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## INTRATHECAL MIDAZOLAM REGULATES SPINAL AMPA RECEPTOR EXPRESSION AND FUNCTION AFTER NERVE INJURY IN RATS

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### Abstract

Spinal  $\gamma$ -aminobutyric acid (GABA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors have been implicated in the mechanisms of neuropathic pain after nerve injury; however, how these two receptors interact at the spinal level remains unclear. Here we show that intrathecal midazolam through activation of spinal GABAA receptors attenuated the expression and function of spinal AMPA receptors in rats following peripheral nerve injury. Thermal hyperalgesia and mechanical allodynia induced by chronic constriction nerve injury (CCI) in rats were attenuated by the short-acting benzodiazepine midazolam (20=10>5  $\mu$ g > vehicle) administered intrathecally once daily for seven postoperative days. CCI-induced upregulation of AMPA receptors within the spinal cord dorsal horn was also significantly reduced by the intrathecal midazolam (10, 20  $\mu$ g) treatment. The inhibitory effects of midazolam (10, 20  $\mu$ g) on neuropathic pain behaviors and AMPA receptor expression were prevented by co-administration of midazolam with the GABAA receptor antagonist bicuculline (3  $\mu$ g), whereas intrathecal treatment with bicuculline (1 or 3  $\mu$ g) alone in naive rats induced the upregulation of spinal AMPA receptor expression and nociceptive responses, indicating a tonic regulatory effect from endogenous GABAergic activity on the AMPA receptor expression and spinal nociceptive processing. These results indicate that modulation of spinal AMPA receptor expression and function by the GABAergic activity may serve as a mechanism contributory to the spinal nociceptive processing.

### Keywords

AMPA receptor; GABA receptor; Midazolam; Nerve injury; Neuropathic pain; Intrathecal

### Introduction

Neuropathic pain after peripheral nerve injury is a chronic pain condition, which remains very difficult to treat. Spinal glutamatergic and  $\gamma$ -aminobutyric acid (GABA)ergic systems are among several proposed mechanisms of neuropathic pain and have been extensively investigated over the last two decades (Dubner, 1991; Dougherty and Willis, 1991; Yamamoto

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and Yaksh, 1992;Mao et al., 1995;Woolf and Mannion, 1999;Hammond 2001). Evidence exists indicating that either activation of the glutamatergic system or a possible loss of GABAergic activity, or both, after peripheral nerve injury contributes to the development and maintenance of neuropathic pain behaviors in rats (Mao et al., 1995;1997;Bridges et al., 2001;Cronin et al., 2004;Polgar et al., 2005;Scholz et al., 2005). These earlier studies suggest a possible interaction between these regulatory systems within the central nervous system.

Ionotropic glutamate receptors including alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are localized both presynaptically and postsynaptically within superficial laminae of spinal cord dorsal horn. AMPA receptors have been shown to play a significant role in the mechanisms of neuropathic pain and spinal nociceptive processing (Mao et al., 1992a,b;Leem et al., 1996;Kondo et al, 2002;Garry et al., 2003). The AMPA receptor consists of several subunits and previous studies have indicated that spinal AMPA receptor expression was altered after either peripheral nerve injury or inflammation, which contributed to the development of pathological pain conditions in rats (Harris et al., 1996;Alvarez et al., 2000).

On the other hand, it has been demonstrated that activation of spinal GABAergic system negatively regulated spinal nociceptive processing (Kontinen and Dickenson, 2000;Hammond 2001;Nishiyama et al., 2003), suggesting an important role for the GABAergic system in the balance between spinal excitatory and inhibitory elements after peripheral nerve injury. Benzodiazepines activate GABAA receptors, reduce excitatory transmitter release presynaptically as well as excitatory activity postsynaptically in spinal dorsal horn neurons (Haefely, 1988). Indeed, intrathecal midazolam (a benzodiazepine-GABAA receptor agonist) attenuated neuropathic pain behaviors (Hwang and Yaksh, 1997). Recently, presynaptic AMPA receptors on GABAergic terminals have been shown to have a bidirectional role in neuronal activity with the superficial spinal cord dorsal horn and contributed to the mechanisms of central sensitization and hyperalgesia (Lu et al, 2005). Moreover, the effect on GABA receptors may interact with that of AMPA receptor antagonists in rats (Nishiyama et al, 1999). Thus, it is possible that there might be interactions at the spinal level between the GABA and AMPA receptors and such interactions may have a functional role in neuropathic pain behaviors.

Utilizing a rat model of chronic constriction nerve injury (CCI) (Bennett and Xie, 1988), we examined whether midazolam (a clinically available short-acting benzodiazepine and GABA analogue) given intrathecally would modulate the expression of spinal AMPA receptors and neuropathic pain behaviors in CCI rats through activation of spinal GABAA receptors.

## Material and Methods

### CCI model

Adult male Sprague Dawley rats weighing 275–325 gm (Charles River Laboratories, Wilmington, MA) were used. The animal room was artificially lighted from 7 AM to 7 PM. The experimental protocol was approved through our Institutional Animal Care and Use committee. Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). CCI was produced by loosely ligating one common sciatic nerve (using 4-0 chromic gut sutures, 4 ligatures) according to the method of Bennett and Xie (1988), and sham operation was performed following the same surgical procedure for CCI except for nerve ligation.

### Intrathecal catheterization and drug delivery

An intrathecal catheter was implanted in each rat under the same surgical condition. A PE-10 catheter was inserted onto the lumbar enlargement (about 8.5 cm from the incision site for this

rat size) according to the method described previously (Yaksh and Rudy, 1976; Mao et al., 2002). Those rat exhibiting postoperative neurological deficits (e.g., paralysis) or poor grooming and eating were excluded from experiments as described previously (Mao et al., 2002). Midazolam was purchased from Sigma (St. Louis, MO) and bicuculline and S-AMPA were purchased from Tocris (Ellisville, MO). Bicuculline was dissolved in 10% DMSO diluted in normal saline (vehicle) and S-AMPA was dissolved in normal saline. Midazolam was diluted in normal saline as indicated. All drugs were injected intrathecally in a 10- $\mu$ l volume followed by a 10- $\mu$ l saline flush. The doses for each agent used in this study are: 1) midazolam (a GABA agonist, 5, 10, 20  $\mu$ g), bicuculline (a GABAA receptor antagonist, 0.3, 1, 3  $\mu$ g), S-AMPA (an AMPA receptor agonist, 0.1  $\mu$ g).

### Behavioral test and statistical analysis

Rats were habituated to the test environment (one 60 min session per day) for two consecutive days before the baseline testing. Testing for thermal hyperalgesia was performed according to a previously published method (Hargreaves et al., 1988). Radian heat was adjusted to ensure the baseline latency at 10–12 sec and the cutoff time of 20 sec. At least two trials were made in each rat. Mechanical allodynia was examined by applying a von Frey filament to the plantar surface of each hindpaw until the filament was bended and threshold force was established by examining the bending force that resulted in at least two clear paw-withdrawals out of five applications (Tal and Bennett, 1994). The cutoff force was 20 gm. All testing was conducted between 9 AM and 1 PM.

