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Nicotinic acetylcholine receptors expressed in the ventral posterolateral thalamic nucleus play an important role in antiallodynic effects

Running title:

NAChRs in the VPL mediate antiallodynic effects

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Summary

Background and purpose: Much interest is currently being focused on the antinociceptive effects mediated by nicotinic acetylcholine (nACh) receptors, including their location and mechanism of action. The purpose of this study is to elucidate these issues using 5-iodo-3-(2(S)-azetidinylmethoxy)pyridine (5IA), a nACh receptor agonist, and [^{125}I]5IA.

Experimental approach: We partially ligated the sciatic nerve of a SD rat to induce neuropathic pain (Seltzer's PSL model). We then examined the changes in central nACh receptor density in the model using [^{125}I]5IA autoradiography and the involvement of nACh receptors in antinociceptive effects in the region where changes occurred.

Key results: Autoradiographic studies showed that the accumulation of [^{125}I]5IA and the number of nACh receptors in the thalamus of PSL rats were increased about 2-fold compared to those in the sham-operated rats. No change was observed in other regions. This is the first report of the upregulation of thalamic nACh receptors under chronic painful conditions. Thus, we investigated the action site of 5IA in the thalamus. Rats receiving an intra-ventral posterolateral thalamic nucleus (VPL) administration of 5IA demonstrated a significant and dose-dependent antiallodynic effect. This effect was completely antagonized by coadministered mecamylamine. The blockade of nACh receptors in the VPL by mecamylamine caused a 70%

decrease in the antiallodynic effect of i.c.v. administered 5IA. Moreover, intra-VPL administered mecamylamine induced significant hyperalgesia.

Conclusions and implications: The findings in the present study suggest that the nACh receptors expressed in the VPL play an important role in the antiallodynic effects produced by exogenous and endogenous agonists.

Keywords:

Nicotinic acetylcholine receptor; Thalamus; Ventral posterolateral thalamic nucleus; 5IA; Antiallodynic effect; Neuropathic pain

Abbreviations:

nACh receptor: nicotinic acetylcholine receptor; VPL: ventral posterolateral thalamic nucleus; 5IA: 5-iodo-3-(2(S)-azetidinylmethoxy)pyridine; MPE: maximal possible effect; PPTg: pedunculopontine tegmental nucleus; NRM: nucleus raphe magnus

Introduction

Numerous studies have shown that nicotinic acetylcholine (nACh) receptor agonists possess analgesic effects (Decker *et al*., 2004; Jain, 2004). Because these effects are not antagonized by opioid antagonists and arise from the activation of nACh receptors (Bannon *et al*., 1998a), much interest has been focused on nACh receptors as a novel target of antinociceptive drugs that do not involve the opioid system. Although morphine is a very well known and effective analgesic, its usefulness for patients with neuropathic pain is limited (Bannon *et al*., 1998c; Sindrup and Jensen, 1999). In contrast, nACh receptor agonists are able to ameliorate neuropathic pain by affecting systems other than the opioids (Bannon *et al*., 1998b; Bannon *et al*., 1998c; Decker *et al*., 1998). Therefore, identification of the sites of action of nACh receptor agonists and the elucidation of their antiallodynic mechanisms are desirable.

One of the most studied nACh receptor-acting analgesics is ABT-594. It is a selective and potent agonist with high affinity for α4β2-nACh receptors (predominant in the brain), low affinity for α7-nACh receptors, and no affinity for α1β1γδ-nACh receptors (Donnelly-Roberts *et al*., 1998; Holladay *et al*., 1998). By virtue of this selectivity, ABT-594 has analgesic efficacy with an improved therapeutic window compared to non-selective agonists such as epibatidine (Bannon *et al*., 1998b).

Recently, 5-iodo-3-(2(S)-azetidinylmethoxy)pyridine (5-iodo-A-85380, 5IA),

an analogue of ABT-594, was synthesized (Saji *et al*., 2002). It is also an α4β2-nACh receptor-specific agonist (Ueda *et al*., 2008) with a relatively good safety profile (Ueda *et al*., 2004; Vaupel *et al*., 2003). In addition, radiolabeled 5IA, [$^{123/125}$I]5IA, was also developed (Saji *et al*., 2002) and was reported to be a promising ligand for imaging nACh receptors in rodents and in humans (Brasic *et al*., 2009; Mamede *et al*., 2004; Mamede *et al*., 2007; Oishi *et al*., 2007; Saji *et al*., 2002). Nonradioactive 5IA and [$^{123/125}$I]5IA can be considered the same compound with regard to their biodistribution or metabolism. Therefore, the pharmacokinetics, receptor occupancy, and binding potential (an index of nACh receptor density) of 5IA are able to be measured readily using [$^{123/125}$I]5IA. Studies using 5IA have the advantage that pharmacodynamic effects can be compared directly with the pharmacokinetic profile of the compound.

Previous studies have demonstrated the upregulation of spinal muscarinic acetylcholine receptors (Chen and Pan, 2003) and thalamic cannabinoid CB_1 receptors (Siegling *et al*., 2001) in neuropathic pain models. It has been reported that the upregulation of muscarinic and CB_1 receptors contributes to the increased analgesic efficacy of each agonist. Since nACh receptor agonists are also effective against neuropathic pain, upregulation of nACh receptors may also occur in the models. We hypothesized that brain regions where changes in the density or function of nACh receptors occurred would play an important role in antinociceptive effects,

provided that such changes were found under neuropathic conditions. In the present study we used a partial sciatic nerve ligation (PSL) model of neuropathic pain (Seltzer *et al*., 1990) and examined the changes in nACh receptors in the PSL rats using [^{125}I]5IA. Furthermore, based on the result, we investigated the involvement of the nACh receptors expressed in the region where changes occurred to the neuropathic pain.

Methods

Animals

Animal experiments were conducted in accordance with our institutional guidelines, and the experimental procedures were approved by the Kyoto University Animal Care Committee.

Male Sprague-Dawley rats weighing 200–250 g were purchased from Japan SLC Co., Ltd. (Hamamatsu, Japan). The rats were kept at a constant ambient temperature under a 12-hr light/dark cycle with free access to food and water.

