20 μ g) before the high-frequency (50 Hz) tetanus produced a long-lasting depression (rather than potentiation) of spinal dorsal horn field potentials. In separate but related Western immunoblot experiments, we also established that the chronic constriction injury was accompanied by significant decreases in the content of the GABA transporter GAT-1. These data demonstrated that GABA-A receptor agonists may effectively influence the expression of long-lasting synaptic plasticity in the spinal dorsal horn, and that an injury-induced loss in GABA transporter content may have contributed to a depletion of GABA from its terminals within the spinal dorsal horn. These data lent further support to the notion that the loss of GABA inhibition may have important consequences for the development of neuropathic pain.

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Keywords: Gamma-aminobutyric acid agonist; Gamma-aminobutyric acid transporter; Long-term potentiation; Neuropathic pain; Nociception; Thermal hyperalgesia; Sciatic nerve; Synaptic plasticity

1. Introduction

Significant changes in spinal nociceptive processing accompany peripheral nerve injury or inflammation to contribute to the development of persistent pain (reviews by Randic, 1996; Millan, 1999; Sandkuhler, 2000; Zimmermann, 2001). The inhibitory action of gamma-aminobutyric acid (GABA) is considered an integral component of numerous neuronal circuits in both the brain and the spinal cord (reviews by Paulsen and Moser, 1998; Eaton, 2000; Hammond, 2001). Consequently, any injury-induced modification of this inhibitory action has the potential to critically modify the

processing of nociceptive information in the spinal dorsal horn. Indeed much evidence suggests that a loss of GABA inhibition may at least partly contribute to the development of neuropathic pain (recent reports by Patel et al., 2001; Moore et al., 2002; Somers and Clemente, 2002). Especially important from a clinical perspective are reports that there is a significant imbalance in the levels of GABA (as well as aspartate and glycine) in human neuropathic pain patients (Mertens et al., 2000), and that local application of GABA or GABA agonists onto the spinal dorsal horn effectively alleviate pain in neuropathic animals (Eaton et al., 1999; Stubbley et al., 2001; Malan et al., 2002).

Using the well-established Bennett and Xie (1988) model of neuropathic pain, we have recently reported that there was

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an important inhibitory contribution of GABA to activity-dependent long-lasting synaptic plasticity in the superficial spinal dorsal horn (Miletic and Miletic, 2001). In the present study, we examined whether the GABA-A receptor agonist muscimol would modify post-tetanic responses in the spinal dorsal horn of rats with chronic constriction injury and by extension, spinal synaptic plasticity and persistent pain.

In addition, in a related but separate study, we sought to establish whether chronic constriction injury would be accompanied by modified levels of the GABA transporter GAT-1. Significant GABA depletion accompanies chronic constriction injury (Ibuki et al., 1997), but it remains unclear whether this depletion results from the actual loss of GABA neurons, or from a loss in content or activity of the GABA synthesizing enzymes glutamic acid decarboxylase (GAD) 65 and 67 (Eaton et al., 1998; Moore et al., 2002) or from a loss in the ability of GABA transporters to recapture and recycle the neurotransmitter (Soudijn and van Wijngaarden, 2000). We surmised that a loss of GABA inhibition following injury may alternatively or additionally result from the lack of an effective recapture process because this in turn may lead to significant depletion of GABA from its terminals in the spinal dorsal horn without a concomitant loss of GABA neurons.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (Harlan, ~300 g) were used. Water and food were provided ad libitum. Experiments were conducted in accordance with guidelines accepted by the International Association for the Study of Pain (Zimmermann, 1983). The animal protocol was approved by the Animal Care Committee of the School of Veterinary Medicine at the University of Wisconsin—Madison.

