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Spinal α_2-adrenergic and muscarinic receptors and the NO release cascade mediate supraspinally produced effectiveness of gabapentin at decreasing mechanical hypersensitivity in mice after partial nerve injury

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1 After partial nerve injury, the central analgesic effect of systemically administered gabapentin is mediated by both supraspinal and spinal actions. We further evaluate the mechanisms related to the supraspinally mediated analgesic actions of gabapentin involving the descending noradrenergic system.

2 Intracerebroventricularly (i.c.v.) administered gabapentin (100 μg) decreased thermal and mechanical hypersensitivity in a murine chronic pain model that was prepared by partial ligation of the sciatic nerve. These effects were abolished by intrathecal (i.t.) injection of either yohimbine (3 μg) or idazoxan (3 μg), α_2-adrenergic receptor antagonists.

3 Pretreatment with atropine (0.3 mg kg^{-1}, i.p. or 0.1 μg, i.t.), a muscarinic receptor antagonist, completely suppressed the effect of i.c.v.-injected gabapentin on mechanical hypersensitivity, whereas its effect on thermal hypersensitivity remained unchanged. Similar effects were obtained with pirenzepine (0.1 μg, i.t.), a selective M_1-muscarinic receptor antagonist, but not with methoctramine (0.1 and 0.3 μg, i.t.), a selective M_2-muscarinic receptor antagonist.

4 The cholinesterase inhibitor neostigmine (0.3 ng, i.t.) potentiated only the analgesic effect of i.c.v. gabapentin on mechanical hypersensitivity, confirming spinal acetylcholine release downstream of the supraspinal action of gabapentin.

5 Moreover, the effect of i.c.v. gabapentin on mechanical but not thermal hypersensitivity was reduced by i.t. injection of L-NAME (3 μg) or L-NMMA (10 μg), both of which are nitric oxide (NO) synthase inhibitors.

6 Systemically administered naloxone (10 mg kg^{-1}, i.p.), an opioid receptor antagonist, failed to suppress the analgesic actions of i.c.v. gabapentin, indicating that opioid receptors are not involved in activation of the descending noradrenergic system by gabapentin.

7 Thus, the supraspinally mediated effect of gabapentin on mechanical hypersensitivity involves activation of spinal α_2-adrenergic receptors followed by muscarinic receptors (most likely M_1) and the NO cascade. In contrast, the effect of supraspinal gabapentin on thermal hypersensitivity is independent of the spinal cholinergic–NO system.
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Abbreviations: ACh, acetylcholine; 5-HT, 5-hydroxytryptamine (serotonin); L-NAME, L-N^G-nitro arginine methyl ester; L-NMMA, L-N^G-nitro arginine methyl acetate; McN-A-343, 4-(*N*-[3-chlorophenyl]-carbamoyloxy)-2-butynyl-trimethylammonium chloride; NA, noradrenaline; NO, nitric oxide; PWL, paw withdrawal latency

Introduction

The antiepileptic agent gabapentin (neurontin) exhibits analgesic effects in patients with several neuropathic conditions, including diabetic neuropathy and postherpetic neuralgia (Segal & Rordorf, 1996; Backonja *et al*., 1998). These observations are supported by animal studies employing various models of thermal and mechanical hypersensitivity (Singh *et al*., 1996; Field *et al*., 1997; Pan *et al*., 1999b; Luo *et al*., 2001).

A number of studies have characterized the pharmacological properties of gabapentin and have provided several lines of evidence to understand the mechanisms underlying its analgesic effects. Of particular interest is the specific binding of gabapentin to the $\alpha_2\delta$-1 auxiliary subunit of the voltage-sensitive Ca^{2+} channel (Gee *et al*., 1996), which may provide a molecular explanation for the inhibition of high-threshold Ca^{2+} channels (Sutton *et al*., 2002), the modulation of synaptic transmission (Patel *et al*., 2000; Shimoyama *et al*., 2000; Bayer *et al*., 2004), and the antiallodynic effects (Luo *et al*., 2001; 2002) observed with gabapentin. These findings

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have undoubtedly contributed to our understanding of the well-established effects of gabapentin on the spinal cord (Hwang & Yaksh, 1997; Kaneko *et al*., 2000; Patel *et al*., 2000; Luo *et al*., 2001; 2002).

In a recent study, we demonstrated that gabapentin acts on supraspinal structures to activate the descending noradrenergic system that terminates in the lumbar spinal cord, where noradrenaline (NA) interacts with α_2-adrenergic receptors to reduce thermal and mechanical hypersensitivity (Tanabe *et al*., 2005a). This novel supraspinally mediated effect, together with the spinal action, provides a substantial explanation of the analgesia produced by systemically administered gabapentin in mice that develop thermal and mechanical hypersensitivity following partial ligation of the sciatic nerve.

Activation of spinal α_2-adrenergic receptors followed by release of acetylcholine (ACh) and nitric oxide (NO) in the spinal cord is considered to play a role in the analgesic effects of intrathecally administered clonidine, an α_2-adrenergic receptor agonist (Detweiler *et al*., 1993; De Kock *et al*., 1997; Pan *et al*., 1998; 1999a; Xu *et al*., 2000), and of systemically administered morphine through the descending noradrenergic system (Xu *et al*., 1997; Song *et al*., 1998; Chen & Pan, 2001). In the current study presented here, we therefore sought to evaluate the involvement of the spinal ACh system, in particular focusing on muscarinic receptors and sequential NO release, in the supraspinally mediated analgesic actions of gabapentin by using a mouse chronic pain model. Our study demonstrates that the supraspinal action of gabapentin employs the descending noradrenergic system coupled with α_2-adrenergic receptors, muscarinic receptors (most likely M_1) and NO release, independently of activation of opioid receptors.

Methods

All of the experimental protocols used here were approved by the Animal Care and Use Committee of Nagoya City University, and were carried out according to the guidelines of the National Institutes of Health and the Japanese Pharmacological Society.

Preparation of the animal model

The surgical procedure was based on that described by Seltzer *et al*. (1990). In brief, 4- or 5-week-old, male ddY mice were anesthetized by intraperitoneal (i.p.) administration of pentobarbital sodium (60 mg kg^{-1}). One-third to one-half of the dorsal aspect of the right sciatic nerve was ligated just distal to its branch to the posterior biceps and semitendinosus muscles. Thermal and mechanical hypersensitivity was assessed 7 days after ligation.

Assessment of thermal hypersensitivity

Thermal hypersensitivity was assessed by the plantar test (Ugo Basile, Comerio, Italy) following a modification of the method of Hargreaves *et al*. (1988). Mice ($n = 5$–6/group) were placed in a clear plastic chamber with a glass floor and allowed to acclimate to their environment before testing. During this time, the mice initially demonstrated exploratory behavior, but subsequently stopped exploring and stood quietly with occasional bouts of grooming. A mobile radiant heat source, which was located under the glass floor, was focused onto the plantar surface of the right hindpaw, and paw withdrawal latencies (PWLs) were recorded. The intensity of radiant heat was adjusted to give a 7–8 s withdrawal latency in naive mice. A cutoff latency of 15 s was imposed to avoid tissue damage. PWLs were measured in duplicate for the right hindpaw of each animal, and the mean of the two values was used for analysis.