Reagents and nomenclature

5IA and [^{125}I]5IA were synthesized according to a previously published method (Saji *et al*., 2002). Mecamylamine hydrochloride, a nACh receptor antagonist,

was purchased from Sigma-Aldrich (St. Louis, MO). All drugs were administered in physiological saline solution. All other chemicals used were of reagent grade.

The nomenclature for receptors follows the guide to receptors and channels (Alexander *et al*., 2008).

Surgical operation

PSL neuropathic pain was established according to a previously published method (Seltzer *et al*., 1990). Under sodium pentobarbital (50 $mg \cdot kg^{-1}$, i.p.) anesthesia, the right sciatic nerve was exposed just distal to the branch leading to the posterior biceps femoris/semitendinosus muscles. A 7-0 silk suture was inserted into the nerve and tightly ligated so that the dorsal 1/3 to 1/2 of the nerve was trapped in the ligature. In the sham-operated rats, the sciatic nerve was exposed, but was left intact.

After recovery from the surgery, the rats were implanted with stainless steel guide cannulae (o.d. 0.7 mm, i.d. 0.38 mm) under sodium pentobarbital (50 $mg \cdot kg^{-1}$, i.p.) anesthesia for i.c.v. or intra-ventral posterolateral thalamic nucleus (VPL) administration. The rats were placed into a stereotaxic apparatus (SR-5, Narishige Co., Ltd., Tokyo, Japan) and unilaterally implanted with a guide cannula above the lateral ventricle (0.8 mm posterior and 1.5 mm lateral to bregma, 2.0 mm below the outer surface of the skull) for i.c.v. administration or above the left VPL, which was

contralateral to the nerve ligation (2.4 mm posterior and 3.3 mm lateral to bregma, 2.0 mm below the outer surface of the skull) for intra-VPL administration. The stereotaxic coordinates were determined following an atlas (Paxinos and Watson, 2005). The guide cannulae were held firmly in place using dental acrylic cement. After surgery, the rats were individually returned to their cages and left to recover for 5 days or more until the experiments.

von Frey Filament Test

Just before and two weeks after the PSL, tactile sensitivity was measured by the up-down method using calibrated von Frey filaments ranging from 0.07–26 g (North Coast Medical, Morgan Hill, CA), in a previously described method with slight modifications (Marcil *et al*., 2006). Briefly, for testing, the rats were individually placed on an elevated wire mesh floor. After a habituation period of 15–30 min, the tactile stimulus was applied to the middle plantar of the each paw by placing the von Frey filament perpendicular to the surface of the paw. The filament was held in this position with enough force to cause slight bending. Each trial involved 10 applications of filaments every 1 to 2 s. The threshold was determined as the filament of the lowest stiffness at which the rat responded (quick paw flick) in one or more of the trial. The rats that showed a lower threshold postoperatively than preoperatively in both paws were considered as demonstrating allodynia and were

used in the following studies.

Effect of 5IA and/or mecamylamine on tactile allodynia

For i.c.v. administration, an injection cannula (o.d. 0.35 mm, i.d. 0.18 mm) was inserted 5.0 mm below the surface of the skull along the guide cannula. Then, various concentrations of 5IA (1–10 nmol/5 μL) or vehicle were infused at 5 μL/rat with a constant rate of 10 μL·min^{-1} using a microsyringe pump (EP-60; Eicom Corporation, Kyoto, Japan). The cannula was retained in place for an additional 1 min to prevent backflow of the drugs. When required, mecamylamine (5 mg·kg^{-1}) was injected subcutaneously 30 min before i.c.v. administration of 5IA.

For intra-VPL administration, an injection cannula was inserted 6.2 mm below the surface of the skull. Subsequently, vehicle, 5IA (1–50 nmol/0.5 μL), mecamylamine (1–10 nmol/0.5 μL), or a mixed solution of 5IA and mecamylamine (10 nmol each/0.5 μL) was infused at 0.5 μL/rat with a constant rate of 0.4 μL·min^{-1}. The cannula was retained in place for an additional 1 min.

For double injection, the cannula for intra-VPL administration was inserted intra-VPL, and mecamylamine (10 nmol/0.5 μL) or vehicle was infused in the same way as described above. Five minutes later, the cannula for i.c.v. administration was inserted, and 5IA (10 nmol/5 μL) or vehicle was infused as described above. Both cannulae were retained in place for an additional 1 min.

Just before and 15, 30, 60, 90, and 120 min after administration, tactile allodynia was evaluated using the von Frey filament test. The results were expressed as a percentage of the maximal possible effect (%MPE) according to the following formula:

$$\%MPE = \frac{\text{Post-drug threshold - Pre-drug threshold}}{\text{Cut-off (26 g) - Pre-drug threshold}} \text{ X } 100$$

Inclined plane test

The inclined plane test was carried out using a sliding apparatus (Medical Agent Co., Ltd., Kyoto, Japan), as described previously (Okada *et al*., 2002). This procedure was used to evaluate the effects of antiallodynic drugs on motor function (Fukui *et al*., 2001; Okada *et al*., 2002; Yasuda *et al*., 2005). It was previously reported that intraperitoneal administration of baclofen, which causes muscle relaxation, significantly reduced the slope angle, thus indicating the appropriateness of this procedure (Fukui *et al*., 2001). Each rat was placed on a stainless steel plate inclined at 30°, and the angle of the plate was increased at a rate of $2° \cdot s^{-1}$. The maximum angle of the plane at which the rat maintained its body position without sliding down was determined.

The rats were habituated to the procedure three times per day. After two days of habituation, the test was performed three times, and the mean of the last two values

was taken as a control. Soon after measuring the control value, drugs or vehicle were administered via each route as described in the section entitled *Effect of 5IA and/or mecamylamine on tactile allodynia*, and the maximum angle before sliding down was determined at 15, 30, 60, 90, and 120 min after administration. Drugs were used at the maximum concentration for each administration route. The data are represented as a percentage of the control value.

Ex vivo autoradiography

Ex vivo autoradiography was performed using a previously described method (Kanegawa *et al*., 2006) with slight modifications. Three groups of rats, a sham-operated group, a 2 week, and a 1 month post-PSL group, were used in this experiment. Each group consisted of 4 to 7 animals.