Data from the thermal hyperalgesia (withdrawal latency in sec) test were analyzed by using repeated measure two-way ANOVA across testing time points to detect overall differences among treatment groups. Whenever applicable, the data were also examined by using repeated measure two-way ANOVA across treatment group to examine overall difference among testing time points. When significant main effects were observed, *post hoc* Newman-Keuls's test were followed. Data from the mechanical allodynia (threshold bending force in gm) test were analyzed using the non-parametric Mann-Whitney test. The statistically significant level was set at  $\alpha = 0.05$ .

### Western blot

Rats were decapitated rapidly (<1 min) under pentobarbital anesthesia and lumbar spinal cord segments (lumbar 5) were removed. Spinal segments were separated into the ipsilateral and contralateral side as well as the dorsal and ventral horn and homogenized in SDS sample buffer containing a mixture of proteinase inhibitors (Sigma). Lumbar segments were harvested because CCI has a major impact at these sites. Protein samples were separated on SDS-PAGE gel (4–15% gradient gel; Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride filters (Millipore, Bedford, MA). The filters were blocked with 3% milk and incubated overnight at 4 °C with a primary antibody (GluR1 - rabbit polyclonal, 1:100, Chemicon, Temecula, CA; GluR2 - mouse monoclonal, 1:250, BD, Franklin Lakes, NJ; GluR3 - mouse monoclonal, 1:300, Chemicon) and then 1 hr at room temperature with HRP-conjugated secondary antibody (1:700, Amersham, Arlington Heights, IL). The blots were visualized in ECL solution (NEN, Boston, MA) for 1 min and exposed onto hyperfilms (Amersham) for 1–10 min. The blots were then incubated in a stripping buffer (67.5 mM Tris, pH 6.8, 2% SDS, 0.7%  $\beta$ -mercaptoethanol) for 30 min at 50 °C and reprobated with a polyclonal rabbit anti- $\beta$ -actin antibody (1:20,000; Alpha Diagnostic International, San Antonio, TX) as loading control. The Western analysis was made in triplicates. The density of a specific band was measured against a corresponding loading control, and differences were compared using repeated measure one-way ANOVA followed by *post hoc* Newman-Keuls's tests.

## Results

### Effects of midazolam on neuropathic pain behaviors: reduction by bicuculline

The effects of midazolam on neuropathic pain behaviors were examined in seven groups of rats including (1) CCI plus vehicle, (2–4) CCI plus 5, 10 or 20 µg midazolam, (5) CCI plus 10 µg midazolam and 3 µg bicuculline, (6) sham plus 10 µg midazolam, and (7) sham plus vehicle. Each agent was given once daily (intrathecally) for seven consecutive postoperative days, beginning immediately after operation. Midazolam (20 = 10µg > 5µg > vehicle) reduced thermal hyperalgesia and mechanical allodynia in the hindpaw ipsilateral to CCI, as compared with vehicle-treated CCI rats on all of postoperative days (Fig. 1,  $p < 0.05$ ;  $n = 6-7$ ). In contrast, midazolam (10 or 20 µg) did not change baseline thermal and mechanical nociceptive responses in sham rats, nor did midazolam (5–20 µg) change thermal and mechanical nociceptive responses in the hindpaw contralateral to CCI (Fig. 1,  $P > 0.05$ ;  $n = 5-7$ ).

The effects of midazolam on neuropathic pain behaviors were primarily mediated through the GABAA receptor, because co-administration of midazolam (10 µg) with the GABAA receptor antagonist bicuculline (3 µg), given once daily for postoperative day 1–7, reversed the attenuation of thermal hyperalgesia and mechanical allodynia by midazolam (Fig. 1,  $P < 0.05$ ,  $n = 5-7$ ). The bicuculline dose was referenced from previous studies (Hwang and Yaksh, 1997; Kaneko and Hammond, 1997; Malan et al., 2002) and a lower dose of bicuculline (0.3 µg) was ineffective in our pilot experiment. The combined treatment of midazolam (10 µg) and bicuculline (3 µg) did not alter the thermal and mechanical nociceptive responses in the hindpaw contralateral to CCI (Fig. 1,  $p > 0.05$ ;  $n = 5-7$ ). These results indicate that intrathecal midazolam attenuated neuropathic pain behaviors in CCI rats, which was mainly mediated through the GABAA receptor within the spinal cord dorsal horn.

### Effects of midazolam on spinal AMPA receptor expression: reduction by bicuculline

The effects of midazolam on the expression of spinal AMPA receptors after CCI were examined using Western blot. CCI but not sham operation induced an upregulation of AMPA receptors (GluR1, GluR2, GluR3) within the spinal cord dorsal horn ipsilateral to CCI on postoperative day 4 and 8 (not day 1) (Fig. 2,  $p < 0.05$ ,  $n = 4-6$ ), while there were no significant changes in the AMPA receptor expression within the contralateral spinal cord dorsal horn (data not shown). Intrathecal treatment with midazolam (10 = 20 µg, but not 5µg, once daily for seven postoperative days) reduced the upregulation of AMPA receptors in CCI rats as compared with CCI rats treated with a vehicle (Fig. 3,  $p < 0.05$ ,  $n = 4-6$ ). Similar to its effects on neuropathic pain behaviors, the GABAA receptor antagonist bicuculline reduced the effect of midazolam on spinal AMPA receptor expression when bicuculline (3 µg) was co-administered intrathecally with midazolam (10µg) once daily for postoperative day 1–7 (Fig. 4,  $p < 0.05$ ,  $n = 5-6$ ). These results indicate that the upregulation of spinal AMPA receptor after CCI was mediated at least in part through the GABAA receptor within the spinal cord dorsal horn.

### Effects of bicuculline on nociceptive behaviors and spinal AMPA receptor expression in naïve rats

In order to examine whether inhibition of GABAA receptor activity within the spinal cord would alter thermal and mechanical nociceptive responses as well as spinal AMPA receptor expression in naïve rats, four groups of rats were used including naïve rats treated with 0.3, 1 or 3 µg bicuculline. Each dose was given once daily to naïve rats for seven consecutive days. Bicuculline (3 µg but not 0.3 µg or 1µg) induced nociceptive responses to thermal and mechanical stimulation in naïve rats (Fig. 5,  $P < 0.05$ ,  $n = 5$ ), similar to that observed after CCI. Interestingly, bicuculline (3 µg but not 0.3 or 1µg) treatment also induced an upregulation of AMPA receptors (GluR1, GluR2, GluR3) within the spinal cord dorsal horn of naïve rats in the absence of CCI (Fig. 6,  $p < 0.05$ ,  $n = 5$ ). Collectively, these results demonstrate a tonic

influence from spinal GABA activity on both AMPA receptor expression and nociceptive responses.