Assessment of mechanical hypersensitivity

Mice ($n = 5$–6/group) were placed in individual transparent Perspex cubicles with a wire mesh bottom, and a series of calibrated von Frey filaments (Semmes-Weinstein monofilaments; Stoelting, Wood Dale, IL, U.S.A.) was used to determine the 50% likelihood of a paw withdrawal response (50% threshold) by the up-down method of Dixon (1980). Eight von Frey filaments, with approximately equal logarithmic incremental bending forces, were chosen (von Frey number: 2.36, 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, and 4.17; equivalent to 0.02, 0.03, 0.07, 0.17, 0.41, 0.69, 1.20, and 1.48 g force, respectively). Testing was initiated with the 0.17 g hair, and each hair was applied perpendicularly to the plantar surface of the right hindpaw, with sufficient force to bend the filament, for 3–4 s. Lifting of the paw indicated a positive response and prompted the use of the next weaker (i.e. lighter) filament. Absence of a paw withdrawal response prompted the use of the next stronger (i.e. heavier) filament. This paradigm was continued until four measurements had been obtained after an initial change in behavior, or until four consecutive negative scores (score of 0.01 g) or five positive scores (score of 1.5 g) had been obtained. The resulting scores were used to calculate the 50% threshold (Chaplan *et al*., 1994).

In the study presented here, mice that exhibited a PWL of less than 5 s in the plantar test and a 50% threshold of 0.1 g in the von Frey test 7 days after partial ligation of the sciatic nerve were considered to be developing thermal and mechanical hypersensitivity.

Effects on acute nociception

The degree of antinociception was determined by the plantar test (see above) and the paw pressure test in normal (nonligated) mice ($n = 5$–6/group).

In the plantar test, a higher intensity of radiant heat than that used in ligated animals was applied. Otherwise, at the weaker intensity used in ligated animals (including before ligation), mice occasionally exhibited PWL values above 10 s, which is close to the cutoff latency of 15 s and prevented us from making a proper evaluation of analgesic effects on acute nociception.

Following the plantar test, mice were subjected to the paw pressure test (Pressure Analgesy-Meter; Muromachi Kikai, Tokyo, Japan) to assess their threshold for acute mechanical nociception. In brief, while the experimenter gently held the body of each mouse, the right hindpaw was exposed to increasing mechanical pressure. The pressure level was increased at a rate of 10 mmHg s^{-1} and the pressure (mmHg) required to elicit a response was determined for each mouse, that pressure being defined as the nociceptive threshold. Paw pressure measurements were made in duplicate, and the mean

of the two values was used for calculations. The cutoff pressure was 200 mmHg.

Drugs

In most of the experiments, experimenters were not blind to the drugs injected. Gabapentin, clonidine hydrochloride (HCl), idazoxan HCl, methoctramine tetrahydrochloride, 4-(*N*-[3-chlorophenyl]-carbamoyloxy)-2-butynyl-trimethylammonium chloride (McN-A-343), neostigmine bromide, L-N^G-nitro arginine methyl ester (L-NAME), L-N^G-nitro arginine methyl acetate (L-NMMA), and naloxone HCl were purchased from Sigma Chemical (St Louis, MO, U.S.A.). Yohimbine HCl and pirenzepine dihydrochloride were purchased from Research Biochemicals International (Natrick, MA, U.S.A.), atropine sulfate monohydrate from Wako Chemical (Tokyo, Japan), and morphine HCl from Shionogi (Osaka, Japan). L-NMMA was dissolved in distilled water. The other drugs were dissolved in 0.9% w v^{-1} physiological saline. When given i.p., the drugs were administered in a volume of 0.1 ml (10 g body weight)$^{-1}$. For i.t. injection, the drugs were administered in a volume of 5 μl *via* a disposable 27-gauge needle, which was inserted into the subarachnoid space through the intervertebral foramen between L5 and L6 according to the method described by Hylden & Wilcox (1980). For intracerebroventricular (i.c.v.) injection, the drugs were also administered in a volume of 5 μl *via* a disposable 27-gauge needle, which was inserted into the lateral ventricle (Haley & McCormick, 1957).

Statistical analysis

All data are expressed as means ± s.e.m. The effects of drugs (gabapentin, clonidine, McN-A-343 and morphine) on the nociceptive threshold in both the plantar and von Frey tests were evaluated with respect to time; the time of administration of drugs was defined as time zero. Unless otherwise mentioned, the antagonists (for α_2-adrenergic, muscarinic, or opioid receptors) or inhibitors (for cholinesterase or NO synthase) were administered 15 min before gabapentin or receptor agonists. Two-tailed non-parametric multiple comparisons with Bonferroni correction following the Kruskal–Wallis test (Glantz, 1992) were used for comparisons between the control and treated groups. The Mann–Whitney *U*-test was used for comparisons between two groups. Differences with $P<0.05$ (two tailed) were considered significant.

Results

Supraspinally administered gabapentin results in activation of spinal α_2-adrenergic receptors

In our previous study with Seltzer model mice, either systemic or intrathecal (i.t.) blockade of α_2-adrenergic receptors, or depletion of central NA content, strongly reduced the analgesic effects of systemically administered gabapentin (Tanabe *et al*., 2005a). The current study began by confirming