Each rat was injected with 2 MBq of [^{125}I]5IA via its tail vein and sacrificed 60 min after radioligand injection. Their brains were quickly removed, frozen in hexane (–80°C), and cut into 20 μm-thick coronal sections with a cryomicrotome (CM3000, Leica, Germany). The sections were exposed to imaging plates (BAS-UR, Fuji Photo Film Co., Ltd., Tokyo, Japan) with ^{125}I autoradiographic microscale standards (Amersham Biosciences, Buckinghamshire, UK). After 25 hr exposure, the [^{125}I]5IA autoradiograms were obtained using a bioimaging analyzer (BAS 3000, Fuji Photo Film Co., Ltd.), and quantitative densitometric analyses were performed with

dedicated software (Image Gauge ver. 3.1, Fuji Photo Film Co., Ltd.). Regions of interest (ROIs) were drawn over eight brain regions bilaterally (frontal cortex, striatum, hippocampus, thalamus, pedunculopontine tegmental nucleus (PPTg), nucleus raphe magnus (NRM), locus coeruleus, and cerebellum). Data were represented as the mean of the ligated and contralateral side.

In vitro autoradiography

Three groups of rats were used as described for the ex vivo autoradiography. Each group consisted of 5 to 7 animals. The rats were sacrificed, and their brains were removed and frozen immediately. Each frozen brain was cut into 20 μm-thick coronal sections, thaw-mounted onto gelatin-coated glass slides, and kept frozen at –80°C until use.

A method for the autoradiographic determination of receptor density was previously published (Suzdak *et al*., 1994; Tanaka *et al*., 1993). A binding assay was conducted using a modification of a previously described method (Mukhin *et al*., 2000). The assay buffer used was 50 mM Tris-HCl (pH 7.0) containing 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1 mM $MgCl_2$. The sections were incubated with 23.75 to 380 pM [^{125}I]5IA (specific activity: 220 Ci·mmol^{-1}) in the same buffer for 1 hr at 25°C, then rinsed twice in ice-cold buffer for 5 min each time, and once in distilled water for 1 min, and dried under a cold air stream. Nonspecific binding was

determined in the presence of 300 μM (–)-nicotine. The sections were exposed to the imaging plates with ^{125}I standards for 20 hr. The autoradiograms were obtained and the quantitative analyses were performed in a similar way to that described above. Values for the maximum density of binding sites (B_{max}) were gained from saturation binding isotherms (one-site binding) of specific binding by means of nonlinear curve fitting (Prism 5.00, GraphPad Software, San Diego, CA).

Statistical Analyses

After the behavioral experiments, the rats were sacrificed, and their brains were removed and frozen immediately. Coronal sections (20 μm) including the VPL were prepared and thaw-mounted onto gelatin-coated glass slides and subjected to Nissl staining. The placements of the tips of the injection cannulae were confirmed by using the atlas (Paxinos and Watson, 2005). Only data from rats with the injection cannulae correctly placed in the VPL were used for the statistical analyses.

The analyses of the data from the von Frey filament test and the inclined plane test were performed using two-way analysis of variance (ANOVA) with repeated measures. If there was a significant difference, a *post hoc* one-way ANOVA followed by a Tukey-Kramer multiple comparison test was performed using each treatment combination as an independent group. Analyses of the data from the autoradiographic studies were performed using a one-way ANOVA followed by a

Tukey-Kramer multiple comparison test. Differences were considered significant when $p < 0.05$.

Results

Antiallodynic effect of i.c.v. administered 5IA

In the present study, the rats presented a bilateral tactile allodynia following the PSL. The paw withdrawal thresholds decreased from 13.4 ± 0.9 g to 3.1 ± 0.3 g and from 13.4 ± 1.0 g to 4.2 ± 0.4 g in the ligated paws and the contralateral paws, respectively. First, we evaluated an antiallodynic effect of i.c.v. administered 5IA, since there has been no previous report regarding whether 5IA is able to palliate neuropathic pain. Figure 1A shows the antiallodynic effect observed in the ligated paws. Two-way ANOVA demonstrated significant main effects of treatment ($F_{3, 90}$ = 5.56, P = 0.007) and time ($F_{4, 90}$ = 7.51, $P < 0.001$) and a significant interaction between treatment and time ($F_{12, 90}$ = 2.85, P = 0.003). Ten nmol of 5IA increased %MPE values significantly at 15 min after administration (Vehicle: –3.4 ± 2, 10 nmol: 38 ± 6). Although the effect peaked at 15 min after administration and decreased gradually, significant increases in %MPE values were also observed in the 3 nmol- and 10 nmol-treated groups 30 min after administration. Similar results were also observed in the contralateral paws (data not shown).

Next, we examined an effect of mecamylamine, a blood-brain barrier permeable nACh receptor antagonist, on the antiallodynic effect of 5IA. Significant main effects of treatment ($F_{3, 95}$ = 6.75, P = 0.003) and time ($F_{4, 95}$ = 5.61, P < 0.001) and a significant interaction between treatment and time ($F_{12, 95}$ = 7.44, P < 0.001) were found. Mecamylamine (5 mg·kg^{-1}, s.c.) blocked the effect of 5IA completely and the %MPE values were reduced to vehicle levels (5IA alone: 38 ± 6 and 24 ± 8, 5IA + MEC: 3.0 ± 7 and 2.9 ± 7 at 15 and 30 min after administration, respectively) (Fig. 1B).

Changes in the accumulation of [^{125}I]5IA in vivo

We compared [^{125}I]5IA accumulation between the PSL model rats and the sham-operated rats using an *ex vivo* autoradiographic method. Representative autoradiograms are shown in Fig. 2A. The thalamic signals of the PSL groups were stronger than those of the sham-operated group, while the cortical and hippocampal signals were similar among the 3 groups. There was no difference between the ligated and contralateral sides of the PSL model rats. A significant increase (170%) in the accumulation of [^{125}I]5IA was observed only in the thalamus in both PSL groups (Sham: 1.5 ± 0.15, 2 weeks: 2.6 ± 0.34, 1 month: 2.6 ± 0.08), and no detectable change was seen in other regions (Fig. 2B).