### Effects of midazolam on nociceptive behaviors induced by S-AMPA in naïve rats

In order to confirm the modulatory effect of spinal GABA activity on AMPA receptor-mediated nociceptive behaviors, the effects of midazolam on nociceptive responses induced by an exogenous AMPA receptor agonist (S-AMPA) were examined in the following groups of naïve rats including (1) 0.1 µg S-AMPA alone, (2) 0.1 µg S-AMPA and 10 µg midazolam, (3) vehicle alone. S-AMPA dose was referenced from a previous study (Kontien and Meert, 2002). Each agent was given intrathecally once daily for seven consecutive days.

S-AMPA (0.1 µg) induced nociceptive responses to thermal or mechanical stimulation, as compared with the vehicle group, when examined on day 8 (Fig. 7,  $P < 0.05$ ,  $n=5-7$ ). The S-AMPA-induced nociceptive behaviors were attenuated by intrathecal co-administration of S-AMPA (0.1 µg) with midazolam (10 µg) once daily for seven days, when also examined on day 8 (Fig. 7,  $P < 0.05$ ,  $n=5-7$ ). Midazolam (10 µg) alone did not alter nociceptive behaviors in sham rats (see Fig. 1). Moreover, S-AMPA (0.1 µg) induced an upregulation of AMPA receptor (GluR1, GluR2, GluR3) within the spinal cord dorsal horn of naïve rats when examined on day 8, which was also significantly reduced by co-administering midazolam (10 µg) with S-AMPA for seven days (Fig 8,  $p < 0.05$ ,  $n=5-7$ ). Similar to that seen in sham rats (Fig. 3), Midazolam (10 µg) alone did not change the baseline AMPA receptor expression (data not shown). These results support a functional relationship between spinal GABA activity and AMPA receptor-mediated nociceptive responses in naïve rats as well.

## Discussion

We have demonstrated that 1) intrathecal treatment with midazolam attenuated the development of thermal hyperalgesia and mechanical allodynia in CCI rats, which was mediated primarily through spinal GABAA receptors, 2) CCI induced an upregulation of spinal AMPA receptor (GluR1, GluR2, and GluR3 subunits) expression, which was also attenuated by midazolam through activation of spinal GABAA receptors, 3) inhibition of spinal GABAA receptors with bicuculline in naïve rats induced an upregulation of spinal AMPA receptor expression and nociceptive responses to thermal and mechanical stimulation, and 4) activation of spinal AMPA receptors with S-AMPA (an exogenous AMPA receptor agonist) induced both nociceptive responses and the upregulation of spinal AMPA receptor expression in naïve rats, preventable by intrathecal midazolam treatment. These results indicate a functional relationship between spinal GABAA and AMPA receptors under both physiological and pathological (nerve injury) conditions, which contributed to the development of neuropathic pain behaviors in rats.

A technical concern is an appropriate intrathecal dose range of midazolam. Previous studies have shown that the effect of intrathecal midazolam on nociceptive behaviors was dose-dependent such that a low dose range (2–4 µg) did not produce significant antinociceptive effects (Taira et al., 2000), whereas a high dose range (10–100 µg) was effective in antinociception (Nishiyama et al., 1999). Previous studies have also shown that intrathecal 30 µg midazolam induced motor dysfunction (Nishiyama et al., 1999) and midazolam at an even higher dose (e.g., 100 µg/day for 20 consecutive days) caused spinal neurotoxicity (Svenson et al., 1995). Considering these potential confounding factors, we used a lower but effective dose range (5, 10, 20 µg) in the present studies and did not observe any motor dysfunction and abnormal behavioral responses in both sham and CCI rats.

The role of spinal AMPA receptors in neuropathic pain behaviors after nerve injury, as demonstrated in the present study, is consistent with previous studies (Mao et al.,

1992a,b;Leem et al., 1996;Kondo et al, 2002;Garry et al., 2003). For instance, activation of spinal AMPA receptors has been shown to induce nociceptive behaviors including thermal hyperalgesia (Yamamoto and Sakashita, 1998;Menéndez et al., 2003) and vocalization (Kontinen and Meert, 2002). Our previous study has indicated that non-NMDA receptors within the spinal cord are necessary for the development of neuropathic pain behaviors after CCI, because inhibition of non-NMDA receptors alone prevented the development of thermal hyperalgesia and spontaneous pain behaviors in CCI rats (Mao et al., 1992a,b). The role of non-NMDA receptor activation in this process may be in part related to its ability to depolarize neuronal membrane potentials thereby facilitating the activation of NMDA receptors (Dubner, 1991;Mao, et al., 1995;Garry et al., 2003).

GABA is an important inhibitory neurotransmitter (Sosnowski and Yaksh, 1990;Hammond 2001). Spinal GABAergic activity mediates synaptic inhibition and diminishes AMPA receptor-mediated acute nociception (Nishiyama et al., 1998), thereby modulating spinal nociceptive processing (Simmons et al., 1998). A growing body of evidence has suggested a functional relationship between GABA and AMPA receptor-mediated activities in spinal nociceptive processing (Izzo et al., 2001;Kovacs et al., 2003;Sanchez et al., 2005). For example, there is a presynaptic modulatory role of AMPA receptors in the GABA release (Engelman et al., 2006) and midazolam regulates excitatory transmission in spinal cord dorsal horn neurons of adult rats (Kohno et al., 2006). In addition, colocalization between GABA and AMPA receptors has been demonstrated in several spinal and supraspinal regions (Kondo et al., 1995;Kerr et al., 1998;Bergersen et al., 2003), and ionotropic glutamate receptors are expressed in GABAergic terminals in the rat superficial dorsal horn (Lu et al., 2006).

In the present study, a short-acting benzodiazepine (midazolam) effectively prevented the development of neuropathic pain behaviors in CCI rats after repeated administration, which was blocked by the GABA<sub>A</sub> receptor antagonist bicuculline. These data provides additional evidence for a regulatory mechanism regarding the expression and function of spinal AMPA receptors through activation of spinal GABA<sub>A</sub> receptors. It should be pointed out that the present study only examined the regulatory role of spinal GABA<sub>A</sub> activity on the AMPA receptor-mediated development of neuropathic pain behaviors after CCI, these results do not rule out the possibility that spinal GABA activity may also regulate the maintenance of neuropathic pain behaviors.

Consistent with previous studies, we observed a tonic GABAergic influence on spinal AMPA receptor expression and spinal nociceptive processing because inhibition of intrinsic GABA activity in naïve rats induced mechanical allodynia and thermal hyperalgesia. However, this endogenous inhibitory GABAergic activity seen in naïve rats must be limited or insufficient under pathological conditions, because 1) both thermal hyperalgesia and mechanical allodynia did develop in CCI rats despite this intrinsic inhibitory GABA activity and 2) activation of spinal AMPA receptors with exogenous AMPA receptor agonist S-AMPA was able to induce nociceptive behaviors and upregulation of spinal AMPA receptor expression in naïve rats, similar to the results observed after CCI. While the endogenous GABAergic activity may play a role in the regulation of spinal nociceptive processing, it is possible that this function could be diminished after nerve injury presumably due to a potential loss of spinal GABAergic interneurons (Sugimoto et al., 1990;Mao et al., 1997;Scholz et al., 2005). These findings add to the discussion concerning the role of spinal GABAergic neurons in the mechanisms of neuropathic pain (Polgar et al., 2005;Scholz et al., 2005). It seems reasonable to suggest that preserving endogenous spinal GABAergic activity alone after nerve injury might be insufficient to counter-balance spinal excitation induced by glutamatergic activities, as indicated by our data showing that AMPA receptor-mediated spinal excitation induced nociceptive responses in naïve rats even in the presence of an intact endogenous GABAergic system (i.e., without nerve injury).