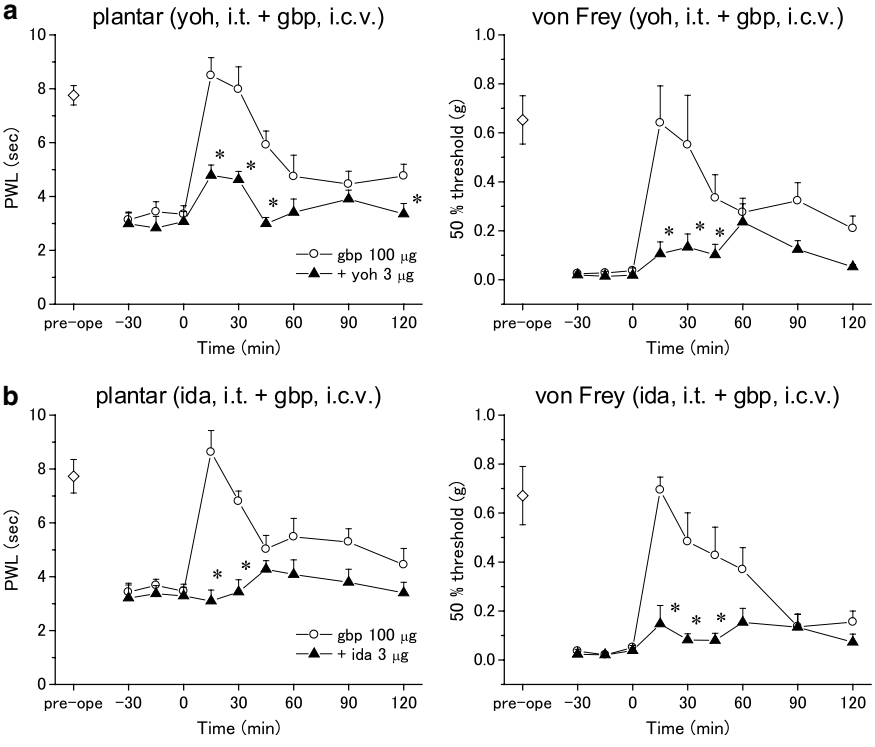

Figure 1 The α_2-adrenergic receptor antagonists yohimbine and idazoxan abolish the analgesic effects of i.c.v.-administered gabapentin on thermal and mechanical hypersensitivity. Thermal and mechanical hypersensitivity was assessed by the plantar and von Frey tests, respectively. Either yohimbine HCl (yoh, 3 μg, i.t. in (a)) or idazoxan HCl (ida, 3 μg, i.t. in (b)) was administered 15 min before the administration of gabapentin (gbp, 100 μg, i.c.v., administered at time zero). Each point represents the mean ± s.e.m. of six separate mice. Ordinates: mean PWLs (plantar test; left) and 50% thresholds (von Frey test; right). Abscissae: 7 days before (pre-ope) and time in minutes after gabapentin administration. The open diamond in each graph shows the mean of pooled PWLs (left in (a) and (b)) or 50% thresholds (right in (a) and (b)) obtained before ligation in the two groups of mice. The asterisks indicate data points for which a significant difference between the gabapentin-only (open circles) and yohimbine or idazoxan-treated (closed triangles) groups was observed, as determined by the Mann–Whitney *U*-test (two tailed, $^*P<0.05$).

whether supraspinally injected gabapentin really leads to activation of spinal α_2-adrenergic receptors. As Figure 1 shows, after i.t. injection of either of the α_2-adrenergic receptor antagonists yohimbine HCl (3 μg) or idazoxan HCl (3 μg), i.c.v. gabapentin (100 μg) did not reduce thermal and mechanical hypersensitivity. Unless otherwise mentioned in the following study, gabapentin (100 μg) was always injected i.c.v. to activate sequentially the descending noradrenergic pathway and spinal α_2-adrenergic receptors, and the spinal downstream mechanisms were further explored.

Spinal muscarinic receptors mediate the supraspinal effect of gabapentin on mechanical hypersensitivity

Recent studies have shown that the cholinergic neurons in the spinal cord work as an important component coupled with the descending noradrenergic pain inhibitory system (Zhuo & Gebhart, 1990; Eisenach, 1999; Pan *et al*., 1999a; Chen & Pan, 2001). We therefore addressed whether the spinal cholinergic system contributes to the supraspinally mediated analgesic effects of gabapentin. Systemic administration of the muscarinic receptor antagonist atropine sulfate (0.1 and 0.3 mg kg^{-1}, i.p.), which alone did not cause changes in the nociceptive thresholds for thermal and mechanical stimuli, selectively reduced the analgesic effect of subsequently i.c.v.-injected gabapentin on mechanical hypersensitivity, whereas the effectiveness of i.c.v. gabapentin at decreasing thermal hypersensitivity was not impaired (Figure 2a). Moreover, after i.t. atropine sulfate (0.1 μg), gabapentin elicited no elevation of the 50% threshold in the von Frey test and only relieved thermal hypersensitivity (Figure 2b).

This observation was further supported by an experiment in which a lower dose of gabapentin (30 μg, i.c.v.) was injected under slight blockade of cholinesterase in the spinal cord. As Figure 3 shows, the reversible cholinesterase inhibitor neostigmine bromide (0.3 ng, i.t.), which alone at this dose did not alter the withdrawal threshold for a thermal or mechanical stimulus, selectively potentiated the analgesic effect of subsequently injected gabapentin on mechanical hypersensitivity. Again, gabapentin, acting on the supraspinal structures, elicits ACh release in the spinal cord which mediates an attenuation of mechanical hypersensitivity.

Consistent with other studies (Paqueron *et al*., 2001; 2003; Koga *et al*., 2004), activation of α_2-adrenergic receptors preceded activation of muscarinic receptors (Figure 4). I.t. injection of the α_2-adrenergic receptor agonist clonidine HCl (0.1 and 0.3 μg) relieved thermal and mechanical hypersensitivity, the latter of which was reduced by atropine sulfate (0.1 μg, i.t.).

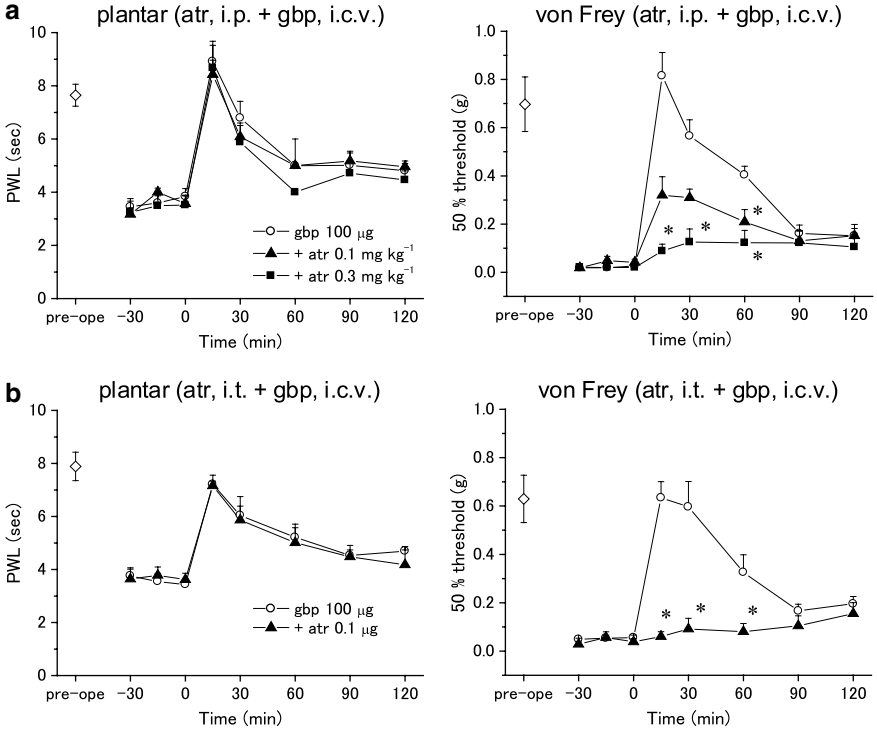

Figure 2 The muscarinic receptor antagonist atropine reduces the analgesic effect i.c.v.-administered gabapentin on mechanical hypersensitivity whereas its effect on thermal hypersensitivity remains unaffected. Atropine sulfate (atr) was administered either i.p. ((a); 0.1 and 0.3 mg kg^{-1}) or i.t. ((b); 0.1 μg) 15 min before the administration of gabapentin (gbp, 100 μg, i.c.v., administered at time zero). Each point represents the mean $\pm$ s.e.m. of six separate mice. Ordinates: mean PWLs (plantar test; left) and 50% thresholds (von Frey test; right). Abscissae: 7 days before (pre-ope) and time in minutes after gabapentin application. The open diamond in each graph shows the mean of pooled PWLs (left in (a) and (b)) or 50% thresholds (right in (a) and (b)) obtained before ligation in the three (a) and two (b) groups of mice. The asterisks indicate data points for which a significant difference between the gabapentin-only (open circles) and atropine-treated (closed triangles and squares) groups was observed, as determined by (a) two-tailed nonparametric Bonferroni-type multiple comparisons following the Kruskal–Wallis test (two comparisons in three groups, $^*P<0.05$) or (b) the Mann–Whitney U-test (two tailed, $^*P<0.05$).