2.2. Sciatic ligation and hindpaw withdrawal latency

Animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and their sciatic nerves loosely ligated as described by Bennett and Xie (1988). The development of thermal hyperalgesia (as a behavioral measure of neuropathic pain) was assessed with the well-established hind paw withdrawal latency test (Hargreaves et al., 1988). Baseline withdrawal latencies were obtained for all animals before they were randomly assigned to control or sciatic ligation groups. Pilot studies have established that withdrawal latencies of sham-operated animals (sciatic nerve exposure without ligation) were statistically indistinguishable from unoperated control animals (i.e. like control animals the sham-operated animals did not develop thermal hyperalgesia). Seven days after surgery (time of maximal hyperalgesia), the hind paw withdrawal latency of all animals was obtained again before they were used in the electrophysiology experiments (a total of 14 sciatic ligation animals) or their spinal dorsal horn tissue

was harvested for Western immunoblots (four control and four sciatic ligation animals).

2.3. Electrophysiological recordings

Recordings were performed as described previously (Miletic and Miletic, 2000, 2001). Briefly, the animals were anesthetized with urethane (1.2–1.5 mg/kg, i.p.) and the lumbar spinal cord was exposed by laminectomy. A bipolar hook electrode was placed proximal to any sciatic ligation, and was used for activating sciatic afferents with single, 0.1 ms long, constant-current pulses at a rate of one stimulus every 5 min. Strength of stimulation was adjusted to achieve a stable maximal field potential amplitude (i.e. increasing stimulation strength beyond this ‘maximal’ current did not result in a further increase in potential height). This ensured that all of the A β and A δ fibers giving rise to a field potential were activated, and that a potentiated post-tetanic response reflected increase in synaptic strength rather than synaptic number. Glass microelectrodes (2 M NaCl, 0.2–2 M Ω) were used for recordings. Electrode impedance was checked routinely throughout each experiment. High-frequency (50 Hz) tetanic stimulation was delivered in one 400 ms train of 20 0.1 ms pulses at maximal current strength (~3 T). After application of saline or muscimol, the exposed spinal cord was periodically covered with warm oil to prevent tissue cooling and drying. At the end of a recording session, and while still deeply anesthetized, the animal was euthanized with an intracardiac injection of saturated potassium chloride. The length of time between induction of anesthesia and termination of the recordings was 7–8 h. Anesthesia with urethane lasted throughout the experiment.

The amplitudes of all individual potentials preceding tetanic stimulation were averaged, and this average height was designated as 100%. Each of the post-tetanic individual potentials was then expressed as the percent change relative to the averaged pre-tetanic response (Fig. 1B). This ensured that pre- and post-tetanic recordings in an individual animal (or a single recording session) could be compared without confounds introduced by between-animal (or between-session) variability in electrode impedance, position of the recording electrode, extracellular milieu at the tip of the electrode, etc.

For statistical analysis, the averaged potentials at each of four post-tetanic time-points (60, 120, 180 and 240 min) were compared using ANOVA. The main emphasis in data analysis was on detecting differences at each of these selected time points. Scheffe’s post hoc test was used when significance of the main effect was noted, and statistical significance was inferred at $p < 0.05$ level.

2.4. Western immunoblots

Animals were deeply anesthetized with isoflurane, euthanized by an intracardiac injection of saturated potassium chloride, and their spinal cords exposed by laminectomy around L5. The cords were excised, divided

