Published in final edited form as:
Anesth Analg. 2008 April ; 106(4): 1296–contents. doi:10.1213/ane.0b013e318164f1e9.

Midazolam administration reverses thermal hyperalgesia and prevents GABA transporter loss in a rodent model of neuropathic pain

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Abstract

Background—Loss of GABA inhibition in the spinal dorsal horn may contribute to neuropathic pain. Here we examined whether systemic administration of the benzodiazepine midazolam would alleviate thermal hyperalgesia due to chronic constriction injury (CCI) of the sciatic nerve.

Methods—Hyperalgesia was evaluated with the thermal paw withdrawal latency test (TWL) before, and 3 and 7 days after CCI. Animals randomly received via osmotic minipump infusion midazolam (2.0 mg/kg/h), flumazenil (0.004 mg/kg/h), midazolam plus flumazenil at the same doses, or saline (0.01 ml/kg/h). Four groups of sham-operated rats (surgery without nerve ligation) received matched treatments. Levels of the GABA transporter 1 (GAT-1) in the lumbar spinal dorsal horn were estimated using western immunoblots 7 days after surgery.

Results—Saline-treated CCI rats developed thermal hyperalgesia on day 3 with a more pronounced effect on day 7. Continuous midazolam infusion prevented thermal hyperalgesia on both days. The anti-hyperalgesic effect of midazolam was reversed by the co-administration of flumazenil. Infusion of flumazenil alone had no effect on the thermal hyperalgesia in CCI rats. Sham-operated rats treated with saline, midazolam or midazolam plus flumazenil exhibited no thermal hyperalgesia. Unexpectedly, TWL in sham animals treated with flumazenil alone were significantly decreased. Changes in GAT-1 levels paralleled the behavior. Midazolam prevented the CCI-associated decreases, and flumazenil reversed midazolam's effect. Flumazenil alone did not modify GAT-1 levels in CCI animals but in sham animals the transporter levels were significantly reduced.

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Implications Statement

This study evaluated the effect of the benzodiazepine, midazolam, on thermal hyperalgesia in a neuropathic pain model. Our results suggested that GABA inhibition may be attenuated following peripheral nerve injury and that this may contribute to resulting hyperalgesia. Our results implied that systemic administration of benzodiazepines prevent loss of the GABA transporter protein and that this class of drugs could be of help in alleviating symptoms of neuropathic pain.

Conclusions—GABA inhibition plays an important role in neuropathic pain. Continuous systemic benzodiazepine administration may prove effective in alleviating neuropathic pain.

Introduction

Neuropathic pain arises from damage to the peripheral or central nervous system. It is associated with spontaneous pain, hyperalgesia, allodynia, and radiation of pain[1-2]. Treatment can be challenging since neuropathic pain is often not responsive to conventional analgesic therapy, e.g. opioids and anti-inflammatory drugs[3]. It has been estimated that 66% of neuropathic pain patients do not experience sufficient pain relief[4]. Laboratory investigations of other drug classes with alternative mechanisms of action may help to resolve unmet needs in neuropathic pain patients[1-4].

Animal models based on injury to the sciatic nerve mimic some of the behavior and symptoms of neuropathic pain[1-2]. These models have established that early and significant changes in spinal cord nociceptive processing accompany injury to peripheral nerves. The changes include activation of glutamatergic NMDA receptors, which play a prominent role in both the initiation and subsequent maintenance of hyperalgesia. In addition to the activation of excitatory pathways, however, the loss of spinal inhibitory controls may also significantly contribute to the development of neuropathic pain[5-8].

The neurotransmitter γ-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the central nervous system and it plays a major role in the central hyperexcitability associated with nerve damage[5-8]. In the spinal cord, GABA is concentrated in the superficial dorsal horn[9]. After peripheral nerve injury there is loss of modulation by spinal GABA-releasing neurons due mostly to an apparent overall reduction in GABA levels[5-8]. Spinal administration of the GABA-A receptor agonists muscimol and isoguvacine attenuate behavioral allodynia and hyperalgesia following nerve injury[10] probably due to the restoration of inhibitory controls[11].