Benzodiazepine receptors are coupled with a subpopulation of GABA receptors and benzodiazepines can enhance spinal GABA activity by increasing  $\text{Cl}^-$  conduction (Unnerstall et al., 1981) and reducing glutamate release in the spinal cord (Haefely, 1988). These interactions between benzodiazepines and GABA receptors may be a pharmacological basis for the midazolam effect that was preventable by the GABAA receptor antagonist bicuculline. Besides its antinociceptive effects (Kontinen and Dickenson, 2000; Nishiyama et al., 2003), intrathecal midazolam exhibited a potent synergistic effect with AMPA receptor antagonists in rats (Nishiyama et al., 1999; Nishiyama et al., 2001). Thus, our data suggest that a combined treatment with a GABA receptor agonist and an AMPA receptor antagonist may be an effective approach for managing clinical neuropathic pain.

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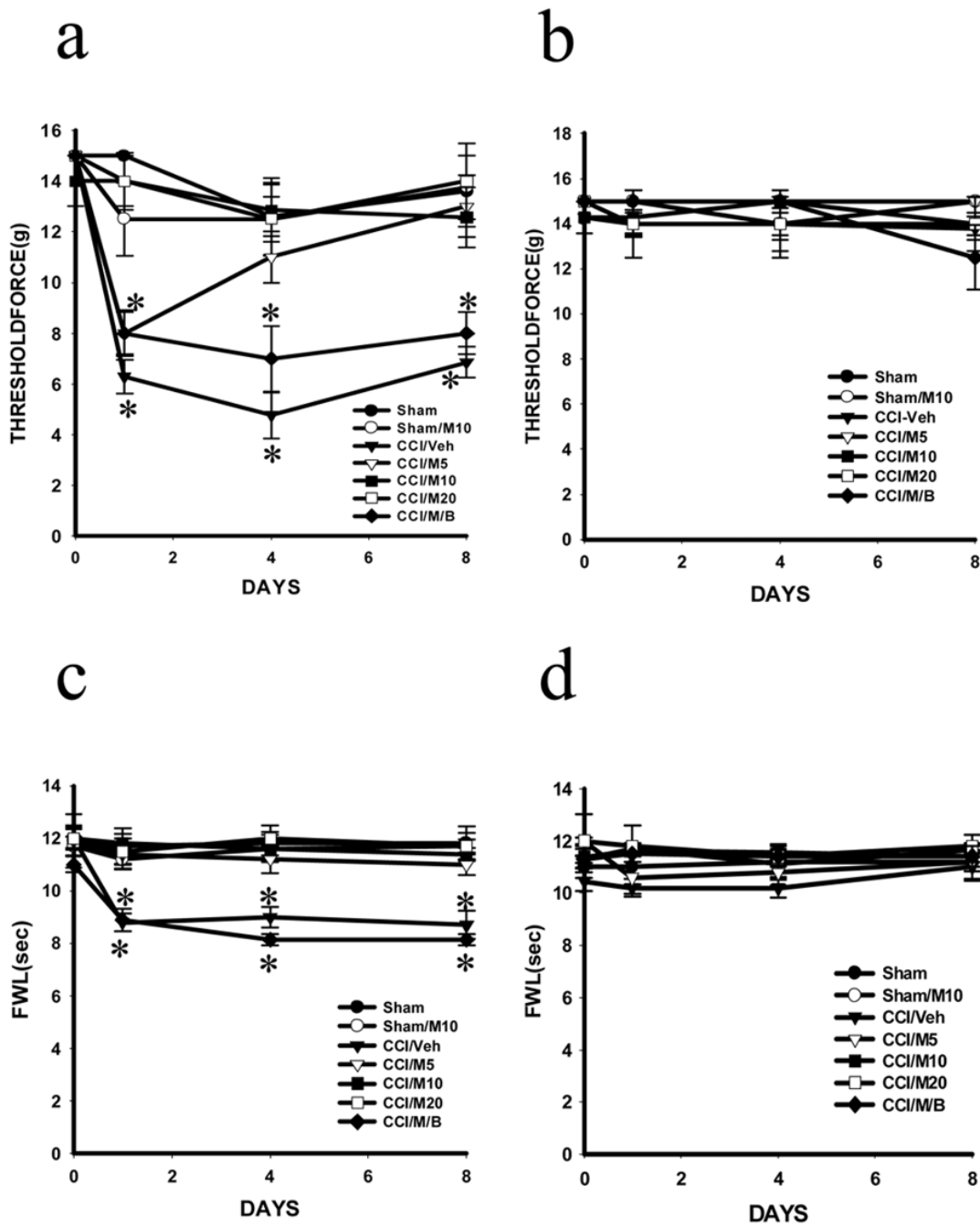
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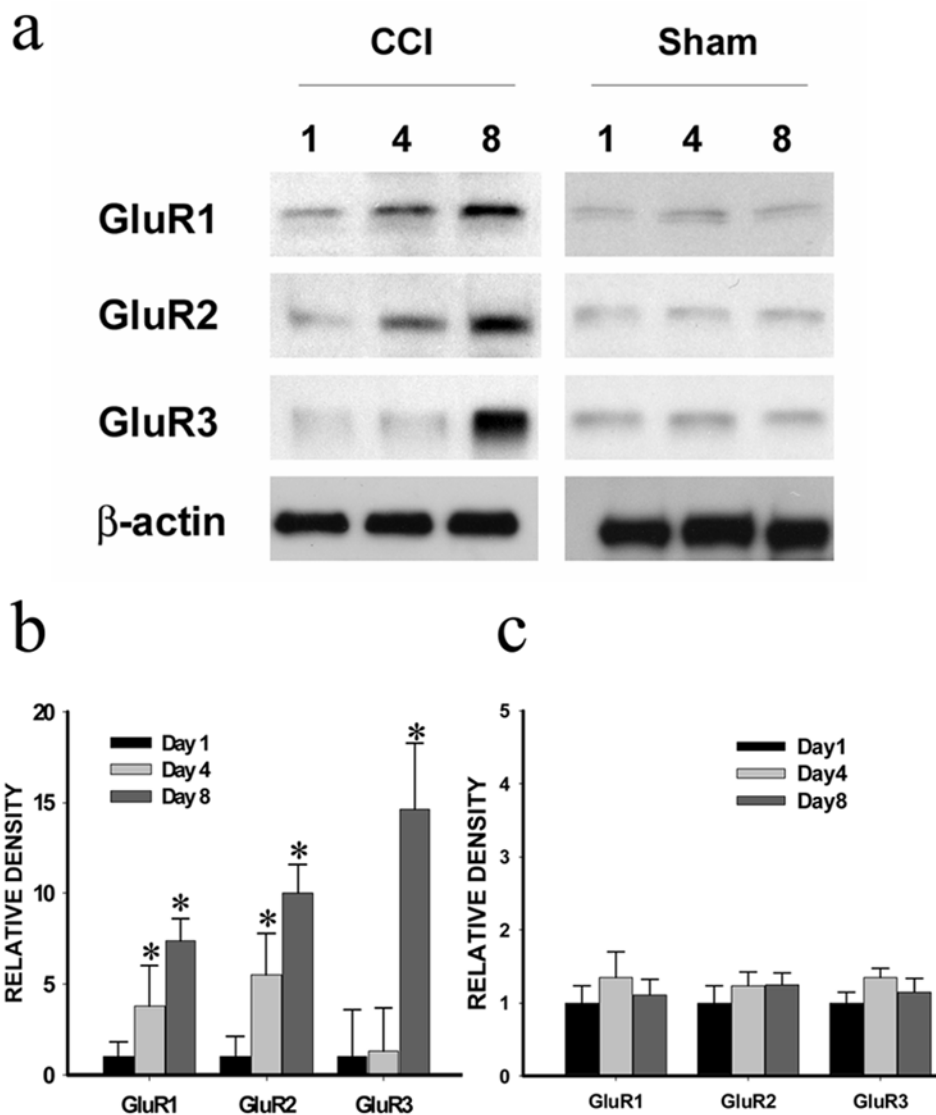
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**Figure 1. Effects of midazolam and bicuculline on mechanical allodynia and thermal hyperalgesia of CCI rat**