Measurement of the density of nACh receptors in vitro

We performed an autoradiographic saturation assay using [^{125}I]5IA as a radioligand. The saturation binding curves are presented in Fig. 3A. Similarly to the *ex vivo* autoradiography, both PSL groups showed a significant increase in B_{max} value that occurred in the thalamus only (Sham: 13 ± 1.9, 2 weeks: 19 ± 0.89, 1 month: 18 ± 0.81) (Fig. 3B). The percentage increase in Bmax values in the thalamus (150%) was similar to those observed in the ex vivo autoradiographic findings.

Antiallodynic effect of intra-VPL administered 5IA

Based on previous reports (Derbyshire *et al*., 1997; Gybels, 2001; Kupers and Gybels, 1993), we predicted that the VPL was involved in the expression of antinociceptive effects and investigated the association between nACh receptors expressed in the VPL and the antiallodynic effect of 5IA. Figure 4A shows the antiallodynic effects that occurred in the ligated paws of rats that received 5IA intra-VPL. Two-way ANOVA demonstrated significant main effects of treatment ($F_{4, 115} = 3.97$, $P = 0.014$) and time ($F_{4, 115} = 13.6$, $P < 0.001$) and a significant interaction between treatment and time ($F_{16, 115} = 3.11$, $P < 0.001$). There was a significant difference between the 50 nmol-treated group and the vehicle- or 1 nmol-treated groups (Vehicle: 0.82 ± 4, 1 nmol: 8.6 ± 5, 50 nmol: 30 ± 9). Although one-way ANOVA revealed a significant difference between the data at 30 min after

administration (P = 0.028), the *post hoc* Tukey test showed no significant difference. Subsequently, we injected a mixed solution of 5IA and mecamylamine (10 nmol each) intra-VPL and performed the von Frey filament test. Two-way ANOVA demonstrated significant main effects of treatment ($F_{3, 85}$ = 11.34, P < 0.001) and time ($F_{4, 85}$ = 5.22, P = 0.001) and a significant interaction between treatment and time ($F_{12, 85}$ = 4.05, P < 0.001). Mecamylamine antagonized the 5IA-induced antiallodynic effect completely, and furthermore, the %MPE values of mecamylamine-treated rats tended to decrease compared to those of the vehicle-treated rats, although the difference was not significant (Fig. 4B).

Involvement of nACh receptors in the VPL in the antiallodynic effect induced by i.c.v. 5IA administration

To explore to what extent nACh receptors in the VPL were involved in the antiallodynic effects arising from central nACh receptor activation, we evaluated the effect of intra-VPL administered mecamylamine on the antiallodynic effects produced by i.c.v. administered 5IA. The results from the ligated paws are illustrated in Figure 4C. Two-way ANOVA demonstrated significant main effects of treatment ($F_{3, 105}$ = 7.91, P = 0.001) and time ($F_{4, 105}$ = 5.65, P < 0.001) and a significant interaction between treatment and time ($F_{12, 105}$ = 2.49, P = 0.008). The rats administered vehicle intra-VPL followed by 10 nmol of 5IA i.c.v. (Vehicle/5IA

group) demonstrated significantly elevated %MPE values at 15 and 30 min after administration (41 ± 20 at 15 min and 35 ± 22 at 30 min). This result was consistent with the antiallodynic effect produced by i.c.v. administered 5IA (Fig. 1). Pretreatment with intra-VPL mecamylamine (10 nmol) significantly inhibited the effect of i.c.v. administered 5IA (10 nmol) (10 ± 4 at 15 min and 3.6 ± 5 at 30 min, in the MEC/5IA group). The %MPE values for rats treated with mecamylamine alone (MEC/Vehicle group) also tended to decrease compared to those of the vehicle treated rats, although the difference was not significant.

Hyperalgesic effect of intra-VPL administered mecamylamine

To examine the effects of mecamylamine on tactile allodynia in more detail, we injected multiple doses of mecamylamine intra-VPL and performed the von Frey filament test. Data from the ligated paws are shown in Figure 5A. There was a significant main effect of treatment ($F_{3, 95} = 6.77$, $P = 0.003$) but no effect of time nor interaction between time and treatment. The post hoc Tukey test demonstrated a significant difference between vehicle-treated group and 5 nmol- or 10-nmol treated groups. The calculated area under the curves of each group clearly represented the dose-dependent hyperalgesic effects of mecamylamine (Fig. 5B).

Effects of 5IA and mecamylamine on motor function

We conducted an inclined plane test to evaluate the effects of 5IA and mecamylamine on motor functions. The maximum angle that the rats were able to endure before slipping down the plane was not significantly different between the vehicle and drug-treated groups at any time point (Table 1, data not shown for mecamylamine).

Discussion and Conclusions

In the present study, we examined the changes in central nACh receptor density in a rat model of neuropathic pain and the involvement of nACh receptors in antinociceptive effects in the region where changes occurred. First, we evaluated the antiallodynic effect of 5IA after i.c.v. administration and found that the antiallodynic effect was expressed in a dose-dependent manner. Because the effect was completely antagonized by mecamylamine, the antiallodynic effect of 5IA must occur via nACh receptors. No significant difference was observed between the vehicle and 5IA treated groups in the inclined plane test suggesting that 5IA is able to show the antiallodynic effect below the dose that causes adverse effects.

We found that bilateral upregulation of thalamic nACh receptors occurred in the PSL model of neuropathic pain. This is the first report of upregulation of thalamic nACh receptors under chronic painful conditions. Because the upregulation of both

muscarinic and cannabinoid CB_1 receptors are reported to contribute to the increased analgesic efficacy of each agonist (Chen and Pan, 2003; Siegling *et al*., 2001), upregulated nACh receptors may also contribute to the potentiation of antiallodynic effects produced by nACh receptor agonists and attenuate neuropathic pain. Consistent with Seltzer's report (Seltzer *et al*., 1990), the unilateral PSL caused bilateral tactile allodynia in the present study; and thus, the upregulation must occur bilaterally. In the present study, we performed an autoradiographic saturation assay to determine the regional Bmax of nACh receptors. Since no report concerning autoradiographic saturation assays using [^{125}I]5IA has been published, it is important to validate the method used. Doura *et al*. performed quantitative autoradiography using [^{125}I]5IA (Doura *et al*., 2008). However, in their study, they incubated brain slices with only a single concentration of [^{125}I]5IA and did not perform a saturation assay. Thus, a direct comparison between our present data and theirs is not possible. Nevertheless, the ratios of the thalamus to the striatum or the cortex of our present data were consistent in the data reported by Doura *et al*., suggesting that the method is valid.