It is unclear whether the loss of GABA inhibition is due to an absolute loss of GABA neurons, decrease in their activity, or depletion of GABA through the loss of GABA synthesizing enzymes[5-8]. GABA loss may also result from the inability of the GABA transporter protein (GAT) to recapture and recycle the neurotransmitter[12], leading to depletion of GABA from nerve terminals. The GABA transporter GAT-1 helps maintain cellular levels of GABA, and is the most copiously expressed GAT in the CNS[13]. We have previously reported that thermal hyperalgesia in the chronic constriction injury (CCI) model was closely associated with a significant loss of GAT-1 levels in the spinal dorsal horn[14].

Benzodiazepines bind to a modulatory site on the GABA-A receptor and increase the opening frequency of the related chloride channel. Midazolam is a short acting benzodiazepine that is widely used as an anxiolytic[15]. It is not regarded as a traditional analgesic but its effect on the GABAergic system might make it useful in approaches to treatment of neuropathic pain[16, 17]. Flumazenil is a receptor-specific reversal agent for the benzodiazepines[15].

The purpose of the present study was to assess the effects of continuous systemic midazolam infusion on thermal hyperalgesia, using the CCI model of neuropathic pain. We hypothesized that midazolam treatment may alleviate or diminish thermal hyperalgesia (by enhancing GABA inhibition), and that this effect would be reversed by co-administration of flumazenil. We also hypothesized that midazolam infusion would attenuate the injury-elicited spinal cord sensitization “marked” as a decrease in GAT-1 protein.

Methods

Chronic constriction injury

Male Sprague-Dawley rats (250−300 g) were used (Harlan Laboratories, Madison WI). Rats were housed individually with food and water ad libitum and artificial lighting with a fixed 12h light/dark cycle. Experiments were conducted in accordance with guidelines accepted by the International Association for the study of Pain[18]. The animal protocol was approved by the Animal Care Committee of the School of Veterinary Medicine at the University of Wisconsin-Madison. Chronic constriction injury was performed as originally described[19]. Briefly, the animals were anesthetized with isoflurane (Abbott, Chicago IL). The skin and musculature of the lateral left thigh were incised to expose the sciatic nerve and the nerve was loosely ligated with 4 simple interrupted 4−0 chromic gut sutures. After checking hemostasis, muscle and overlying skin were closed routinely. Sham surgeries were performed by exposing, but not ligating, the sciatic nerve.

Thermal hyperalgesia

Thermal hyperalgesia was assessed using a commercially available paw withdrawal apparatus (Stoelting, Wood Dale, IL). Testing was performed between 8:00 am and 10:00 am on each day that data were collected. Animals were placed in the plexiglass chamber and given 15 minutes to acclimate. A thermal stimulus was positioned under the plantar side of the left hind paw and when an animal withdrew its paw an automatic timer shut off the heat source. This provided an accurate record of the paw withdrawal latency (TWL). The assessment of thermal sensitivity was performed before surgery (baseline), on day 3 (onset of hyperalgesia) and day 7 (maximal hyperalgesia) after surgery.

Groups and drug treatments

Animals were randomly assigned to eight groups. The four CCI groups consisted of eight animals each and received, respectively, midazolam (2.0 mg/kg/h), flumazenil (0.004 mg/kg/h), midazolam (2.0 mg/kg/h) plus flumazenil (0.004 mg/kg/h), and saline (0.01 ml/kg/h). Sham-operated animals were similarly divided into four matched groups (n=4 in each group) and received the same respective treatments. All drugs were administered via subcutaneously implanted osmotic minipumps (Model 2ML1, 10 μl/hr, 7 days, 2 ml total volume; Alzet® Duracet Laboratories, Cupertino CA), such that the same volume of drug (0.010 ml) was delivered per hour continuously for 7 days. The minipumps were implanted after baseline thermal testing and just before sciatic exposure or ligation as described previously[20]. Drug doses for midazolam and flumazenil were extrapolated from clinically recommended doses for these drugs. Flumazenil was obtained as a commercially available injectable solution (Romazicon®, Roche, Nutley, NJ) at a concentration of 0.1 mg/ml. Midazolam (50 mg/ml) was a gift (Roche Laboratories, Switzerland).