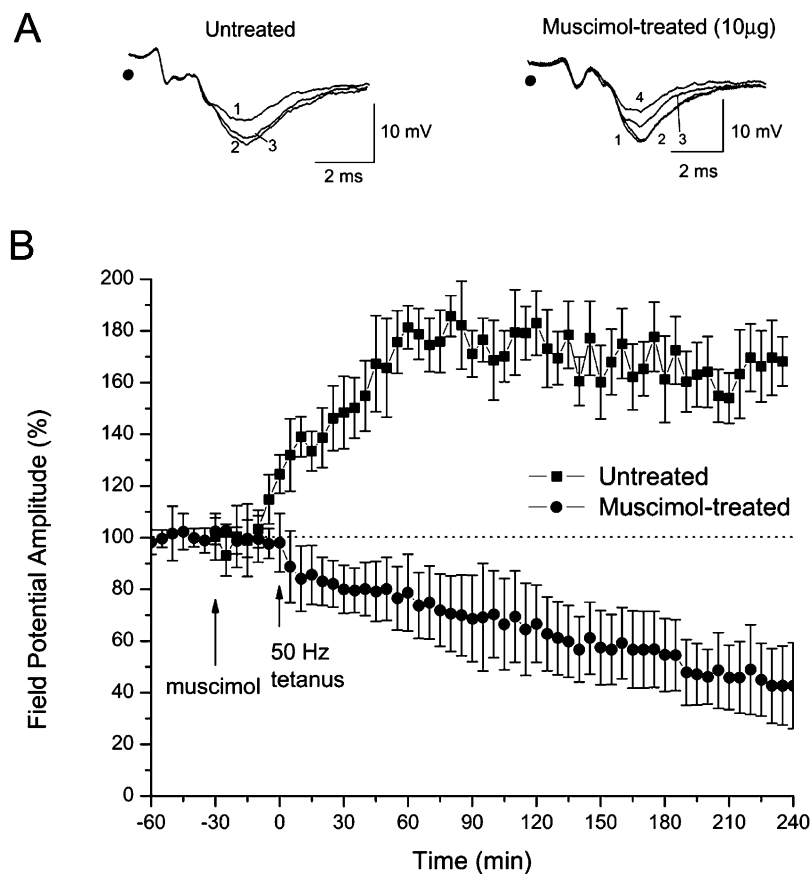


Fig. 1. Local muscimol application prevented the long-lasting post-tetanic potentiation to high-frequency stimulation of A-fiber evoked superficial dorsal horn field potentials in chronic constriction injury animals. (A) Sciatic-evoked A-fiber dorsal horn field potentials recorded in two individual animals. For the untreated animal, trace 1 is an example of a recording before tetanus, trace 2 was recorded about 90 min post-tetanus, and trace 3 in the last 30 min of the experiment. For the muscimol-treated animal (10 μ g), trace 1 was recorded before muscimol application, trace 2 was recorded after muscimol application but before tetanus, trace 3 was recorded about 90 min post-tetanus and trace 4 in the last 30 min of the experiment. The filled circle denotes the time of stimulation. (B) Sciatic afferents were stimulated every 5 min, and baseline responses were recorded before and after muscimol application. Tetanic stimulation was delivered at 50 Hz (one 400 ms train of 20 pulses each 0.1 ms in duration), and post-tetanic responses were again recorded every 5 min for up to 4 h post-tetanus. Note that in untreated animals, tetanic stimulation produced an immediate and long-lasting potentiation in evoked activity. In contrast, muscimol application prevented the potentiation and instead elicited a long-lasting depression following the same high-frequency tetanus. Post-tetanic field potentials are expressed as percent change over the averaged pre-tetanic baseline (which is denoted as 100%). Plotted values are averages of six untreated and eight muscimol-treated animals. For the latter, the data were pooled from groups given 5 μ g ($n = 3$), 10 μ g ($n = 4$) or 20 μ g ($n = 1$) because the post-tetanic responses were the same. Error bars represent the SEM.

into a dorsal and a ventral half, and the dorsal half weighed, homogenized and centrifuged at 7000g for 15 min. The 50 mM Tris-HCl (pH 7.4) homogenizing buffer also contained 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% NP-40, 0.25% sodium deoxycholate, and 5 μ g/ml of a mixture of protease inhibitors [4-(2-aminoethyl) benzene-sulfonyl fluoride, pepstatin A, trans-epoxysuccinyl-L-leucyl-amido(4-guanidino) butane, bestatin, aprotinin, leupeptin]. Total protein content in the homogenates was determined with a commercially available kit (Pierce, # 23236).