Mechanical allodynia (a, b) was dose-dependently attenuated in the ipsilateral hindpaw (a) in CCI rats treated with midazolam (5, 10 and 20  $\mu$ g; CCI/M5, CCI/M10 and CCI/M20) but not vehicle (CCI/Veh) given intrathecally once daily for postoperative day 1–7. Thermal hyperalgesia (c, d) were attenuated in the ipsilateral hindpaw (c) in CCI rats treated with midazolam (same as above) but not vehicle (CCI/Veh) given intrathecally once daily for postoperative day 1–7. Midazolam did not alter baseline nociceptive response in sham rats (Sham/M10). There were no changes in nociceptive responses in the contralateral hindpaw (b, d). The attenuation by midazolam (10  $\mu$ g) of mechanical allodynia (a) and thermal hyperalgesia

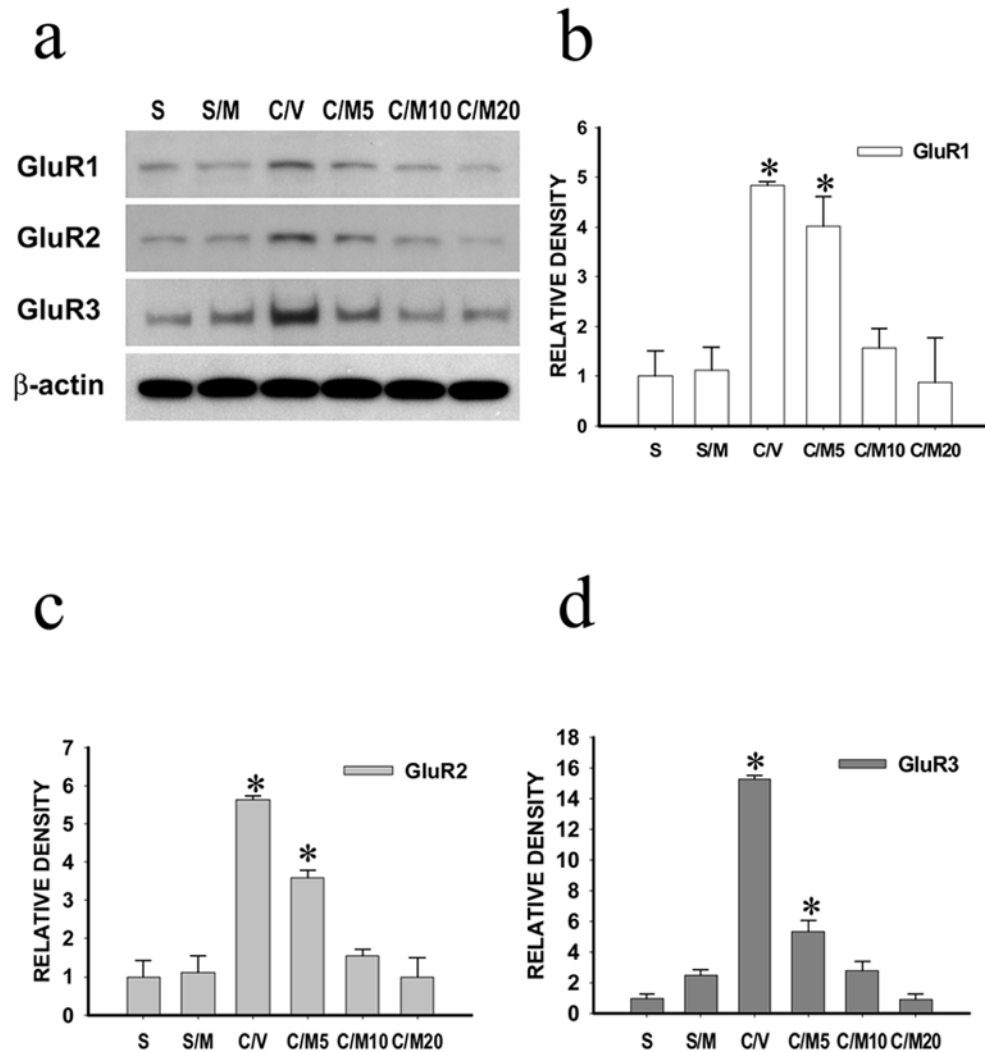
(c) in the ipsilateral hindpaw of CCI rats was reduced by bicuculline (3  $\mu$ g; CCI/M/B), given intrathecally once daily for postoperative day 1–7. The combined treatment with midazolam and bicuculline did not alter the nociceptive responses in the contralateral hindpaw \*  $P < 0.05$  as compared with the corresponding sham group. FWL: foot-withdrawal latency; Threshold force: a measurement for mechanical allodynia (also in other figures). For all figures, the number of rats per group was shown in the main text.