GAT-1 immunoblots

Following TWL testing on day 7, animals were deeply anesthetized with isoflurane and euthanized by an intracardiac injection of super-saturated potassium chloride (2−3%). A laminectomy rapidly (<2 min) exposed the lumbar spinal cord at L5 and about 1 cm of the cord was excised and cut first into dorsal and ventral halves and then the dorsal half was further divided into ipsilateral and contralateral quadrants. All tissues were immediately placed into dry-ice cooled collecting tubes, and stored at −80°C until use. The quadrants were then processed for Western immunoblots as described previously[14]. The GAT-1 protein levels were estimated from optical density measurements of scanned images of the respective bands using Image J (NIH, Bethesda, MD), a public domain Java image processing program based on NIH Image for the Macintosh. The protein levels in CCI or sham-operated animals were normalized

to those in control, uninjured animals, i.e., control values served as baseline reference values. These control samples were obtained from normal, non-painful animals used in other studies. The average density of the protein bands in the control animals was denoted as 100% and the average density of the bands in the CCI or sham-operated animals was expressed as a percent change from this control.

Midazolam Serum Concentration Determination

Blood samples were collected for analysis of serum midazolam concentrations on day 3 and day 7. For purposes of blood collection, rats were briefly anesthetized with isoflurane after thermal latency data had been obtained. Blood was collected (0.25−0.5 ml) from the ventral tail artery. On day 3 rats were recovered from anesthesia and returned to their holding cages. On day 7, rats were then euthanized as described above for spinal cord collection. Midazolam was extracted from serum samples using liquid-liquid extraction, and assayed by fractional chromatography and mass spectrophotometry[21]. The limit of quantification for this assay was approximately 2 ng/ml.

Statistical analysis

Data for TWL and GAT-1 content were normally distributed. Repeated measures ANOVA was used for the statistical data analysis with Systat® (Richmond, CA). The main emphasis was on detecting changes in the dependent measures (TWL and GAT-1 levels) both within and between groups. All significant effects were further analyzed with Scheffe's post-hoc test. Significance was inferred at the $p \leq 0.05$ level.

Results

Midazolam prevented the development of thermal hyperalgesia in CCI rats

As expected, CCI rats treated with saline developed thermal hyperalgesia on day 3 and this became more pronounced on day 7, $F(2, 14)=56.2$, $p<0.001$ (Fig. 1). There was a significant decrease in TWL between baseline (11.0±0.9s, mean±SD) and day 3 (7.9±1.6s, $p<0.001$), and a further significant decrease between day 3 and day 7 (5.6±0.7s, $p=0.005$). Midazolam infusion prevented any CCI-associated decreases in TWL between baseline (10.7±1.4s), day 3 (10.0 ±1.1s) or day 7 (9.0±1.7s), $F(2,14)=3.3$, $p=0.09$. Co-administration of flumazenil with midazolam reversed midazolam's anti-hyperalgesic effect, $F(2,14)=24.0$, $p<0.001$. In these CCI animals the TWL significantly decreased on day 3 (7.4±2.0, $p=0.003$) when compared to baseline (11.1±0.8s), and the TWL continued to remain decreased on day 7 (6.9±0.6s), i.e. there were no significant differences between day 3 and 7 latencies ($p=0.47$). The infusion of flumazenil alone did not modify the significant ligation-associated decreases in TWL, $F(2,14) =49.5$, $p<0.001$. As in saline-treated animals, the TWL decreased significantly between baseline (10.3±1.0s) and day 3 (8.0±1.4s, $p=0.02$), and then further between day 3 and day 7 (4.6±0.8s, $p<0.001$).

Between group ANOVA established that there were no differences in baseline latencies among the CCI groups, $F(3,28)=1.0$, $p=0.39$. On the other hand the groups differed both on day 3, $F(3,28)=5.8$, $p=0.003$ and on day 7, $F(3,28)=27.7$, $p<0.001$. Scheffe's post-hoc test indicated that this difference was attributable to the significant anti-hyperalgesic effect of midazolam when compared to saline, flumazenil alone, or midazolam plus flumazenil either on day 3 ($p=0.03$, $p=0.04$, $p=0.08$, respectively) or on day 7 ($p<0.001$, $p<0.001$, $p=0.003$, respectively).