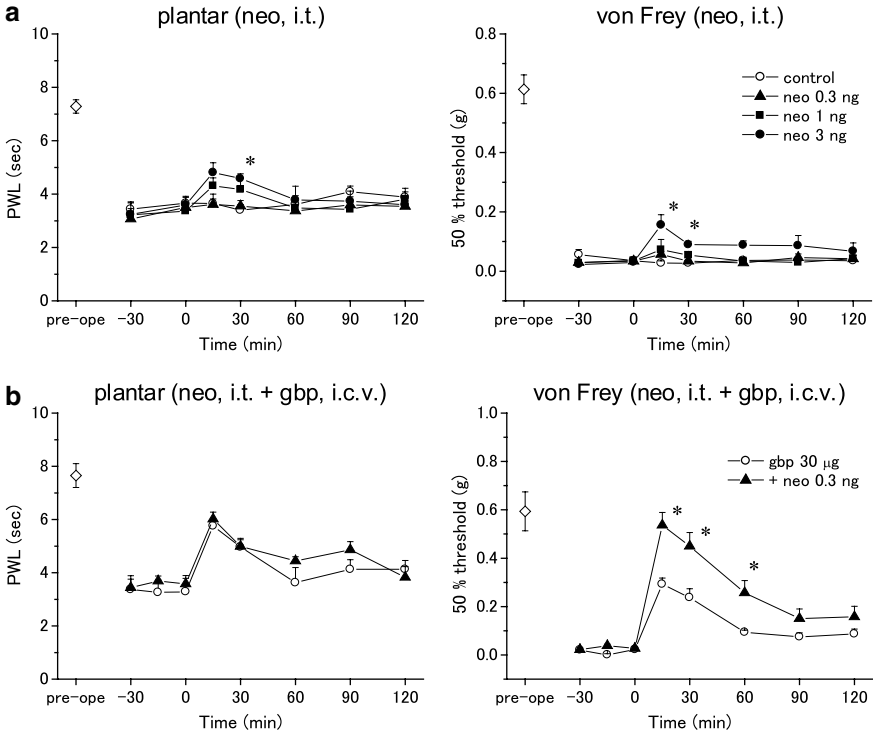

Figure 3 The cholinesterase inhibitor neostigmine at a dose with no behavioral effects preferentially potentiates the analgesic effect of i.c.v. administered gabapentin on mechanical hypersensitivity. In (a), a dose-dependent analgesic effect of neostigmine alone (neo, 0.3, 1 and 3 ng, i.t., injected at time zero) was established. In (b), a low dose of neostigmine (neo, 0.3 ng, i.t.) was injected 15 min before the administration of gabapentin (gbp, 30 μg, i.c.v., administered at time zero). Each point represents the mean $\pm$ s.e.m. of five or six separate mice. Ordinates: mean PWLs (plantar test; left) and 50% thresholds (von Frey test; right). Abscissae: 7 days before (pre-ope) and time in minutes after neostigmine (a) and gabapentin (b) application. The open diamond in each graph shows the mean of pooled PWLs (left in (a) and (b)) or 50% thresholds (right in (a) and (b)) obtained before ligation in the four (a) and two (b) groups of mice. The asterisks indicate data points for which a significant difference between (a) the control (open circles) and neostigmine-treated (closed triangles, squares and circles) groups or (b) the gabapentin-only (open circles) and neostigmine-treated (closed triangles) groups was observed, as determined by (a) two-tailed nonparametric Bonferroni-type multiple comparisons following the Kruskal–Wallis test (three comparisons in four groups, $*P<0.05$) or (b) the Mann–Whitney U-test (two tailed, $*P<0.05$).

Spinal M_1- but not M_2-muscarinic receptors appear to mediate the analgesic effect of i.c.v.-injected gabapentin on mechanical hypersensitivity

We next assessed which muscarinic receptor subtypes are involved in the supraspinally mediated analgesic effect of gabapentin on mechanical hypersensitivity by using the subtype-specific antagonists pirenzepine (M_1) and methoctramine (M_2). I.t. injection of the selective M_1-muscarinic receptor antagonist pirenzepine HCl (0.1 μg) significantly reduced the effectiveness of i.c.v. gabapentin at reducing mechanical hypersensitivity, whereas its analgesic effect against a thermal stimulus was unaltered (Figure 5a). By contrast, i.t. application of the selective M_2-muscarinic receptor antagonist methoctramine tetrahydrochloride, at the equivalent dose (0.1 μg, i.t.), did not affect the supraspinal actions of gabapentin against both thermal and mechanical stimuli (Figure 5b). At 1 μg, however, methoctramine tetrahydrochloride significantly suppressed the analgesic effect against a mechanical stimulus; this is presumably attributable to its affinity for M_1-muscarinic receptors (Dörje *et al*., 1991).

Consistently, i.t. injection of McN-A-343, a specific agonist of M_1-muscarinic receptors, produced a relatively selective relief of mechanical hypersensitivity. As shown in Figure 6a, McN-A-343 (3 and 10 μg) slightly but significantly elevated the PWL in the plantar test ($P<0.05$ at 10 μg) and markedly elevated the 50% threshold in the von Frey test ($P<0.05$ at 3 and 10 μg). By contrast, McN-A-343 (10 μg, i.t.) did not exhibit any antinociceptive effects against acute thermal and mechanical nociception as assessed by the plantar and paw pressure tests, respectively, in nonligated mice (Figure 6b).

Spinal NO release mediates the analgesic effect of i.c.v.-injected gabapentin on mechanical hypersensitivity

Finally, we assessed whether spinal NO synthesis is involved in the supraspinally mediated analgesic effect of gabapentin on mechanical hypersensitivity. The analgesia elicited by activation of spinal muscarinic receptors has been demonstrated to involve enhanced NO production (Zhuo *et al*., 1993; Iwamoto & Marion, 1994; Chen *et al*., 2001). As Figure 7 shows, a low dose of L-NMMA (10 μg, i.t.), an irreversible nonselective NO synthase inhibitor with no affinity to muscarinic receptors (Buxton *et al*., 1993; Golkar *et al*., 2000), suppressed the analgesic effect of i.c.v. gabapentin on mechanical but not thermal hypersensitivity. At 30 and 100 μg, i.t. injected L-NMMA alone caused a significant elevation of withdrawal thresholds in the plantar and von Frey tests. Similar effects were obtained with L-NAME, a nonselective NO synthase inhibitor (data not shown). L-NAME alone (10 μg, i.t.)