**Figure 2. Time course of AMPA receptor expression after CCI**

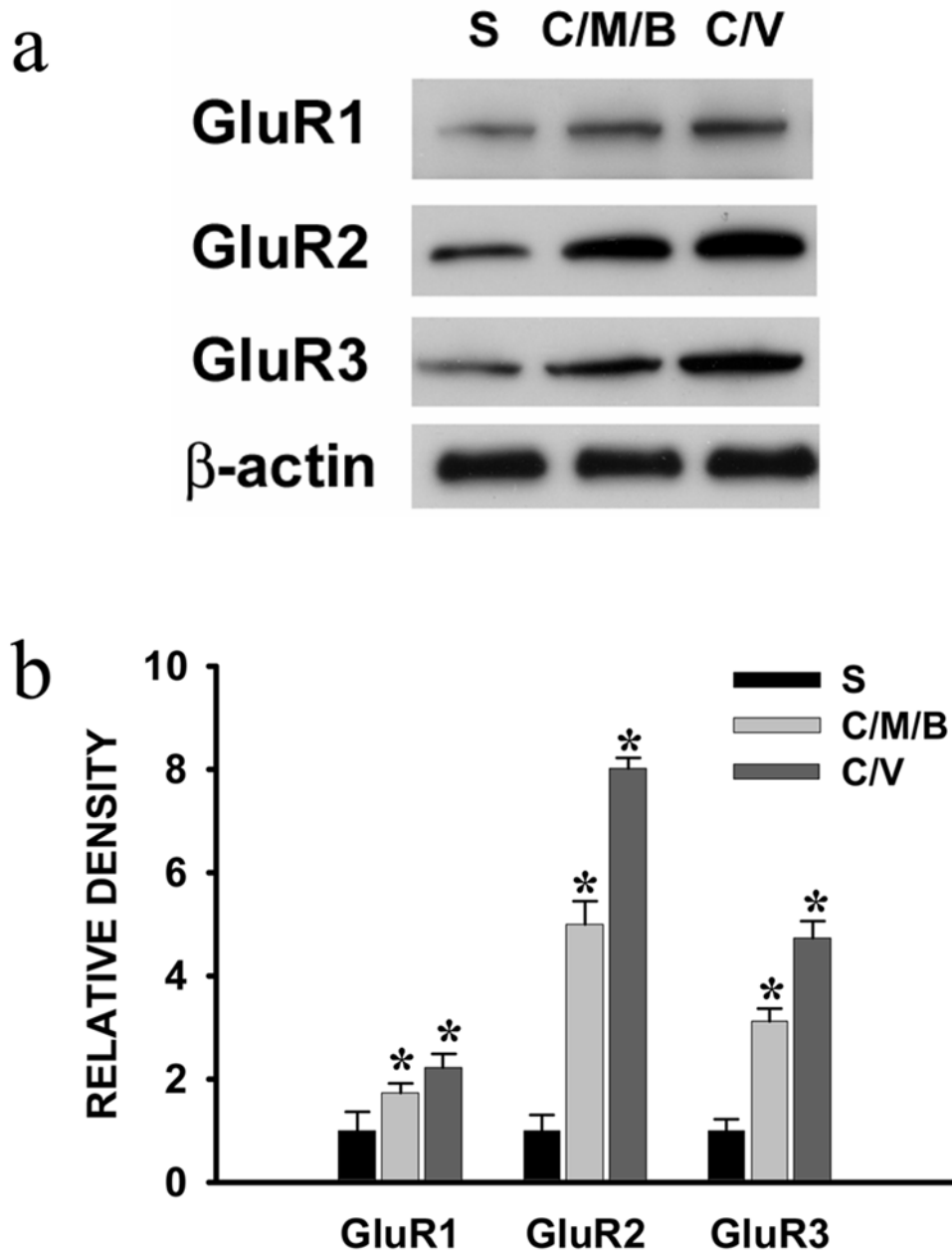
The AMPA receptor (GluR1, GluR2, GluR3) expression (Western blot) within the ipsilateral spinal cord dorsal horn was upregulated in CCI rats but not in sham rats on postoperative day 4 and 8 (not day 1). a: Western blots;  $\beta$ -actin is a loading control. B, c: Statistical data analysis.

\*  $P < 0.05$  as compared with the corresponding sham control.



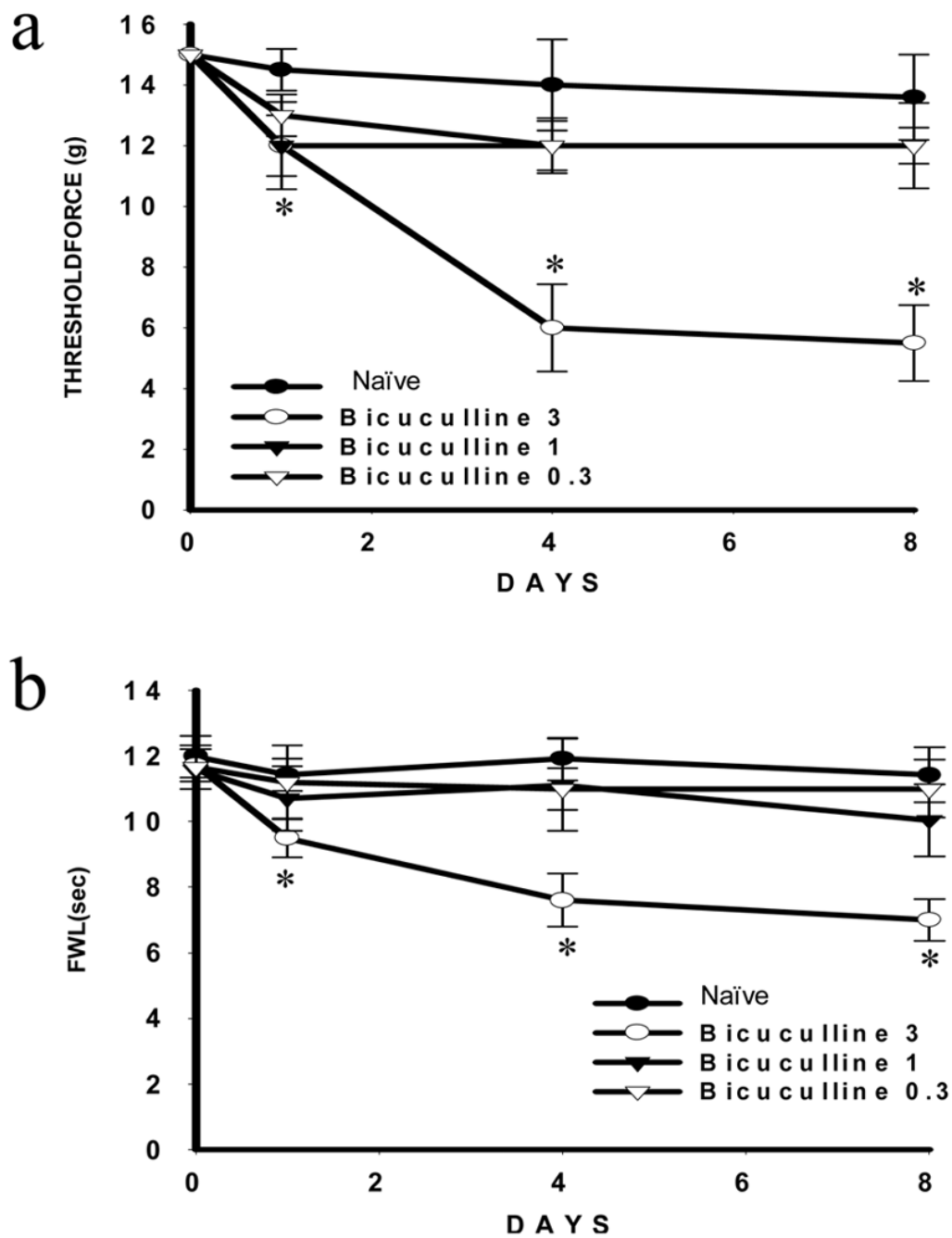
### Figure 3. Effects of midazolam on spinal AMPA receptor expression

The AMPA receptor (GluR1, GluR2, GluR3) expression (Western blot) within the ipsilateral spinal cord dorsal horn was upregulated in CCI rats, which was reduced by intrathecal midazolam (10 or 20  $\mu$ g, not 5 $\mu$ g) given once daily for postoperative day 1–7. Samples were taken on day 8. a: Western blots;  $\beta$ -actin is a loading control. b-d: Statistical data analysis. S: sham; S/M; sham rats with 10  $\mu$ g midazolam; C/M5, C/M10 or C/M20: CCI rats with 5, 10 or 20  $\mu$ g midazolam; C/V: CCI rats with vehicle. \*  $P < 0.05$  as compared with the corresponding sham group.