A previous PET study showed that the increase in cerebral blood flow in the VPL positively correlated with pain intensity, suggesting the involvement of the VPL in pain transmission (Derbyshire *et al*., 1997). Furthermore, electrical stimulation of the VPL has produced pain alleviation in both rat models and patients with

neuropathic pain (Gybels, 2001; Kupers and Gybels, 1993). These findings suggest that the VPL is involved in the expression of antinociceptive effects. Indeed, we demonstrated that 5IA administered locally into the VPL significantly and dose-dependently reversed tactile allodynia. This effect was antagonized by coadministered mecamylamine. Since no significant difference was observed between the vehicle and 5IA treated groups in the results of an inclined plane test, the changes in %MPE values observed in the 5IA treated groups were considered to reflect the analgesic effect, not motor dysfunction. These findings suggest that the nACh receptors expressed in the VPL were involved in the antiallodynic effect that occurred after nACh receptor agonist administration. This was consistent with the finding that blockade of nACh receptors in the VPL caused a decrease in the antiallodynic effect of i.c.v.-administered 5IA. Moreover, intra-VPL administered mecamylamine induced significant decreases in %MPE values. Mecamylamine is not an inverse agonist, but a non-competitive antagonist (Jensen *et al*., 2005). Thus, during neuropathic pain, an intrinsic antiallodynic mechanism by which ACh activated nACh receptors expressed in the VPL may be present, and antagonism by mecamylamine cause a hyperalgesic effect. That is to say, the nACh receptors expressed in the VPL may participate in antiallodynic effects produced not only by exogenous but also endogenous agonists.

The present findings do not negate the involvement of nACh receptors in

antiallodynic effects outside of the VPL. Previous pharmacological studies reported that the central sites involved in nACh receptor-mediated antinociception were the NRM and the PPTg. Iwamoto *et al*. reported that the antinociception produced by nicotinic stimulation of the PPTg or the NRM depended upon muscarinic cholinergic, serotonergic, and adrenergic systems at the level of the lumbar spinal cord (Iwamoto and Marion, 1993). Curzon *et al*. showed that microinjection of nACh receptor agonists (epibatidine and A-85380) into the NRM produced antinociception against heat stimuli, and these effects were prevented by coadministration of mecamylamine into the NRM (Curzon *et al*., 1998). Indeed, we demonstrated that VPL blockade by mecamylamine before i.c.v. administration of 5IA decreased the antiallodynic effect by up to approximately 70%, not 100%. Thus, the remainder of the antiallodynic effect is possibly caused by the binding of 5IA to the NRM and/or the PPTg.

Recently, Mogg *et al*. have reported that 5IA can functionally activate the α4β2-nACh and α6β2-nACh receptors (Mogg *et al*., 2004). On the other hand, Perry *et al*. reported that the nACh receptors expressed in the thalamus were mainly of the α4β2 (Perry *et al*., 2002) and that the α6 subunit was present in less than 4% of the thalamic nACh receptors (Perry *et al*., 2007). Therefore, the antiallodynic effect is probably mediated via the α4β2-nACh receptors, at least when 5IA was administered into the VPL. Whereas many results have shown that α4 subunit-containing nACh receptors play an important role in nACh receptor-mediated antinociception (Bitner *et*

al., 1998; Bitner *et al*., 2000; Marubio *et al*., 1999), the contribution of the α6β2-nACh receptors to the antinociceptive effect is unknown. Since A-186253 has recently been developed as a specific antagonist of the α4β2-nACh receptors (Itier *et al*., 2004), it may be possible to investigate α6β2-nACh receptor-mediated antinociception using both 5IA and A-186253.

In summary, we have demonstrated for the first time that upregulation of thalamic nACh receptors occurs under chronic painful conditions. Moreover, we revealed that intra-VPL administration of 5IA attenuates tactile allodynia in a dose-dependent manner. This effect was completely antagonized by coadministered mecamylamine. The blockade of nACh receptors in the VPL by mecamylamine caused a decrease in the antiallodynic effect of i.c.v.-administered 5IA. These findings indicate that the nACh receptors expressed in the VPL are a potential site of the antinociceptive action produced by 5IA. Furthermore, intra-VPL administered mecamylamine induced a hyperalgesic effect. This effect is likely to be responsible for the mecamylamine antagonism of the intrinsic antiallodynic mechanism induced by endogenous acetylcholine. These findings suggest that the nACh receptors expressed in the VPL play an important role in the antiallodynic effects produced by exogenous and endogenous agonists.

Acknowledgements:

We thank Dr. Takayuki Nakagawa (Department of Molecular Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University) for his technical support and invaluable discussion regarding the behavioral tests. This work was supported in part by a Grant-in-Aid for Scientific Research (A) (19209041) and a Grant-in-Aid for Young Scientists (B) (19790869) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from the Smoking Research Foundation.