Prolonged flumazenil treatment was associated with thermal hyperalgesia in sham animals

There were no differences between baseline (11.4±0.8s), day 3 (10.5±0.8s) and day 7 (9.7 ±0.5s) latencies in saline-treated sham-operated rats confirming the absence of thermal

hyperalgesia in these animals, F(2,6)=4.2, p=0.07 (Fig. 2). Similarly, there were no significant differences in baseline, day 3 and day 7 latencies in the sham animals treated with midazolam (11.9±1.0s vs. 10.7±1.8s vs. 9.1±0.9s), F(2,6)=2.9, p=0.13, or midazolam plus flumazenil (10.6 ±0.5s vs. 9.7±0.3s vs. 8.4±0.7s), F(2,6)=4.6, p=0.06. In contrast, and unexpectedly, sham-operated flumazenil-treated rats exhibited a substantial reduction in TWL by day 7. Within group ANOVA confirmed a significant effect of the time of testing, F(2,6)=15.4, p=0.004. This effect was not due to a significant difference in TWL between baseline (11.8±0.9s) and day 3 (10.4±2.1s, p=0.35). However, there was a significant decrease in TWL between day 3 and day 7 (5.9±0.9s, p=0.03). In fact, these day 7 latencies in sham animals were statistically indistinguishable from those of day 7 saline-treated CCI rats, F(1,10)=0.5, p=0.48, suggesting that the two groups exhibited equivalent thermal hyperalgesia.

Between group ANOVA confirmed that there were no differences in TWL among the sham groups either at baseline, F(3,12)=2.2, p=0.14, or on day 3, F(3,12)=0.3, p=0.81. However, on day 7 there was a significant between group difference, F(3,12)=17.6, p<0.001. Scheffe's post-hoc analysis indicated that this difference was due to the significantly reduced latencies in animals treated with flumazenil alone when compared to saline (p<0.001), midazolam (p=0.001) or midazolam plus flumazenil (p=0.006).

There were no signs of toxicity, CNS depression, or ataxia in any of the tested animals. Specifically, in the sham-operated and CCI animals we saw no evidence of sedation, motor impairment, or muscle relaxation after treatment with midazolam. All sham-operated as well as CCI animals remained alert and active and groomed normally. Some of the CCI animals exhibited guarding of the affected limb, but no signs of significant stress (e.g. porphyrin staining) were observed.

Changes in GAT-1 levels in the dorsal horn paralleled the behavior

Sciatic ligation was associated with a substantial decrease in GAT-1 protein levels in the ipsilateral dorsal horn of saline-treated CCI rats (37±9%, Fig. 3). In contrast, in the midazolam-treated CCI animals the GAT-1 levels were essentially the same as those in control, uninjured animals (103±24%). The preventive effect of midazolam was blocked by co-administration of flumazenil (52±9%). Flumazenil by itself did not prevent the injury-elicited decreases in GAT-1 (38±14%).

ANOVA indicated a significant difference in GAT-1 protein levels among the four groups, F (3,12)=16.3, p<0.001. Scheffe's post-hoc analysis established that this difference was due to the control-like levels of GAT-1 in midazolam-treated CCI animals when compared to the significant reductions observed in saline (p<0.001), flumazenil (p<0.001) or midazolam plus flumazenil (p=0.005) treated animals.

There were no significant differences in GAT-1 levels between the ipsilateral and contralateral dorsal horn in any of the tested groups. For example, in agreement with our previous study[14], GAT-1 levels in the contralateral dorsal horn of saline-treated animals were also reduced (42±9%, p=0.6), and midazolam treatment prevented this reduction (109±8%, p=0.7).

As expected, GAT-1 levels in the ipsilateral dorsal horn of saline-treated sham-operated animals were the same as those in uninjured controls (105±6%, Fig. 4). Similarly, GAT-1 levels were unchanged in animals treated with midazolam alone (113±17%) or midazolam plus flumazenil (106±11%). On the other hand, in sham-operated rats treated with flumazenil alone there was a significant decrease in GAT-1 levels (39±8%).

Statistical analysis confirmed a significant between group difference, F(3,12)=39.3, p<0.001. Scheffe's post-hoc test established that this difference was due to the significantly reduced

levels of GAT-1 in the flumazenil-treated animals when compared to saline, midazolam or midazolam plus flumazenil treatment (all three at $p<0.001$).

