

## RESEARCH PAPER

## Nicotinic acetylcholine receptors expressed in the ventral posterolateral thalamic nucleus play an important role in anti-allodynic effects

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**Background and purpose:** Much interest is currently being focused on the anti-nociceptive effects mediated by nicotinic acetylcholine (nACh) receptors, including their location and mechanism of action. The purpose of this study was to elucidate these issues using 5-iodo-3-(2(S)-azetidylmethoxy)pyridine (5IA), a nACh receptor agonist, and [<sup>125</sup>I]5IA.

**Experimental approach:** We partially ligated the sciatic nerve of Sprague-Dawley rat to induce neuropathic pain [Seltzer's partial sciatic nerve ligation (PSL) model]. We then examined the changes in nACh receptor density in the CNS using [<sup>125</sup>I]5IA autoradiography and the involvement of nACh receptors in anti-nociceptive effects in the region where changes occurred.

**Key results:** Autoradiographic studies showed that the accumulation of [<sup>125</sup>I]5IA and the number of nACh receptors in the thalamus of PSL rats were increased about twofold compared with those in the sham-operated rats. No change was observed in other brain regions. Rats injected in the ventral posterolateral thalamic nucleus (VPL) with 5IA demonstrated a significant and dose-dependent anti-allodynic effect and this effect was completely antagonized by mecamylamine, injected with 5IA, into the VPL. The blockade of nACh receptors in the VPL by mecamylamine decreased by 70% the anti-allodynic effect of 5IA, given i.c.v. Moreover, mecamylamine given intra-VPL by itself, induced significant hyperalgesia.

**Conclusions and implications:** Our findings suggest that the nACh receptors expressed in the VPL play an important role in the anti-allodynic effects produced by exogenous and endogenous agonists.

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**Keywords:** nicotinic acetylcholine receptor; thalamus; ventral posterolateral thalamic nucleus; 5IA; anti-allodynic effect; neuropathic pain

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

## 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 receptor antagonists and arise from the activation of nACh receptors (Bannon *et al.*, 1998a; nomenclature follows Alexander *et al.*, 2008), much interest has been focused on nACh receptors as a novel target of anti-nociceptive 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,c; Decker *et al.*, 1998). Therefore,

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identification of the sites of action of nACh receptor agonists and the elucidation of their anti-allodynic mechanisms are desirable.

One of the most studied nACh receptor-acting analgesics is ABT-594. This compound is a selective and potent agonist with high affinity for  $\alpha 4\beta 2$ -nACh receptors (predominant in the brain), low affinity for  $\alpha 7$ -nACh receptors and no affinity for  $\alpha 1\beta 1\gamma\delta$ -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 with non-selective agonists such as epibatidine (Bannon *et al.*, 1998b).

Recently, 5-iodo-3-(2(S)-azetidylmethoxy)pyridine (5-iodo-A-85380, 5IA), an analogue of ABT-594, was synthesized (Saji *et al.*, 2002). It is also an  $\alpha 4\beta 2$ -nACh receptor-specific agonist (Ueda *et al.*, 2008) with a relatively good safety profile (Vaupel *et al.*, 2003; Ueda *et al.*, 2004). In addition, radiolabelled 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 (Saji *et al.*, 2002; Mamede *et al.*, 2004; Mamede *et al.*, 2007; Oishi *et al.*, 2007; Brasic *et al.*, 2009). Non-radioactive 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 up-regulation of spinal muscarinic acetylcholine receptors (Chen and Pan, 2003) and thalamic cannabinoid CB<sub>1</sub> receptors (Siegling *et al.*, 2001) in neuropathic pain models. It has been reported that the up-regulation of muscarinic and CB<sub>1</sub> receptors contributes to the increased analgesic efficacy of each agonist. As nACh receptor agonists are also effective against neuropathic pain, up-regulation of nACh receptors may also occur in such models. We hypothesized that brain regions where changes in the density or function of nACh receptors occurred would play an important role in anti-nociceptive 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 as a result of the model of neuropathic pain.

## Methods

### Animals

Animal care and experimental procedures 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 h light/dark cycle with free access to food and water.

### Surgical operation

The PSL neuropathic pain model was established according to a previously published method (Seltzer *et al.*, 1990). Under sodium pentobarbital (50 mg·kg<sup>-1</sup>, i.p.) anesthesia, the right sciatic nerve was exposed just distal to the branch leading to the posterior biceps femoris/semiotendinosus muscles. A 7–0 silk suture was inserted into the nerve and tightly ligated so that the dorsal 1/3–1/2 of the nerve was trapped in the ligation. 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·kg<sup>-1</sup>, 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 2 weeks after the PSL, tactile sensitivity was measured by the up-down method using calibrated von Frey filaments ranging from 0.07 to 26 g (North Coast Medical, Morgan Hill, CA, USA), 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 area 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 1–2 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 mecamlamine 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 in 5  $\mu$ L) or vehicle were infused at 5  $\mu$ L per rat with a constant rate of 10  $\mu$ L·min<sup>-1</sup> 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, mecamlamine (5 mg·kg<sup>-1</sup>) 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 in 0.5 µL), mecamlamine (1–10 nmol in 0.5 µL) or a mixed solution of 5IA and mecamlamine (10 nmol each in 0.5 µL) was infused at 0.5 µL per rat with a constant rate of 0.4 µL·min<sup>-1</sup>. The cannula was retained in place for an additional 1 min.