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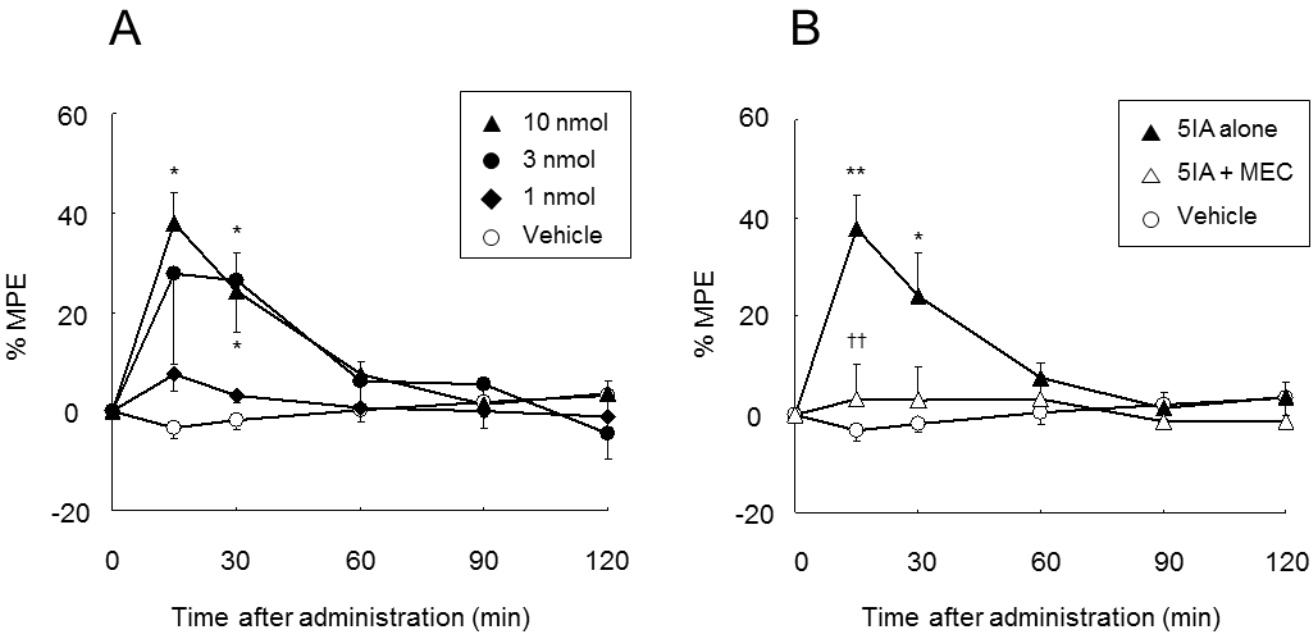
Tables

Table 1. Effects of 5IA on motor functions

	Time after administration				
Drug	15 min.	30 min.	60 min.	90 min.	120 min.
(i.c.v.)					
Vehicle	98 ± 1	102 ± 4	98 ± 2	92 ± 3	94 ± 3
5IA	93 ± 2	97 ± 3	88 ± 4	96 ± 3	93 ± 1
(intra-VPL)					
Vehicle	99 ± 3	99 ± 2	97 ± 4	100 ± 2	97 ± 3
5IA	100 ± 4	97 ± 2	99 ± 2	97 ± 1	102 ± 2

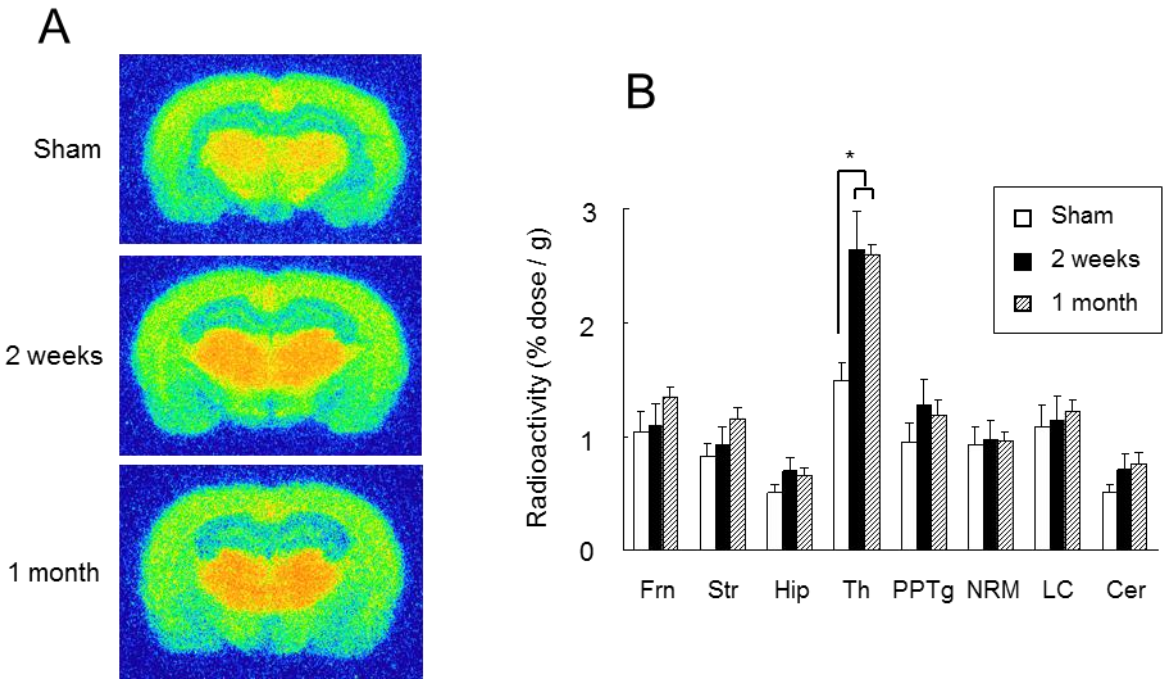
Data are expressed as a percentage of the control level, which was determined before drug administration in each animal, and represent the mean ± SEM of five to six animals per group. No significant difference was observed between the two groups (two-way ANOVA with repeated measures).