Proteins were separated by SDS-PAGE electrophoresis (10 μ g of total protein per well), and transferred onto PVDF membranes. The membranes were placed in a blocking solution (Tris-buffered saline with 0.02% Tween and 5% non-fat dry milk) for 1 h, and incubated overnight in rabbit primary antibodies to GAT-1 (1:300, Chemicon). After washing, incubation in appropriate peroxidase-conjugated

secondary antibodies (1:10000) for 1 h, and washing again, GAT-1 was detected by chemiluminescence. The content of GAT-1 in the spinal dorsal horn was estimated from optical density plots of the scanned images of the GAT-1 bands using Scion Image software (Scion Corporation, Frederick, MD). ANOVA was used for data analysis and statistical significance was inferred at the $p < 0.05$ level.

3. Results

3.1. Application of muscimol prevented the long-lasting potentiation elicited by the 50 Hz tetanus in sciatic ligation animals

All of the sciatic ligation animals used in the electrophysiology experiments ($n = 14$) exhibited significantly

shortened withdrawal latencies 7 days after surgery in the affected leg, 4.8 ± 0.3 (mean \pm SEM) vs. 8.4 ± 0.2 s, $F(1, 13) = 334.6$, $p < 0.001$. This is the time of maximal hyperalgesia for the chronic constriction injury model in our hands.

The sciatic-evoked field potentials were recorded at a microdrive depth of 150–450 μ m. Previous recordings confirmed that both A β and A δ sciatic afferents contributed to the dorsal horn field potentials (Miletic and Miletic, 2001). We focused on A-fiber potentials because changes in the activity or distribution of these primary afferents may significantly contribute to alterations in central processing following peripheral nerve injury (Kajander and Bennett, 1992; Nakamura and Myers, 1999).

The mean electrical stimulation threshold for evoking the dorsal horn field potentials was 99 ± 17 μ A ($n = 14$). Maximal amplitudes were obtained at ~ 300 μ A (3 T). The mean peak latency of the most prominent negative component of the field potential was 4.2 ± 0.9 ms ($n = 14$). The conduction distance between the stimulating and recording electrodes was ~ 8 cm. Based on the latency of afferent fiber volleys as well as the peak and time of onset and disappearance of the field potentials, the conduction velocity of the fibers contributing to the field potentials was estimated to be in the A-range (~ 15 –45 m/s).

Six sciatic ligation animals served as untreated ‘no muscimol’, controls. In these rats, the 50 Hz tetanus was delivered following a 30 min baseline recording period, and the recordings continued at baseline strength and stimulation for another 4 h after tetanus. As reported previously (Miletic and Miletic, 2000), in these animals exhibiting thermal hyperalgesia 7 days after sciatic ligation surgery, tetanic stimulation at 50 Hz elicited a robust and persistent increase in the amplitude of the A-fiber evoked dorsal horn field potentials (Fig. 1A, B).

The potential involvement of GABA inhibitory activity was examined in eight additional animals exhibiting maximal thermal hyperalgesia 7 days after loose ligation of the sciatic nerve. In these animals, after a 30 min baseline recording period, muscimol was applied locally on the exposed spinal dorsal horn using a 10 μ l syringe at 5 μ g ($n = 3$), 10 μ g ($n = 4$), or 20 μ g ($n = 1$). Post-muscimol recordings continued for another 30 min before the 50 Hz tetanus was delivered. Recordings then continued at baseline strength and stimulation for another 4 h after tetanus.

In contrast to the ‘no muscimol’ animals, in the muscimol-treated sciatic ligation animals, the high-frequency tetanus failed to elicit an increase in dorsal horn field potentials at any post-tetanic time. Rather, an immediate, progressive and sustained decrease in field potentials was recorded after tetanic stimulation, and the depression persisted throughout the remainder of the experiment (Fig. 1A, B). Because the timeline and degree of post-tetanic depression in all muscimol-treated animals were the same, the data were pooled and statistically compared to the post-tetanic responses in saline-treated animals.