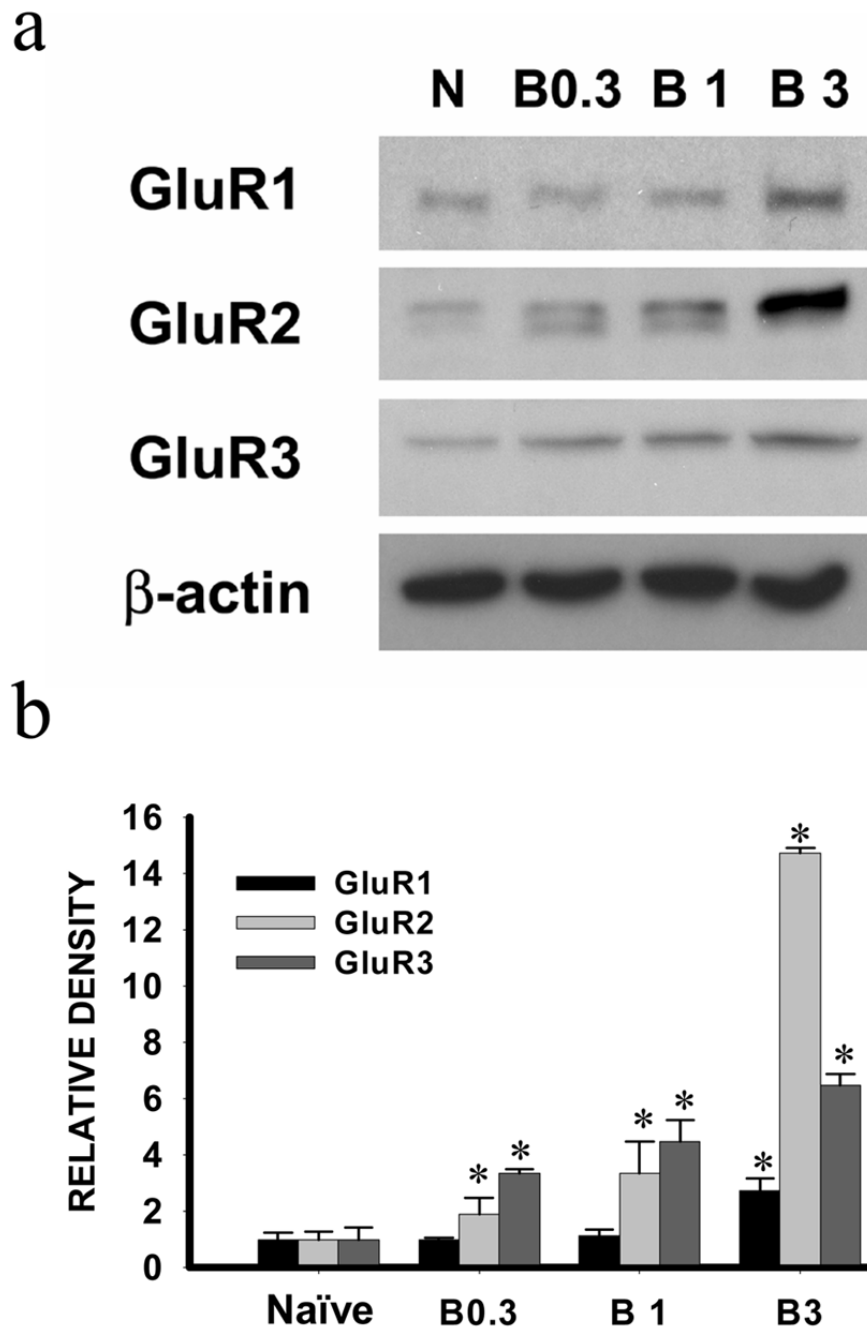


**Figure 4. Effects of bicuculline on spinal AMPA receptor expression**

The effect of midazolam (10  $\mu$ g) on the AMPA-R expression within the ipsilateral spinal cord dorsal horn after CCI was attenuated by co-administration of bicuculline (3  $\mu$ g) once daily for seven days. Samples were taken on day 8. C/M/B: CCI rats with midazolam and bicuculline; C/V: CCI rats with vehicle. \*  $P < 0.05$  as compared with the corresponding sham group.