Figure 1



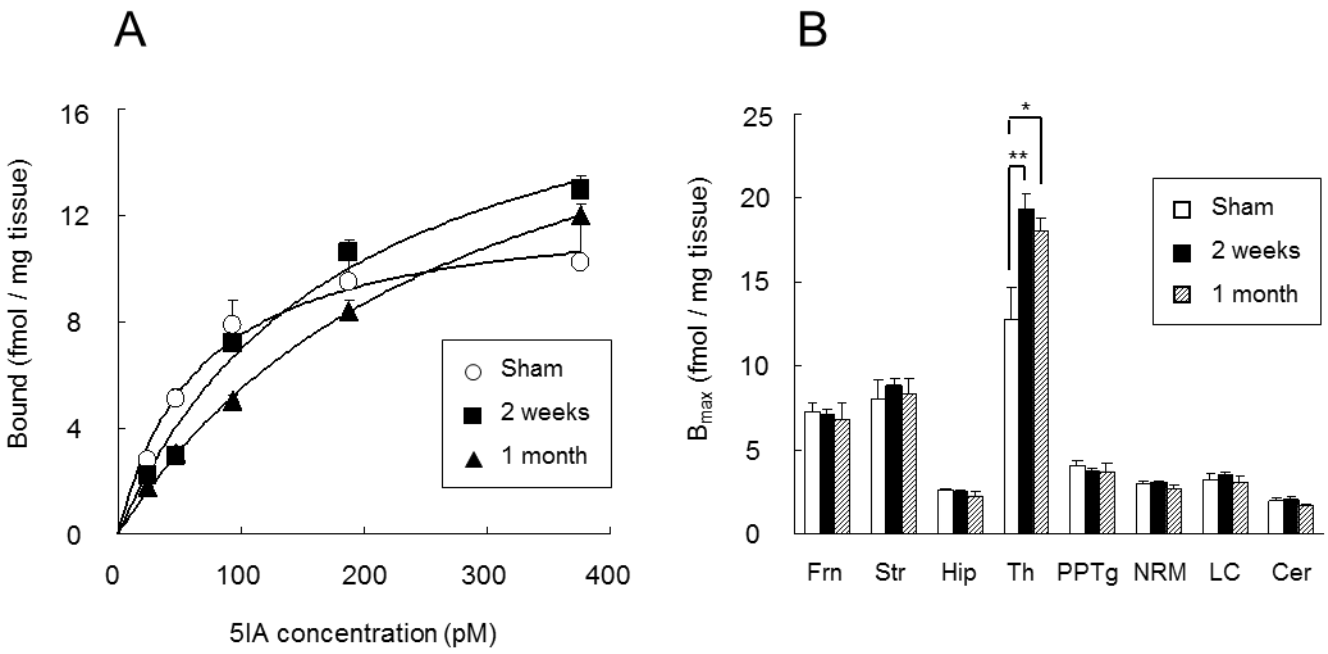
(A) Effect of i.c.v. administered 5IA on neuropathic tactile allodynia. Data are presented as a percentage of the maximum possible effect. Each point represents the mean ± SEM of the ligated paws of five to six animals per group. *$p < 0.05$ vs. vehicle at the same time point. (B) Effect of mecamylamine (MEC, 5 mg·kg^{-1}, s.c., 30 min prior) on the antiallodynic effect induced by 5IA (i.c.v.). Data are presented as a percentage of the maximum possible effect. Each point represents the mean ± SEM of the ligated paws of five to six animals per group. **$p < 0.01$ vs. vehicle, #$p < 0.05$, ##$p < 0.01$ vs. MEC alone, and †$p < 0.05$, ††$p < 0.01$ vs. 5IA alone at the same time point.

Figure 2



(A) Representative autoradiograms of sham-operated and PSL rats. (B) Changes in the regional accumulation of [^{125}I]5IA in Seltzer's PSL model of neuropathic pain. A significant increase was observed only in the thalamus, and no detectable change occurred in other regions. Each column represents the mean ± SEM of four to seven animals per group. *p < 0.05 vs. sham. Abbreviations: Frn: frontal cortex; Str: striatum; Hip: hippocampus; Th: thalamus; PPTg: pedunculopontine tegmental nucleus; NRM: nucleus raphe magnus; LC: locus coeruleus; Cer: cerebellum.