The percent reduction in GAT-1 levels in flumazenil-treated sham animals was the same as that in the saline-treated CCI animals and the two groups were statistically identical, $F(1,6) =0.04$, $p=0.84$. Thus both with respect to behavior and GAT-1 levels the flumazenil-treated sham-operated animals resembled the saline-treated CCI animals.

Serum midazolam concentrations

There was no significant difference in serum concentrations of midazolam in rats administered midazolam alone or midazolam plus flumazenil. Midazolam concentrations did not differ between CCI and sham-operated rats (123±14.2 vs. 121.7±17.1 ng/ml). Midazolam was not detected in any of the groups that received saline or flumazenil alone.

Discussion

Our results confirmed that robust thermal hyperalgesia develops after CCI. Our results also established that this thermal hyperalgesia can be prevented by continuous systemic infusion of midazolam.

Previous investigations into the potential use of midazolam as an analgesic have produced conflicting results. Some studies have reported that this potentiator of GABA inhibition is analgesic[22-25] whereas others that it has weak antinociceptive properties[26] or that it is hyperalgesic[27]. This disparity in results is most likely due to differences in experimental protocols (e.g., dose) and animal models of nociception. In a comparable neuropathic pain model midazolam was reported to reduce A-δ and C-fiber evoked activity and reverse cold and mechanical allodynia following spinal nerve ligation (SNL)[16]. More recently, in the same CCI model it was reported that thermal hyperalgesia and mechanical allodynia were attenuated by intrathecal administration of midazolam once daily for 7 postoperative days[17]. This effect of midazolam was prevented by the co-administration of the GABA-A antagonist bicuculline.

There is limited clinical evidence in the literature to suggest that midazolam, and related benzodiazepines, are effective for chronic pain of neuropathic or non-neuropathic origin when administered intrathecally. Most of these studies have involved a relatively small population of patients, where visual analogue scales of pain or verbal accounts of pain improvement where reported[28-30]. One clinical case study reported complete disappearance of severe lightning pain secondary to spinal analgesia in a man with neuropathic pain of central origin after IV midazolam administration on 2 separate occasions, while IV lidocaine or morphine were ineffective[31]. To our knowledge there have been no clinical studies performed to assess the systemic analgesic effect of midazolam in neuropathic or non-neuropathic pain.

In our study, midazolam was similarly effective in preventing thermal hyperalgesia in the CCI model, and co-administration of the benzodiazepine antagonist, flumazenil, completely reversed midazolam's inhibitory effect. Flumazenil alone had no analgesic effect. However, and unexpectedly, continuous administration of flumazenil alone for 7 consecutive days decreased thermal withdrawal latencies in sham-operated animals suggesting the development of thermal hyperalgesia. GABA receptor antagonists are known to elicit dose-dependent increases in hyperalgesia[32], and to enhance spinal cord central sensitization[33]. Administration of the GABA-A antagonist bicuculline directly onto the spinal cord produces signs of allodynia and mechanical hyperalgesia[8,34]. In our study, chronic flumazenil infusion might have blocked the benzodiazepine binding site at the GABA receptor thereby eliciting an overall reduction in GABA inhibition perhaps by interfering with the action of endogenous

benzodiazepines[35]. Midazolam's anti-hyperalgesic effect is probably mediated via the same benzodiazepine binding site on the GABA-A receptor[22,24].

In agreement with our previous study[14] we demonstrated that spinal dorsal horn levels of the GABA transporter GAT-1 were reduced in CCI animals exhibiting thermal hyperalgesia. GAT-1 plays an important role in recapturing GABA from the synaptic cleft[12]. Decreases in this GABA transporter may contribute to reductions of GABA content and, ultimately, to the loss of GABA inhibition[14]. In the current study, midazolam infusion in CCI rats prevented the loss of the protein suggesting that GAT-1 levels were significantly influenced by activity at the GABA receptor. Re-uptake transport is one of several pathways in the complex regulation of GABA activity[12]. An increase in GAT-1 protein would improve the ability of interneurons to recycle GABA and would, indirectly, increase the efficiency of inhibitory pathways. Likewise, reductions in GAT-1 protein would reduce GABA recycling in the dorsal horn and may lead to an overall reduction in inhibition in the spinal cord. Interestingly, in the study reported here, chronic infusion of flumazenil decreased concentrations of GAT-1 protein in sham-operated animals, which also demonstrated unexpected hyperalgesia. These data offer support to the hypothesis that reduced GAT-1 activity may lead to hyperalgesia.