The high-frequency elicited changes in the amplitude of sciatic-evoked field potentials proved to be significantly different between muscimol-treated and untreated animals. Specifically, in the six untreated sciatic ligation animals, the field potential amplitude was increased at all post-tetanic time points, i.e. 60, 120, 180 and 240 min (Fig. 2). In contrast, the field potential amplitude was decreased in all eight muscimol-treated sciatic ligation animals. The two groups differed significantly at all four of the analyzed post-tetanic time points, i.e. 60 min, $F(1, 12) = 78.9$, $p < 0.001$, 120 min, $F(1, 12) = 56.9$, $p < 0.001$, 180 min, $F(1, 12) = 70.8$, $p < 0.001$ and 240 min, $F(1, 12) = 67.3$, $p < 0.001$ (Fig. 2).

3.2. Decreases in the content of GAT-1 accompany thermal hyperalgesia following chronic constriction injury

Sciatic ligation animals used for Western immunoblot procedures ($n = 4$) exhibited significantly shortened withdrawal latencies 7 days after surgery in the affected leg, 5.3 ± 0.5 (mean \pm SEM) vs. 7.9 ± 0.1 s, $F(1, 3) = 23.3$, $p = 0.02$. In contrast, there were no significant differences between baseline and day 7 latencies in the control animals used for the Western immunoblot procedures ($n = 4$), 8.5 ± 0.3 vs. 8.1 ± 0.4 s, $F(1, 3) = 0.9$, $p < 0.5$. In addition, on the day of the second test, the latencies of 7 days sciatic ligation animals were significantly shorter than those of control animals, $F(1, 6) = 21.4$, $p < 0.005$.

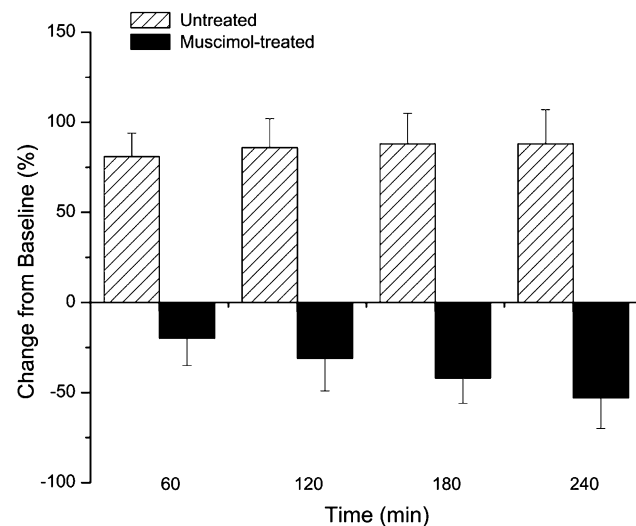


Fig. 2. Post-tetanic responses were significantly different between muscimol-treated and untreated animals. Summary plot of the field potentials following the 50 Hz tetanic stimulation at four statistically analyzed time points. Because the timeline and degree of post-tetanic depression in all muscimol-treated animals were the same, the data were pooled and statistically compared to the post-tetanic responses in saline-treated animals. Note the substantial increase in the A-fiber evoked superficial spinal dorsal horn field potentials at 60, 120, 180 and 240 min in the untreated animals ($n = 6$). Note in contrast the marked decrease in the field potentials at these same time points in the muscimol-treated group ($n = 8$). Values are expressed as percent change from the baseline (which is denoted as 100%). Error bars represent the SEM.

The levels of the GABA transporter GAT-1 appeared lower in animals exhibiting thermal hyperalgesia (as a behavioral sign of neuropathic pain) 7 days after loose ligation of the sciatic nerve (Fig. 3A). Statistical analysis indicated that the decreased amount of GAT-1 ($39 \pm 3\%$) in the spinal dorsal horn of these animals was significantly different from controls, $F(1,6) = 373.9$, $p < 0.001$ (Fig. 3B).

Given that the injury was unilateral, we also examined whether decreases in GAT-1 levels were confined to the corresponding ipsilateral spinal dorsal horn. For these experiments we divided the lumbar spinal dorsal horn into ipsilateral and contralateral quadrants in four additional animals exhibiting thermal hyperalgesia 7 days after sciatic ligation surgery. Results indicated that the GAT-1 content decreased similarly in both the injured, ipsilateral and the contralateral quadrants in these ligated animals (Fig. 3A). Statistical analysis confirmed this conclusion, $F(1,3) = 1.5$, $p = 0.38$. This was altogether not surprising given that the

sciatic ligation model produces variable changes in withdrawal latencies of the contralateral leg (Bennett and Xie, 1988).