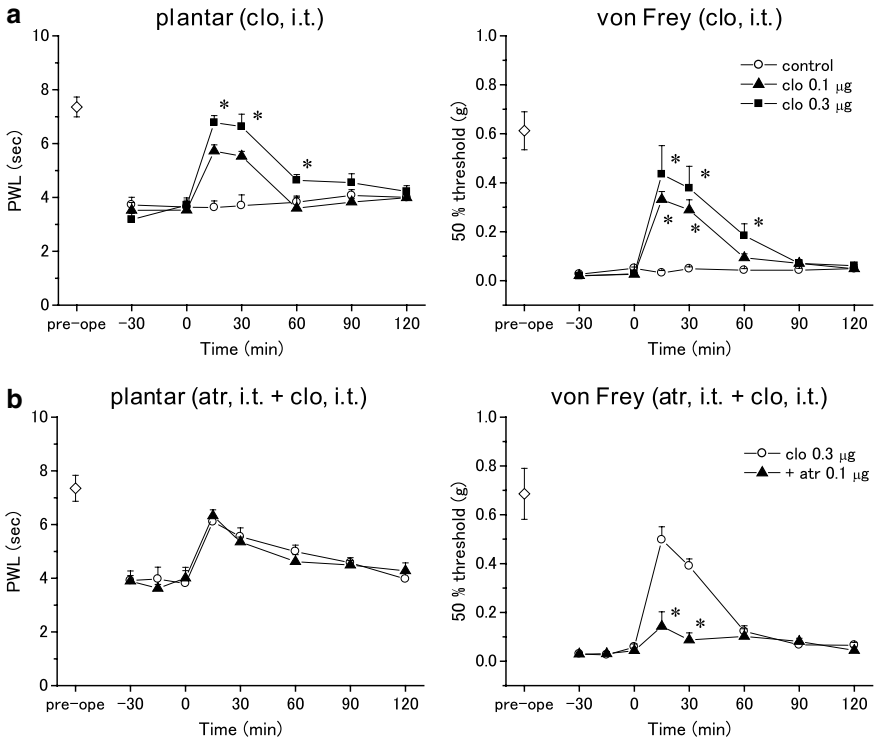

Figure 4 Activation of spinal α_2-adrenergic receptors precedes activation of muscarinic receptors. Clonidine HCl (clo, 0.1 and 0.3 μg, i.t. administered at time zero) reduced thermal and mechanical hypersensitivity (a). Atropine sulfate (atr, 0.1 μg, i.t.), administered 15 min before the administration of clonidine HCl (0.3 μg, i.t.) preferentially reduced the analgesic effect of clonidine on mechanical hypersensitivity (b). Each point represents the mean $\pm$ s.e.m. of five or six separate mice. Ordinates: mean PWLs (plantar test; left) and 50% thresholds (von Frey test; right). Abscissae: 7 days before (pre-ope) and time in minutes after clonidine application. The open diamond in each graph shows the mean of pooled PWLs (left in (a) and (b)) or 50% thresholds (right in (a) and (b)) obtained before ligation in the three (a) and two (b) groups of mice. The asterisks indicate data points for which a significant difference between (a) the control (open circles) and clonidine-treated (closed triangles and squares) groups or (b) the clonidine-only (open circles) and atropine-treated (closed triangles) groups was observed, as determined by (a) two-tailed nonparametric Bonferroni-type multiple comparisons following the Kruskal–Wallis test (three comparisons in four groups, $^*P<0.05$) or (b) the Mann–Whitney U-test (two tailed, $^*P<0.05$).

exhibited analgesic effects in the plantar and von Frey tests, whereas at a lower dose (3 μg) it selectively reduced the analgesic effect of i.c.v. gabapentin on mechanical hypersensitivity. However, the muscarinic receptor-blocking effects of L-NAME (Buxton *et al*., 1993) appear to be taken into consideration.

Gabapentin acts on targets other than opioid receptors in supraspinal structures

As we have demonstrated, the supraspinal action of gabapentin employs the descending noradrenergic pain inhibitory system coupled with spinal α_2-adrenergic receptors, muscarinic receptors, and NO release, an analgesic mechanism that may be shared by the supraspinal action of opioids (Bodnar *et al*., 1990; Zhuo & Gebhart, 1991; Xu *et al*., 1997; Song *et al*., 1998; Chen & Pan, 2001). Therefore, we assessed the possible involvement of opioid receptors in the supraspinal action of gabapentin. However, the opioid receptor antagonist naloxone HCl (10 mg kg^{-1}, i.p.) did not alter the supraspinal analgesic effects of gabapentin (Figure 8a). By contrast, this dose of naloxone HCl completely abolished the analgesic effects of morphine HCl (10 mg kg^{-1}, i.p.) on thermal and mechanical hypersensitivity (Figure 8b). Together, these observations indicate that the supraspinally mediated analgesic actions of gabapentin are independent of activation of opioid receptors.

Discussion

We previously used a mouse neuropathic pain model (the Seltzer model) to demonstrate that gabapentin supraspinally activates the descending noradrenergic system coupled with spinal α_2-adrenergic receptors to generate its analgesic effects on thermal and mechanical hypersensitivity (Tanabe *et al*., 2005a). Herein, we further evaluated the pain inhibitory mechanisms involved in the supraspinal action of gabapentin. The current results indicate that spinal muscarinic receptors (most likely M_1) and the NO system are recruited after activation of α_2-adrenergic receptors to reduce mechanical hypersensitivity. By contrast, the supraspinally mediated relief of thermal hypersensitivity after gabapentin administration is independent of the spinal cholinergic system.

With Seltzer model mice, we have previously demonstrated that either systemic or i.t. blockade of α_2-adrenergic receptors, or depletion of central NA content, strongly reduce the analgesic effects of systemically administered gabapentin (Tanabe *et al*., 2005a). In the present study, we have further established the relation between the supraspinal action of gabapentin and the resultant activation of spinal α_2-adrenergic receptors in reducing thermal and mechanical hypersensitivity. This was attained by i.t. injection of α_2-adrenergic receptor antagonists followed by i.c.v. gabapentin, an experiment that had not been performed in our previous study (Figure 1).