Data from a recently completed study[36] suggested that early after injury (4h) in the CCI model there was a significant loss of potassium-chloride co-transporter (KCC2) protein in the ipsilateral spinal dorsal horn without a concomitant loss of the GABA-A receptor subunit alpha-1 protein. The KCC2 loss appeared mediated at least in part by BDNF. However, 7 days after the sciatic ligation, the levels of KCC2 returned to control levels in the CCI animals which nevertheless continued to exhibit behavioral signs of neuropathic pain. These data provided further support for the notion that nerve injury-associated pain exhibits early, intermediate and late phases of development which may be mediated by different mechanisms.

The anti-hyperalgesic effects of midazolam, and its apparent ability to prevent the loss of GABA inhibition in the spinal dorsal horn, suggest that continual systemic administration of benzodiazepines may provide relief of pain that is neuropathic in origin. Better understanding of basic excitatory and inhibitory mechanisms of neuropathic pain should provide inroads into more rational pharmacotherapy for this challenging syndrome.

Acknowledgements

Supported by grants RR018802 and NS034870 from the National Institutes of Health, Bethesda, MD, and the Companion Animal Research Foundation, School of Veterinary Medicine, University of Wisconsin-Madison.

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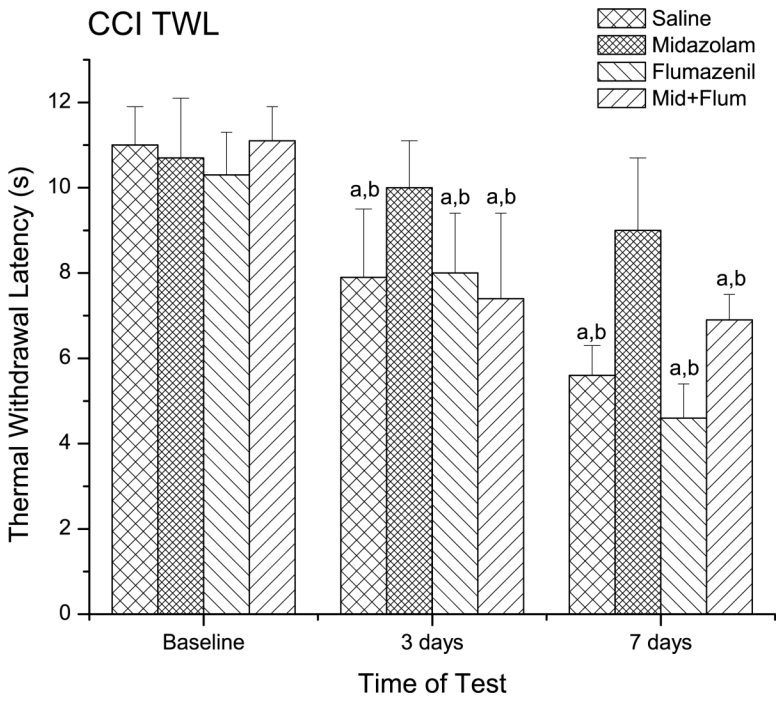

Figure 1. Behavioral signs of neuropathic pain accompanied CCI in rats

Baseline TWL were obtained for all animals before surgery, and then again 3 and 7 days after the ligation. Note that CCI animals treated with saline exhibited thermal hyperalgesia as a behavioral sign of neuropathic pain as their withdrawal latencies significantly decreased on day 3 and especially day 7. In contrast, the TWL of midazolam-treated animals were not significantly different from baseline on either day 3 or 7. Note also that flumazenil co-administration prevented the anti-hyperalgesic effect of midazolam, but that flumazenil alone did not modify the CCI-elicited decreases in TWL. The withdrawal latencies are shown for the ipsilateral (operated) leg. Each group consisted of 8 animals. Error bars represent the SD; a=significantly different from baseline within that group; b=significantly different from midazolam-treated CCI rats on that day.