4. Discussion

These data demonstrated that GABA-A receptor agonists may effectively influence the expression of long-lasting synaptic plasticity in the superficial spinal dorsal horn. The data also demonstrated that an injury-induced loss in GABA transporter content may have contributed to a depletion of GABA from its terminals within the spinal dorsal horn, and ultimately to the loss of GABA inhibition and the development of neuropathic pain.

Inhibitory activity of GABA neurons is considered critical in setting the conditions for activity-dependent plasticity in the hippocampus, and the greater the degree of this inhibition the less likely it is for long-lasting excitability to develop (Paulsen and Moser, 1998). This may be a consequence of the failure to reach the threshold for initiating the cascade of events that ultimately lead to long-term increases in synaptic efficacy. Our results with muscimol suggested that GABA-mediated inhibition plays an important role in spinal synaptic plasticity as well. As we speculated before (Miletic and Miletic, 2001) the activation of inhibitory inputs may be necessary to prevent bursts of high-frequency afferent activity from eliciting long-lasting increases in synaptic strength because the latter may signify the development of neuropathic pain. In other words, sciatic ligation in particular, and nerve injury in general, may give rise to persistent pain at least in part as a result of the loss of GABA-mediated inhibition. With no feedback inhibitory activity by GABA to silence the nociceptive input, only long-lasting increases in the excitability of spinal dorsal horn neurons would be elicited by the bursts of high-frequency discharges typically associated with peripheral nerve injury. Repetitive stimulation of primary afferents would then not only lead to the development of neuropathic pain but also be reflected in an enhanced sensitivity to both innocuous and noxious stimuli, i.e. allodynia and hyperalgesia.

These data confirmed previous reports on the importance of GABA inhibitory activity to the processing of sensory information in the spinal dorsal horn (Eaton, 2000; Hammond, 2001). These data also extended previous observations by providing an electrophysiological basis for studies reporting that application of GABA locally onto the spinal dorsal horn effectively alleviated neuropathic pain (Eaton et al., 1999; Stubley et al., 2001; Malan et al., 2002). Our observation that a single application of muscimol was effective in preventing tetanic-elicited long-lasting increases in excitability further supported the observation that a single intrathecal injection of GABA successfully reversed the neuropathic pain (Eaton et al., 1999). This observation also further supported the notion that