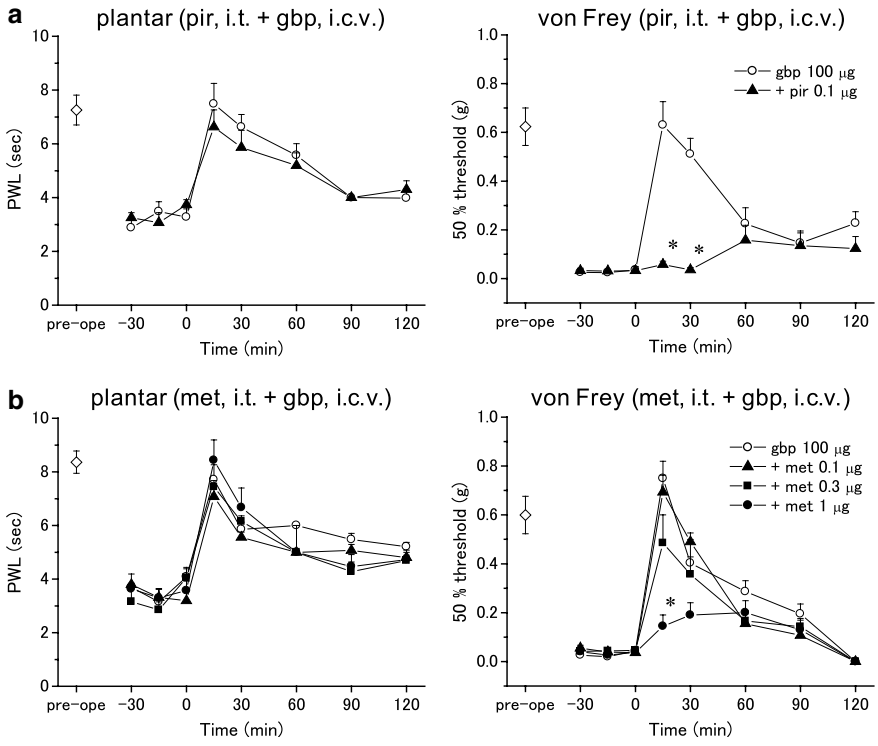

Figure 5 The M_1-muscarinic receptor antagonist pirenzepine, but not the M_2-muscarinic receptor antagonist methoctramine, abolishes the analgesic effect of gabapentin on mechanical hypersensitivity whereas its effect on thermal hypersensitivity remains unaltered. Either pirenzepine HCl (pir, 0.1 μg, i.t. in (a)) or methoctramine tetrahydrochloride (met, 0.1, 0.3, and 1 μg, i.t. in (b)) was administered 15 min before the administration of gabapentin (gbp, 100 μg, i.c.v., administered at time zero). Each point represents the mean $\pm$ s.e.m. of six separate mice. Ordinates: mean PWLs (plantar test; left) and 50% thresholds (von Frey test; right). Abscissae: 7 days before (pre-ope) and time in minutes after gabapentin application. The open diamond in each graph shows the mean of pooled PWLs (left in (a) and (b)) or 50% thresholds (right in (a) and (b)) obtained before ligation in the two (a) and four (b) groups of mice. The asterisks indicate data points for which a significant difference between (a) the gabapentin-only (open circles) and pirenzepine-treated (closed triangles) groups or (b) the gabapentin-only (open circles) and methoctramine-treated (closed triangles, squares and circles) groups was observed, as determined by (a) the Mann–Whitney *U*-test (two tailed, $^*P<0.05$) or (b) two-tailed nonparametric Bonferroni-type multiple comparisons following the Kruskal–Wallis test (three comparisons in four groups, $^*P<0.05$).

The clear demonstration that blockade of spinal α_2-adrenergic receptors by i.t. injection of either yohimbine or idazoxan abolished the analgesic effects of i.c.v. gabapentin further supports our previous findings (Tanabe *et al*., 2005a).

The observation that i.t. injection of muscarinic receptor antagonists reduced the supraspinally mediated analgesic effect of gabapentin on mechanical hypersensitivity (Figures 2 and 5) indicates that i.c.v. injection of gabapentin results in ACh release and subsequent activation of muscarinic receptors in the spinal cord. The specific relation between the attenuation of mechanical hypersensitivity by i.c.v. gabapentin and the spinal cholinergic system was demonstrated further by the important finding that the cholinesterase inhibitor neostigmine, at a small dose eliciting no behavioral effects, preferentially potentiated the analgesic effect of a lower dose of i.c.v. gabapentin (30 μg) on mechanical hypersensitivity (Figure 3). The modality-specific effects of manipulating the spinal ACh system on the analgesic action of i.c.v. gabapentin indicate that ACh release takes place after activation of α_2-adrenergic receptors. This is also supported by the observations that blockade of spinal α_2-adrenergic receptors abolished the analgesic effects of i.c.v. gabapentin on both thermal and mechanical hypersensitivity (Figure 1) and that blockade of spinal muscarinic receptors with atropine selectively abolished the analgesic effect of i.t.-injected clonidine, an α_2-adrenergic receptor agonist, on mechanical hypersensitivity (Figure 4). Consistently, i.t. injection of clonidine causes analgesic effects and increases the ACh concentration in lumbar cerebrospinal fluid, and clonidine-induced analgesia is suppressed by i.t. treatment with atropine in normal and/or neuropathic pain animals (Detweiler *et al*., 1993; Pan *et al*., 1999a; Koga *et al*., 2004). By contrast, i.t. pretreatment with yohimbine does not influence the antinociceptive effects produced by i.t. injection of the muscarinic receptor agonist carbachol (Zhuo & Gebhart, 1991).

The behavioral consequence of ACh release in the spinal cord after i.c.v. injection of gabapentin is a selective relief of mechanical hypersensitivity. This agrees well with studies by others demonstrating a role of spinal ACh in the generation of the analgesic effect against mechanical stimuli. Paqueron *et al*. (2001; 2003) have demonstrated that either destruction of spinal cholinergic neurons or blockade of spinal muscarinic receptors suppresses the analgesic effect of i.t. clonidine against mechanical but not thermal stimulus in rats with spinal nerve ligation. It is likely that spinal ACh released after activation of α_2-adrenergic receptors decreases mechanical hypersensitivity without any effect on a reduced withdrawal threshold to thermal stimuli. We should keep in mind that spinal ACh release is also regulated by the descending serotonergic system, and activation of spinal serotonin 5-HT_2