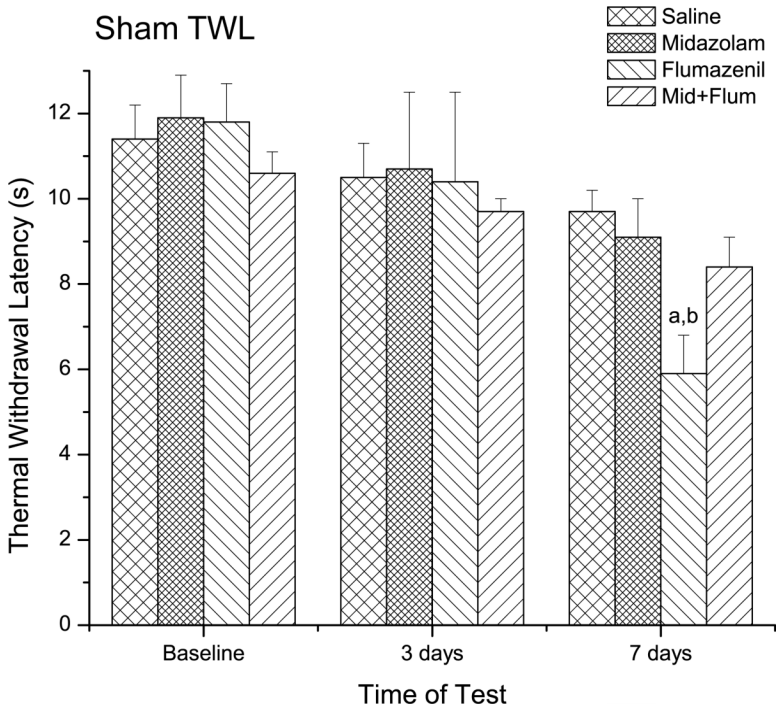

Figure 2. Sham-operated animals did not exhibit behavioral signs of neuropathic pain except for those animals treated with flumazenil alone

Baseline withdrawal latencies were obtained for all animals before surgery, and then again 3 and 7 days later. Note that there were no differences between the baseline and post-surgery withdrawal latencies in the animals treated with saline, midazolam, or midazolam plus flumazenil. Note also that flumazenil treatment alone elicited an unexpected decrease in TWL. The withdrawal latencies are shown for the ipsilateral (operated) leg. Each group consisted of 4 animals. Error bars represent the SD; a=significantly different from baseline within that group; b=significantly different from saline, midazolam or midazolam plus flumazenil-treated rats on that day.

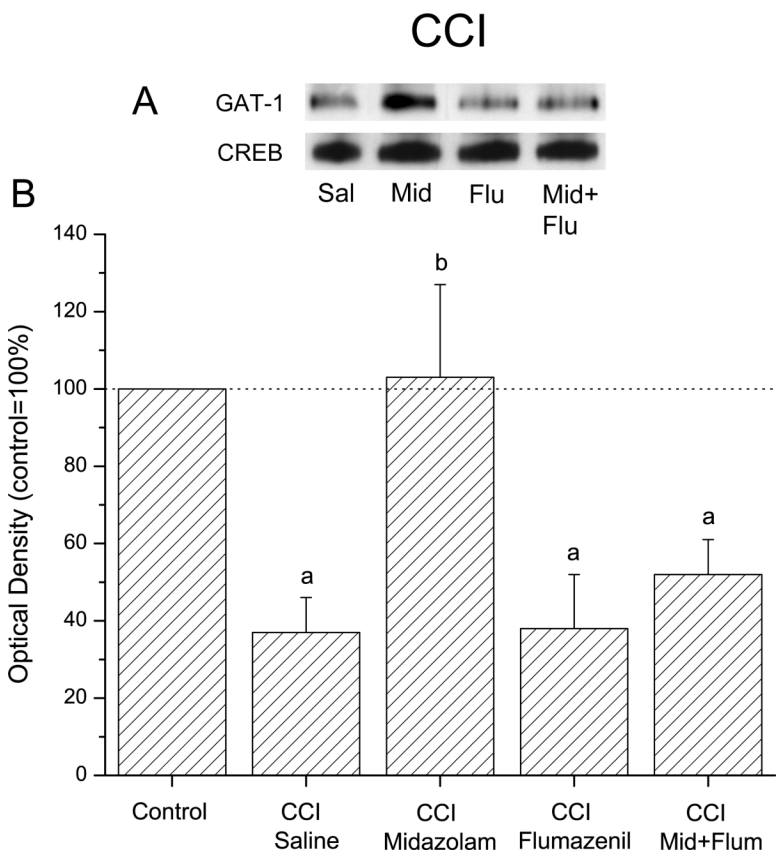

Figure 3. Midazolam treatment prevented the CCI-elicited decreases in GAT-1 protein levels in the ipsilateral spinal dorsal horn