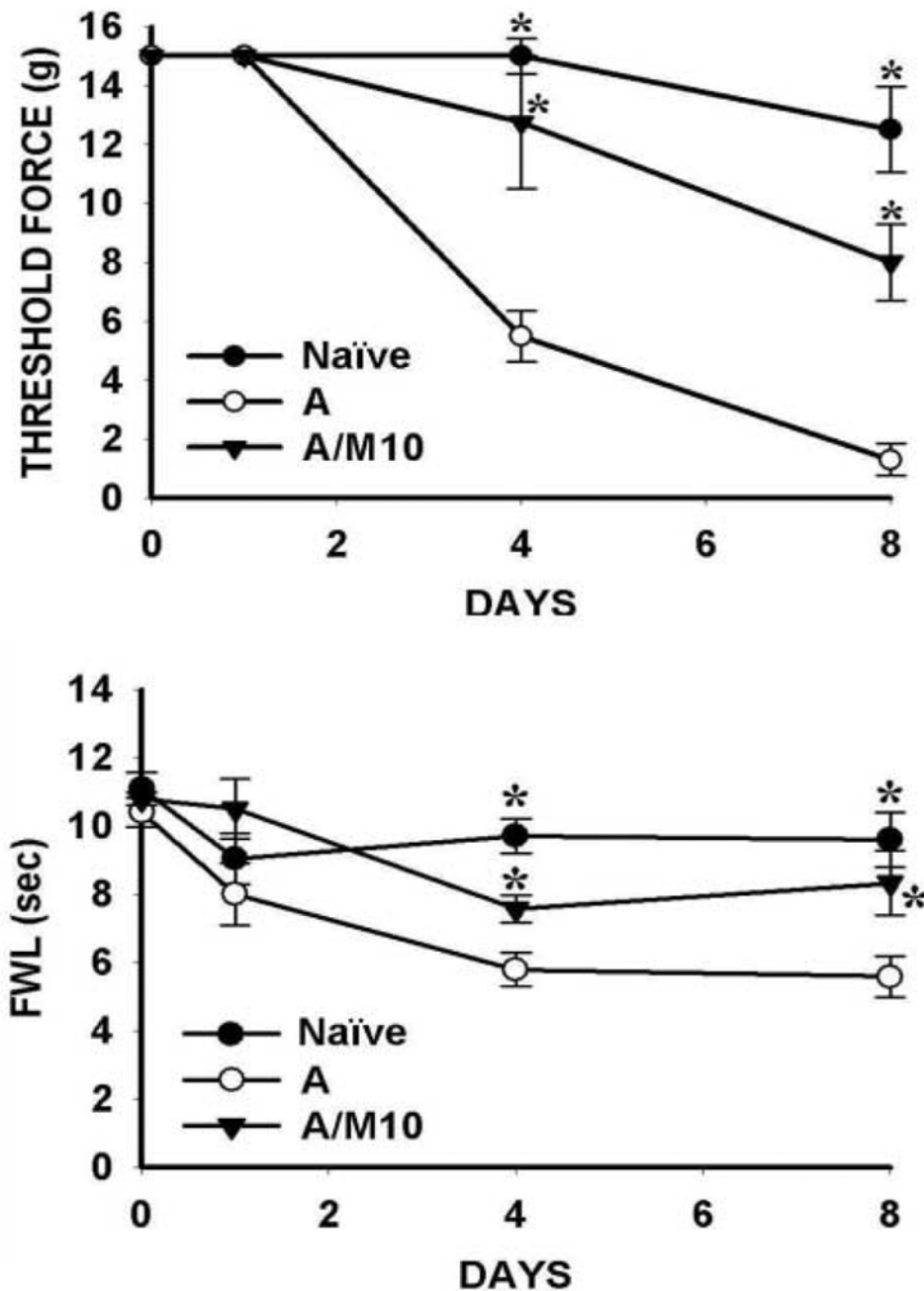


**Figure 5. Effect of bicuculline on nociceptive behaviors in naïve rats**  
 Intrathecal treatment with bicuculline (3 µg but not 0.3 µg and 1 µg) given once daily for seven days induced nociceptive responses to mechanical (a) and thermal (b) stimulation. The behavioral tests were made on day 8. \* P < 0.05 as compared with the naïve group.



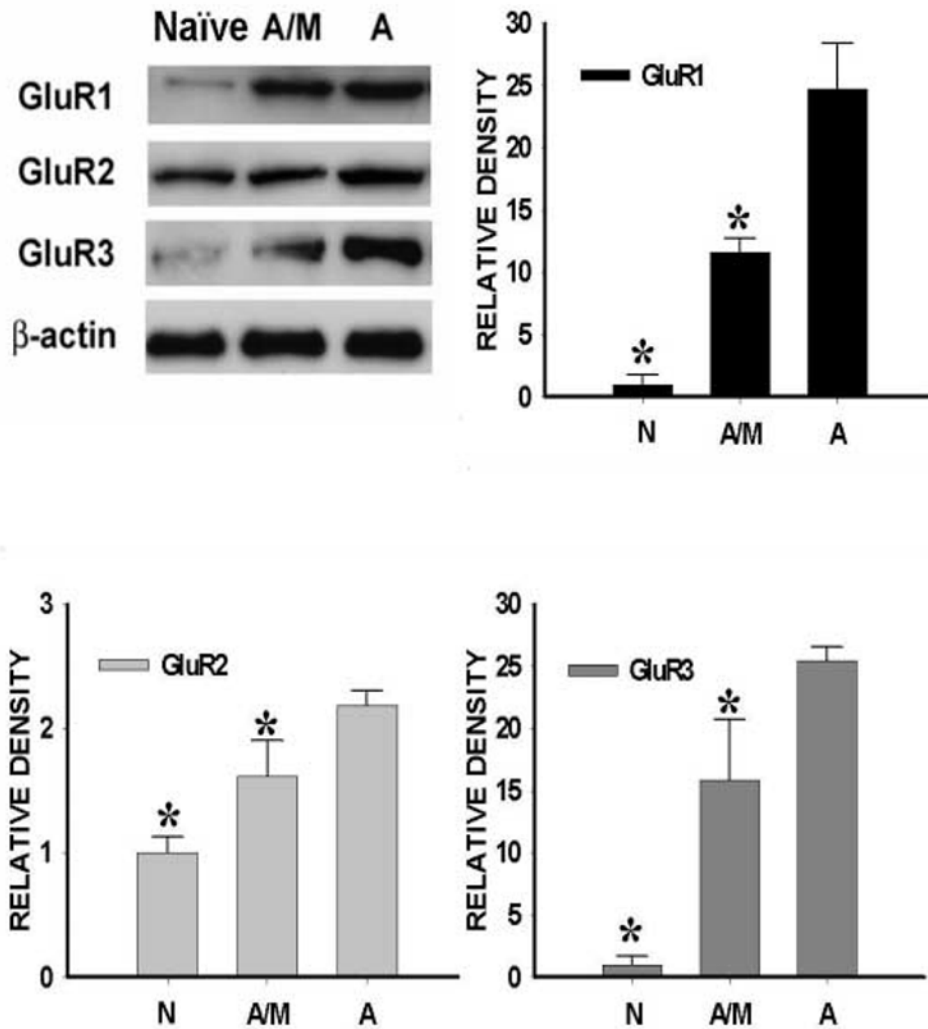
**Figure 6. Effect of bicuculline on spinal AMPA receptor expression in naïve rats**  
 Intrathecal treatment with bicuculline (3  $\mu$ g but not 0.3 or 1  $\mu$ g) given once daily for seven days induced the AMPA receptor (GluR1, GluR2, GluR3) upregulation within the spinal cord dorsal horn of naïve rats. Samples were taken on day 8. a: Western blot; b: statistical analysis. \*  $P < 0.05$  as compared with the naïve rat group.





**Figure 7. Effect of S-AMPA and midazolam on nociceptive behaviors in naïve rats**

Intrathecal treatment with the AMPA receptor agonist S-AMPA (0.1  $\mu$ g) given once daily for seven days induced nociceptive responses to mechanical (a) and thermal (b) stimulation, when examined on day 8. The S-AMPA-induced nociceptive responses were attenuated by intrathecal co-administration of S-AMPA (0.1  $\mu$ g) with midazolam (10  $\mu$ g) given once daily for seven days. \*  $P < 0.05$  as compared with the S-AMPA alone group. A/M: S-AMPA +midazolam; A: S-AMPA alone.



**Figure 8. Effect of S-AMPA and midazolam on spinal AMPA receptor expression in naïve rats**  
 The AMPA receptor upregulation induced by S-AMPA (0.1  $\mu$ g) was attenuated by intrathecal co-administration of S-AMPA (0.1  $\mu$ g) with midazolam (10  $\mu$ g) given once daily for seven days. Samples were taken on day 8. a: Western blot; b–d: statistical analysis. \*  $P < 0.05$  as compared with the S-AMPA treated group. A/M: S-AMPA+midazolam; A: S-AMPA alone.