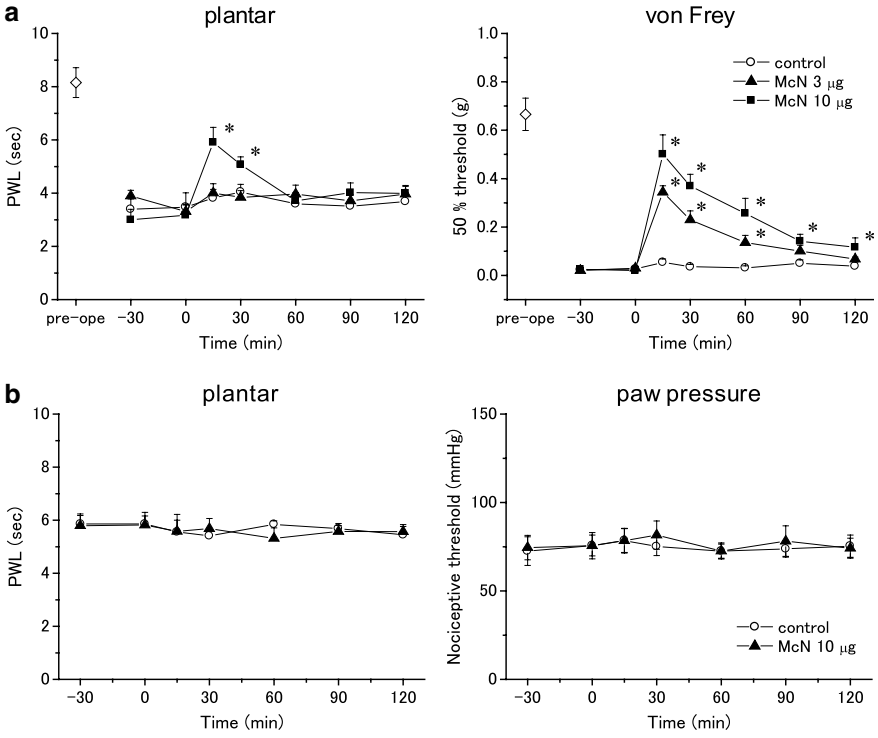

Figure 6 The M_1-muscarinic receptor agonist McN-A-343 relieves thermal and mechanical hypersensitivity. In (a), a dose-dependent analgesic effect of McN-A-343 (McN, 3 and 10 μg, i.t.) assessed in ligated mice was established. By contrast, McN-A-343 (10 μg, i.t.) did not produce antinociceptive effects against acute thermal and mechanical nociception, assessed by the plantar and paw pressure tests, respectively, in nonligated mice (b). Note that a higher intensity of radiant heat than that used in ligated animals was applied in the study on acute thermal nociception. McN-A-343 was administered at time zero. Each point represents the mean $\pm$ s.e.m. of five or six separate mice. Ordinates: mean PWLs (plantar test; left) and 50% thresholds (von Frey test; right in (a)) or nociceptive threshold (paw pressure; right in (b)). Abscissae: 7 days before (pre-ope) and time in minutes after McN-A-343 injection. The open diamond in each graph shows the mean of pooled PWLs (left in (a)) or 50% thresholds (right in (a)) obtained before ligation in the three groups of mice. The asterisks indicate data points for which a significant difference between the control (open circles) and McN-A-343-treated (closed triangles and squares) groups was observed, as determined by two-tailed nonparametric Bonferroni-type multiple comparisons following the Kruskal–Wallis test (two comparisons in three groups, $^*P<0.05$).

receptors relieves thermal and mechanical hypersensitivity by a mechanism involving ACh release and muscarinic receptors (Obata *et al*., 2002; Sasaki *et al*., 2003). In our preliminary experiments, the analgesic effects of i.c.v. gabapentin were not affected by the 5-HT_2 receptor antagonist ketanserin (data not shown), suggesting that i.c.v. gabapentin is not likely to activate the descending serotonergic system.

The present results with muscarinic receptor antagonists suggest that M_1-muscarinic receptors in the spinal cord most likely mediate the analgesic effect of i.c.v.-injected gabapentin on mechanical hypersensitivity. This is in line with the study by Koga *et al*. (2004) demonstrating that spinal M_1-muscarinic receptors participate in the antiallodynic effect of i.t.-injected clonidine in mice with streptozotocin-induced diabetes. However, activation of spinal M_1-muscarinic receptors with the specific agonist McN-A-343 relieved thermal as well as mechanical hypersensitivity (Figure 6). This may imply that only a subpopulation of spinal M_1-muscarinic receptors is activated after i.c.v. injection of gabapentin and that other M_1-muscarinic receptors mediate the ameliorating effect on thermal hypersensitivity or the analgesic effect against both thermal and mechanical stimuli. Accordingly, it is plausible that the analgesic effect produced by higher doses of neostigmine alone (Figure 3) is attributable to the action of ACh on M_1-muscarinic receptors (Hwang *et al*., 1999; but see Chen & Pan, 2003).

A role of spinally released NO has been suggested in analgesia involving a cascade of NA and ACh (Zhuo *et al*., 1993; Xu *et al*., 1997; Pan *et al*., 1998; Song *et al*., 1998; Chen *et al*., 2001; Chen & Pan, 2001). However, most previous studies have suggested a pronociceptive role for spinal NO in the development and/or maintenance of chronic pain after nerve injury or inflammation (Inoue *et al*., 1998; Mabuchi *et al*., 2003; Tao *et al*., 2003; 2004; Tegeder *et al*., 2004). Consistently, in our recent study with Seltzer model mice, blockade of NO synthesis with L-NAME relieved thermal and mechanical hypersensitivity (Tanabe *et al*., 2005b). It is likely that NO has a dual effect on nociception; a small increase in NO reduces nociception and a large increase in NO results in hyperalgesia (Sousa & Prado, 2001; Tegeder *et al*., 2002). In the present study, we injected small doses of L-NMMA or L-NAME, and they at the higher dose indeed reduced thermal and mechanical hypersensitivity. Of importance is the finding that L-NMMA or L-NAME at a smaller dose that alone elicited no behavioral effects selectively reduced the analgesic effect of i.c.v. gabapentin on mechanical hypersensitivity (Figure 7). This suggests that NO is spinally released in a cascade involving i.c.v. gabapentin and

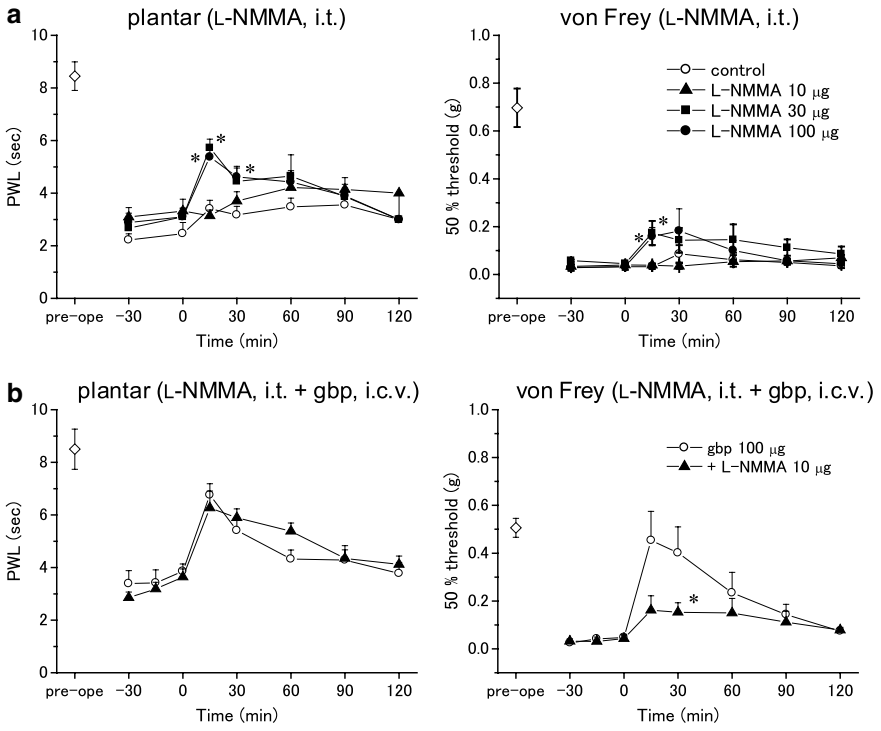

Figure 7 The NO synthase inhibitor L-NMMA suppresses the analgesic effect of i.c.v.-administered gabapentin on mechanical but not thermal hypersensitivity. In (a), a dose-dependent analgesic effect of L-NMMA (10, 30, and 100 μg, i.t., injected at time zero) was established. In (b), a low dose of L-NMMA (10 μg, i.t.) was injected 15 min before the administration of gabapentin (gbp, 100 μg, i.c.v., administered at time zero). Each point represents the mean $\pm$ s.e.m. of five or six separate mice. Ordinates: mean PWLs (plantar test; left) and 50% thresholds (von Frey test; right). Abscissae: 7 days before (pre-ope) and time in minutes after L-NMMA (a) and gabapentin (b) application. The open diamond in each graph shows the mean of pooled PWLs (left in (a) and (b)) or 50% thresholds (right in (a) and (b)) obtained before ligation in the four (a) and two (b) groups of mice. The asterisks indicate data points for which a significant difference between (a) the control (open circles) and L-NMMA-treated (closed triangles, squares and circles) groups or (b) the gabapentin-only (open circles) and L-NMMA-treated (closed triangles) groups was observed, as determined by (a) two-tailed nonparametric Bonferroni-type multiple comparisons following the Kruskal–Wallis test (three comparisons in four groups, $^*P<0.05$) or (b) the Mann–Whitney U-test (two-tailed, $^*P<0.05$).

spinal α_2-adrenergic and muscarinic (most likely M_1) receptors, supporting the observations that spinal NO mediates the antiallodynic effect of i.t. clonidine (Pan *et al*., 1998) and neostigmine (Chen *et al*., 2001) in rat models of neuropathic pain. More importantly, our results imply that a small additional release of NO from a specific group of spinal intrinsic neurons could be effective in reducing mechanical hypersensitivity even in chronic pain states in which NO synthesis is one of the key factors in maintaining this pathologic condition.