A: GAT-1 immunoblots from CCI animals treated with saline, midazolam, flumazenil or midazolam plus flumazenil. Each band represents a different animal. **B:** Plots of the estimated protein content. Note that the levels of GAT-1 in the saline-treated animals were significantly lower in the injured, ipsilateral dorsal horn. Note also that midazolam treatment prevented the CCI-induced decreases, and that flumazenil blocked the midazolam effect. Note further that flumazenil treatment alone exerted no significant effect on the CCI-elicited reduction in GAT-1 protein levels. All immunoblots were from ipsilateral dorsal horn samples. CREB served as the loading control[36]. Each group consisted of 4 animals. Error bars represent the SD; a=significantly different from control; b=significantly different from saline, flumazenil or midazolam plus flumazenil-treated animals.

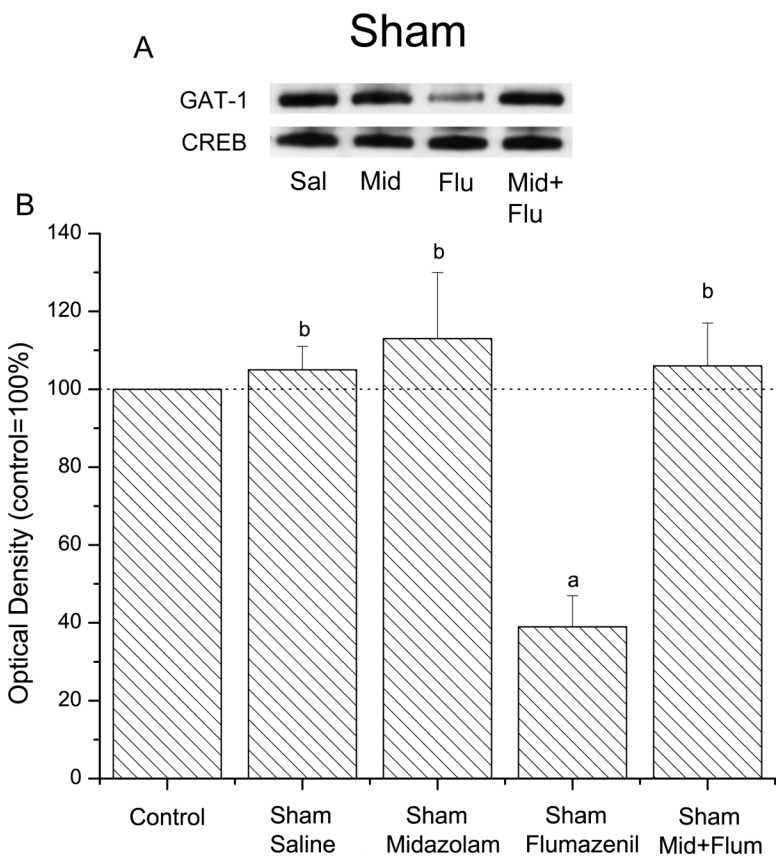

Figure 4. Flumazenil treatment elicited decreases in GAT-1 levels in sham-operated animals

A: GAT-1 immunoblots from sham-operated animals treated with saline, midazolam, flumazenil or midazolam plus flumazenil. Each band represents a different animal. **B:** Plots of the estimated protein content. Note that there were no differences in GAT-1 levels in saline, midazolam or midazolam plus flumazenil-treated animals when compared to controls. Note however that the GAT-1 levels in the dorsal horn of flumazenil-treated animals were significantly decreased. All immunoblots were from ipsilateral dorsal horn samples. CREB served as the loading control[36]. Each group consisted of 4 animals. Error bars represent the SD; a=significantly different from control; b=significantly different from flumazenil-treated animals.