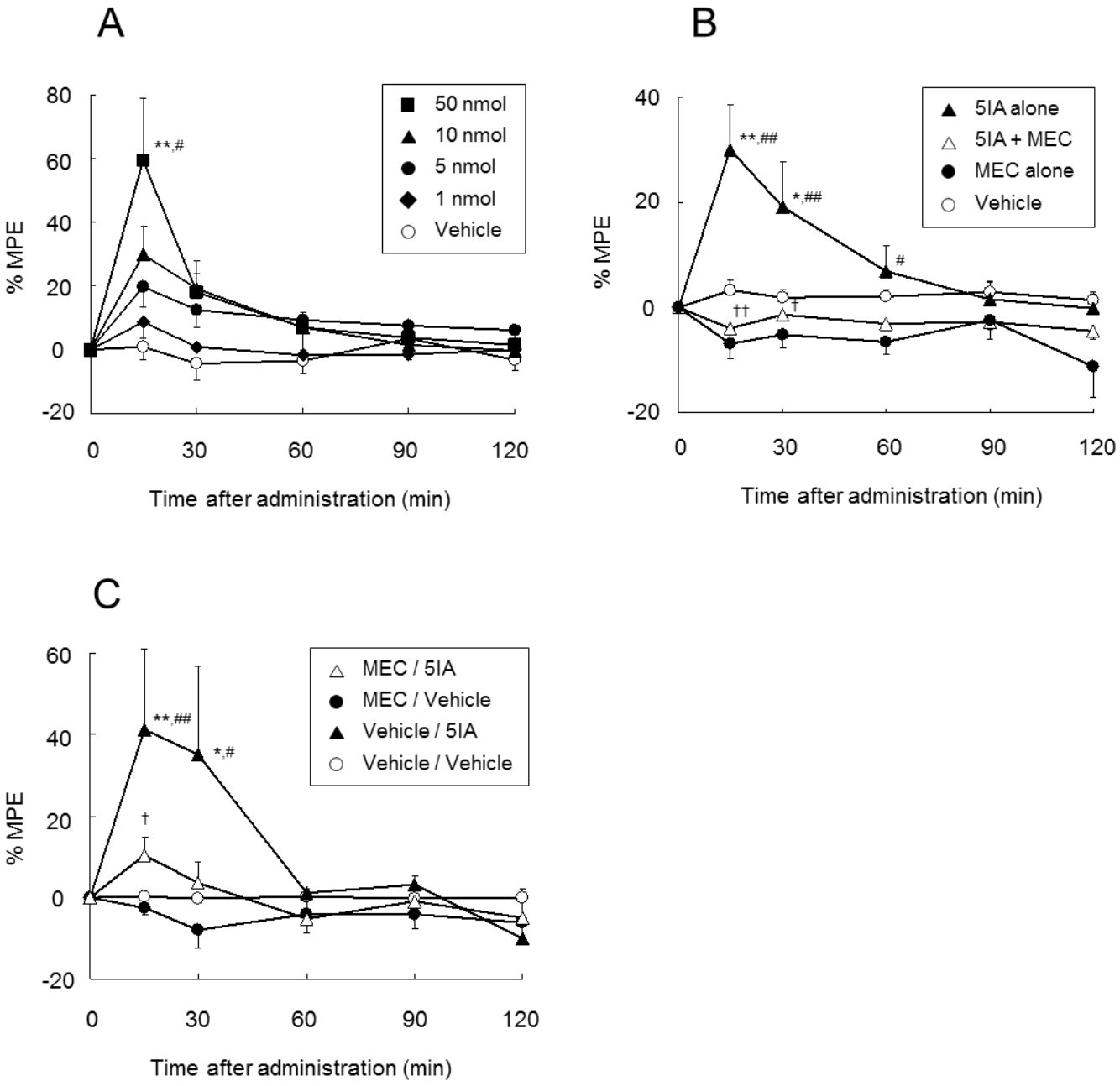
Figure 3



(A) Saturation curves of [^{125}I]5IA binding to sham-operated and PSL rat thalamus. Each point represents the mean ± SEM of five to seven animals per group. The B_{max} values estimated by nonlinear regression analysis of these mean data were 12.5, 19.9, and 21.2 fmol/mg protein in sham-operated, 2-week post-PSL, and 1-month post-PSL group, respectively. (B) Changes in nACh receptor density in Seltzer's PSL model of neuropathic pain. A significant increase was observed only in the thalamus, and no detectable change occurred in other regions. Each column represents the mean ± SEM of five to seven animals per group. *p < 0.05, **p < 0.01 vs. sham. Abbreviations: Frn: frontal cortex; Str: striatum; Hip: hippocampus; Th: thalamus;

PPTg: pedunculopontine tegmental nucleus; NRM: nucleus raphe magnus; LC: locus coeruleus; Cer: cerebellum.

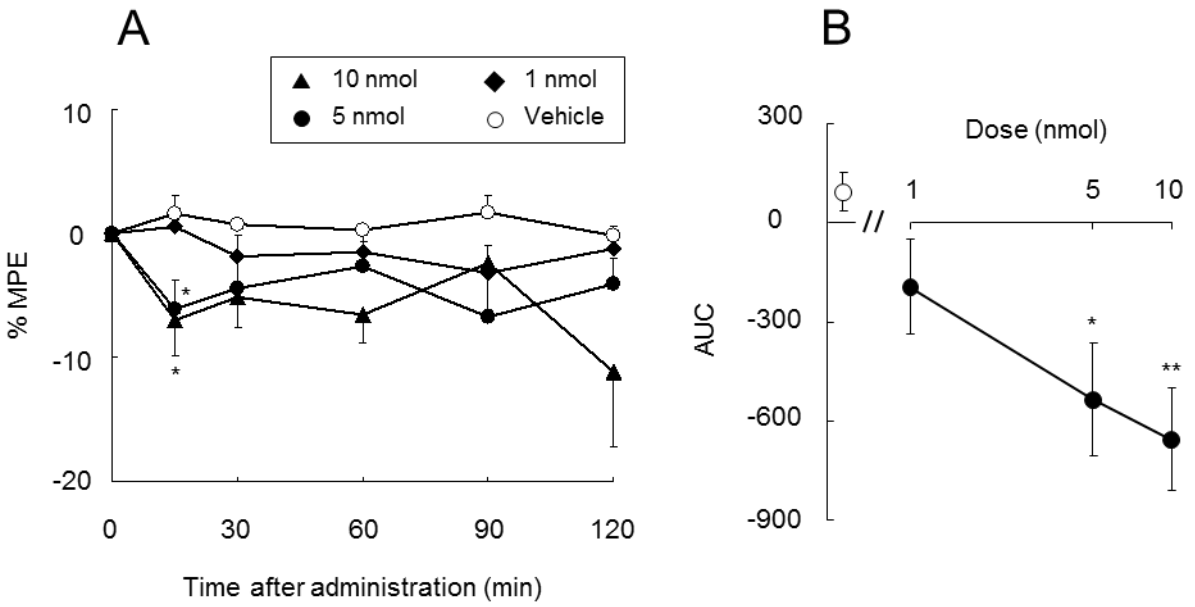
Figure 4



(A) Effect of intra-VPL administered 5IA on neuropathic tactile allodynia. Data are presented as a percentage of the maximum possible effect. Each point represents the mean ± SEM of the ligated paws of five to six animals per group. *$p < 0.05$, **$p <$

0.01 vs. vehicle, and #$p < 0.05$ vs. 1 nmol of 5IA at the same time point. (B) Effect of mecamylamine (MEC, 10 nmol/0.5 μL) administered concurrently with 5IA in an intra-VPL manner on the antiallodynic effect produced by 5IA (10 nmol/0.5 μL, intra-VPL). Data are presented as a percentage of the maximum possible effect. Each point represents the mean ± SEM of the ligated paws of five to six animals per group. *$p < 0.05$, **$p < 0.01$ vs. vehicle, #$p < 0.05$, ##$p < 0.01$ vs. MEC alone, and †$p < 0.05$, ††$p < 0.01$ vs. 5IA alone at the same time point. (C) Effect of mecamylamine (MEC, 10 nmol/0.5 μL, intra-VPL, 5 min prior) on the antiallodynic effect produced by 5IA (10 nmol/5 μL, i.c.v.). Data are presented as a percentage of the maximum possible effect. Each point represents the mean ± SEM of the ligated paws of four to seven animals per group. *$p < 0.05$, **$p < 0.01$ vs. vehicle/vehicle, #$p < 0.05$, ##$p < 0.01$ vs. MEC/vehicle, and †$p < 0.05$ vs. vehicle/5IA at the same time point.

Figure 5



(A) Effect of intra-VPL administered mecamylamine on neuropathic tactile allodynia. Data are presented as a percentage of the maximum possible effect. Each point represents the mean ± SEM of the ligated paws of five to six animals per group. *p < 0.05 vs. vehicle at the same time point. (B) Dose-response curve of mecamylamine-induced hyperalgesia. The open symbol represents the AUC of the vehicle-treated group, and the closed symbols represent that of each concentration of the mecamylamine-treated group. *p < 0.05, **p < 0.01 vs. vehicle.

Conflicts of interest

The authors state that they have no conflicts of interest.