The analgesic signal cascade initiated by the supraspinal action of gabapentin that we have demonstrated here could be shared by opioids. It is well established that morphine stimulates the descending noradrenergic pain inhibitory system that is coupled with spinal α_2-adrenergic receptors (Sawynok & Reid, 1987; Wigdor & Wilcox, 1987; Tseng & Tang, 1989; Bodnar *et al*., 1990). Activation of this descending noradrenergic system by morphine leads to increases in the concentrations of NA, ACh, and NO in the spinal dorsal horn (Xu *et al*., 1997). Hence, the final set of experiments reported here was intended to assess whether opioid receptors mediate the analgesic effect of i.c.v. gabapentin. The analgesic effects of i.c.v. gabapentin on thermal and mechanical hypersensitivity were unaltered by the opioid receptor antagonist naloxone (Figure 8), indicating that the supraspinal action of gabapentin is independent of the opioidergic system. Our results support other studies in which the systemic effect of gabapentin was assessed (Field *et al*., 1997; Dixit & Bhargava, 2002). This is important in the management of clinical pain, since independence from the opioidergic system results in minimum side effects, including tolerance and/or withdrawal symptoms, and is favorable for the treatment of chronic opioid-insensitive pain states.

Interestingly, McN-A-343 did not affect acute thermal and mechanical nociception in nonligated mice at a dose that produced an analgesic effect in ligated animals (Figure 6). Similarly, clonidine has been shown to produce more potent analgesic effects in a model of chronic pain (Paqueron *et al*., 2003). Such increases in potency of clonidine and McN-A-343 may be ascribed to alterations in the function and/or expression of both α_2-adrenergic and M_1-muscarinic receptors after nerve injury, and more importantly may explain why gabapentin generates an analgesic effect only in chronic pain states, as demonstrated by our previous study and by others (Field *et al*., 1997; Laughlin *et al*., 2002; Suzuki *et al*., 2005; Tanabe *et al*., 2005a). Peripheral nerve injury has been demonstrated to cause modification of several channels, receptors, and signal pathways in the spinal dorsal horn, which appear to constitute molecular basis for central sensitization (Ji *et al*., 2003; Yang *et al*., 2004). This may contribute to changes in sensitivity of the cascade involved in the analgesic effects of i.c.v. gabapentin.

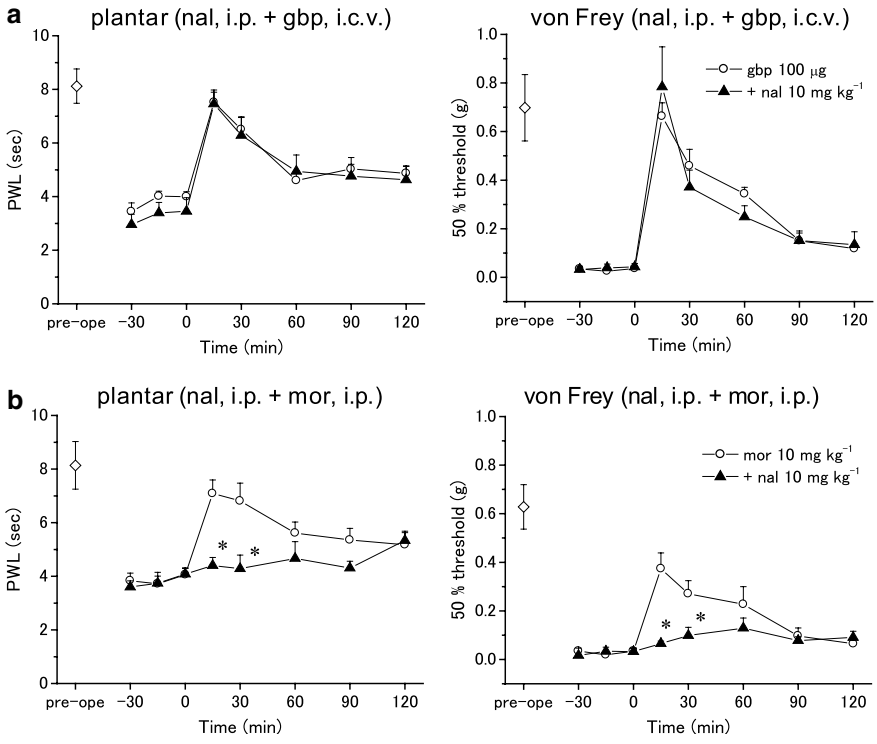

Figure 8 The opioid receptor antagonist naloxone does not affect the analgesic effects of i.c.v.-administered gabapentin on thermal and mechanical hypersensitivity. Naloxone HCl (nal, 10 mg kg^{-1}, i.p.) was administered 15 min before the administration of gabapentin (gbp, 100 μg, i.c.v., administered at time zero in (a)) and morphine HCl (mor, 10 mg kg^{-1}, i.p., administered at time zero in (b)). Naloxone was only effective on the analgesic effect of morphine, indicating that the supraspinal action of gabapentin is independent of the opioidergic system. Each point represents the mean $\pm$ s.e.m. of six separate mice (open circle: gabapentin only (a) and morphine only (b); closed triangle: naloxone treated). Ordinates: mean PWLs (plantar test; left) and 50% thresholds (von Frey test; right). Abscissae: 7 days before (pre-ope) and time in minutes after gabapentin (a) and morphine (b) application. The open diamond in each graph shows the mean of pooled PWLs (left in (a) and (b)) or 50% thresholds (right in (a) and (b)) obtained before ligation in the two groups of mice. The asterisks indicate data points for which a significant difference between the morphine-only (open circles) and naloxone-treated (closed triangles) groups was observed, as determined by the Mann–Whitney *U*-test (two-tailed, $^*P<0.05$).

Based on the present results, we conclude that the spinal muscarinic receptors (most likely M_1) and the NO cascade are recruited after activation of the descending noradrenergic system and spinal α_2-adrenergic receptors in the supraspinal action of gabapentin on mechanical hypersensitivity. By contrast, its ameliorating effect on thermal hypersensitivity is independent of the spinal cholinergic system. This modality-specific interaction obtained between supraspinal gabapentin and the spinal cholinergic system could carry important clinical implications for the treatment of pain states including tactile allodynia and more importantly spontaneous ongoing pain. Further studies combined with electrophysiological and morphological methods may clarify the precise mechanisms underlying the ameliorating effects of gabapentin acting on supraspinal structures.

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