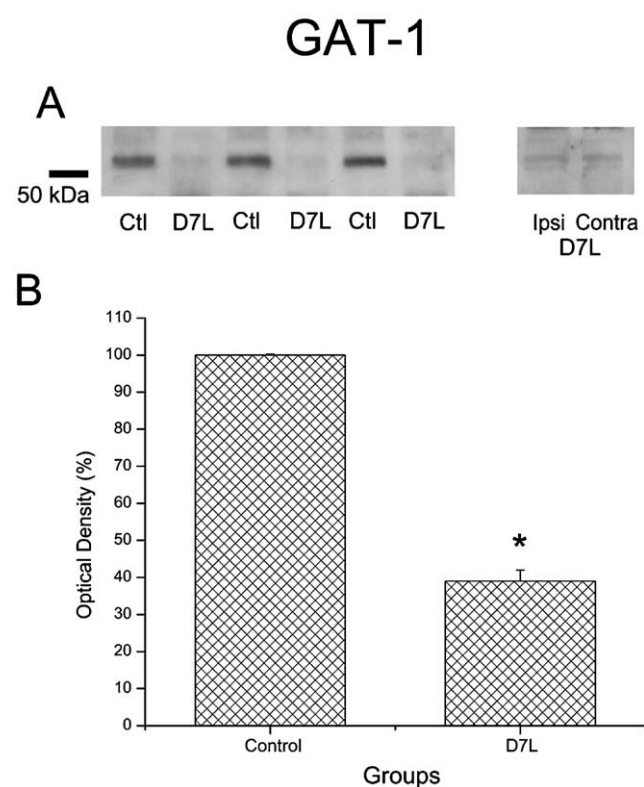


Fig. 3. Decreases in GAT-1 content accompanied thermal hyperalgesia in animals with chronic constriction injury of the sciatic nerve. A. Representative immunoblots of GAT-1 from controls (Ctl) and sciatic ligation animals exhibiting maximal thermal hyperalgesia 7 days after loose ligation surgery (D7L). Also, representative immunoblots of GAT-1 from ipsilateral (Ipsi) and contralateral (Contra) quadrants of a sciatic ligation animal exhibiting maximal thermal hyperalgesia D7L. Note the decreases in GAT-1 content in the sciatic ligation animals, and note that these decreases were similar on both sides of the dorsal horn. B. Summary plots of the estimated content of GAT-1. Note that there was a significant decrease in GAT-1 levels in the sciatic ligation animals ($n = 4$) when compared to control animals ($n = 4$). Error bars represent the SEM. * $p < 0.001$.

the pre-tetanic degree of GABA inhibitory activity critically determined whether long-lasting increases or decreases in excitability ensued (Paulsen and Moser, 1998). The degree of GABA inhibition may have served to control the balance between potentiation and depression after repetitive stimulation. Sciatic ligation-induced loss of GABA inhibition tipped the balance towards potentiation. On the other hand, muscimol-elicited GABA inhibition tipped the balance towards depression. This implies that GABA inhibition was important for the initiation of the post-tetanic response, but that the maintenance of that response was dependent upon other processes (e.g. activation of various protein kinases). Overall, these data lent further support to the notion that the loss of GABA inhibition may have important consequences for the eventual development of neuropathic pain (Eaton, 2000; Hammond, 2001).

Previous reports suggested that a significant depletion of GABA content in the spinal dorsal horn accompanied chronic constriction injury (Ibuki et al., 1997). While it appears clear that sciatic injury is accompanied by cell death in the spinal dorsal horn (Maione et al., 2002; Whiteside and Munglani, 2001), it is less clear if this loss includes GABA neurons. Obviously, GABA depletion may also result from an injury-elicited decrease in the content or activity of GABA synthesizing enzymes GAD-65 and GAD-67, but it also remains unclear how the content of one or both of these isozymes is modified following chronic constriction injury (Eaton et al., 1998; Moore et al., 2002).

Our data provided an alternative explanation for GABA depletion following chronic constriction injury by demonstrating that the content of the GABA transporter GAT-1 was significantly reduced in the spinal dorsal horn of sciatic ligation animals. GABA transporters play a critical role in recapturing GABA following synaptic activation (Soudijn and van Wijngaarden, 2000), and the observed injury-induced decrease in GAT-1 content may have contributed to a significant depletion of GABA content within its terminals in the spinal dorsal horn without a concomitant loss of GABA neurons. This loss of GABA content, and the attendant loss of GABA inhibition, would then allow for the expression of long-lasting synaptic potentiation, and by extension, the development of neuropathic pain in chronic constriction injured animals. Recent data support this notion as they suggest a significant injury-induced down-regulation of GABA transporter genes following spinal cord contusion (Song et al., 2001; Tachibana et al., 2002).

In summary, our data demonstrated that GABA-A receptor agonists may effectively influence the expression of long-lasting synaptic plasticity in the superficial spinal dorsal horn. The data also suggested that an injury-induced loss in GABA transporter content may have contributed to depletion of GABA from its terminals, and ultimately to the loss of GABA inhibition. These data lent further support to the notion that the loss of GABA inhibition in the spinal dorsal horn may have important consequences for the development of neuropathic pain.

Acknowledgements

This study was supported in part by NIH grant NS 34870 and the Christopher Reeve Paralysis Foundation.

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