For double injections, the cannula for intra-VPL administration was inserted intra-VPL, and mecamlamine (10 nmol in 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 in 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:

$$\%MPE = \frac{\text{post-drug threshold} - \text{pre-drug threshold}}{\text{cut-off (26 g)} - \text{pre-drug threshold}} \times 100$$

#### *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 anti-allodynic drugs on motor function (Fukui *et al.*, 2001; Okada *et al.*, 2002; Yasuda *et al.*, 2005). Intraperitoneal administration of baclofen, which causes muscle relaxation, significantly reduced the slope angle at which rats were no longer able to maintain their position and slid down the table, 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°·s<sup>-1</sup>. 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 2 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 above, 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 four to seven animals.

Each rat was injected with 2 MBq of [<sup>125</sup>I]5IA via its tail vein and killed 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 <sup>125</sup>I autoradiographic microscale standards (Amersham Biosciences, Buckinghamshire, UK). After 25 h exposure, the [<sup>125</sup>I]5IA autoradiograms were obtained using a bioimaging analyzer (BAS 3000, Fuji Photo Film Co., Ltd., Tokyo, Japan), and quantitative densitometric analyses were performed with dedicated software (Image Gauge ver. 3.1, Fuji Photo Film Co., Ltd., Tokyo, Japan). Regions of interest were drawn over eight brain regions bilaterally [frontal cortex, striatum, hippocampus, thalamus, pedunculo-pontine 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 five to seven animals. The rats were killed 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 (Tanaka *et al.*, 1993; Suzdak *et al.*, 1994). 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<sub>2</sub> and 1 mM MgCl<sub>2</sub>. The sections were incubated with 23.75–380 pM [<sup>125</sup>I]5IA (specific activity: 220 Ci·mmol<sup>-1</sup>) in the same buffer for 1 h 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. Non-specific binding was determined in the presence of 300 µM (–)-nicotine. The sections were exposed to the imaging plates with <sup>125</sup>I standards for 20 h. 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<sub>max</sub>) 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, USA).

#### *Statistical analyses*

After the behavioural experiments, the rats were killed, 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  $P < 0.05$ .

### Materials

5IA and [<sup>125</sup>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, USA). The sodium pentobarbital for injection was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). All drugs were administered in physiological saline solution. All other chemicals used were of reagent grade.

## Results

### Anti-allodynic 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  $13.4 \pm 0.9$  g to  $3.1 \pm 0.3$  g and from  $13.4 \pm 1.0$  g to  $4.2 \pm 0.4$  g in the ligated paws and the contralateral paws respectively. First, we evaluated an anti-allodynic effect of i.c.v. administered 5IA, as there has been no previous report regarding whether 5IA is able to palliate neuropathic pain. Figure 1A shows the anti-allodynic 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 ( $F_{12,90} = 2.85$ ,  $P = 0.003$ ). Injection of 5IA (10 nmol) increased % MPE values significantly at 15 min after administration (Vehicle:  $-3.4 \pm 2$ , 10 nmol:  $38 \pm 6$ ). 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).

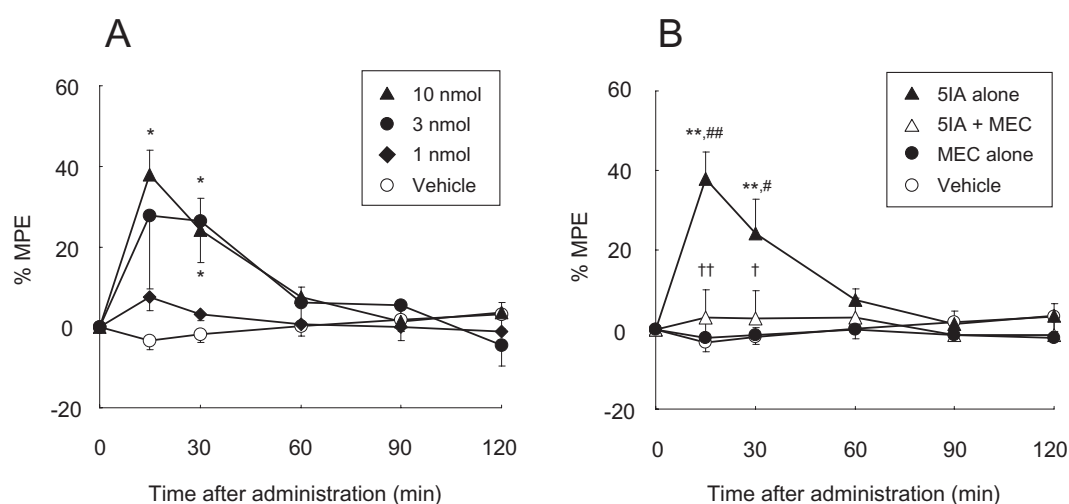
Next, we examined an effect of mecamylamine, a blood-brain barrier permeable nACh receptor antagonist, on the anti-allodynic effect of 5IA. Significant main effects of treatment ( $F_{3,95} = 6.75$ ,  $P = 0.003$ ) and time ( $F_{4,95} = 5.61$ ,  $P < 0.001$ ) and a significant interaction between treatment and time ( $F_{12,95} = 7.44$ ,  $P < 0.001$ ) were found. Mecamylamine ( $5 \text{ mg}\cdot\text{kg}^{-1}$ , s.c.) blocked the effect of 5IA completely and the % MPE values were reduced to levels obtained after treatment with vehicle (Figure 1B).

### Changes in the accumulation of [<sup>125</sup>I]5IA in vivo

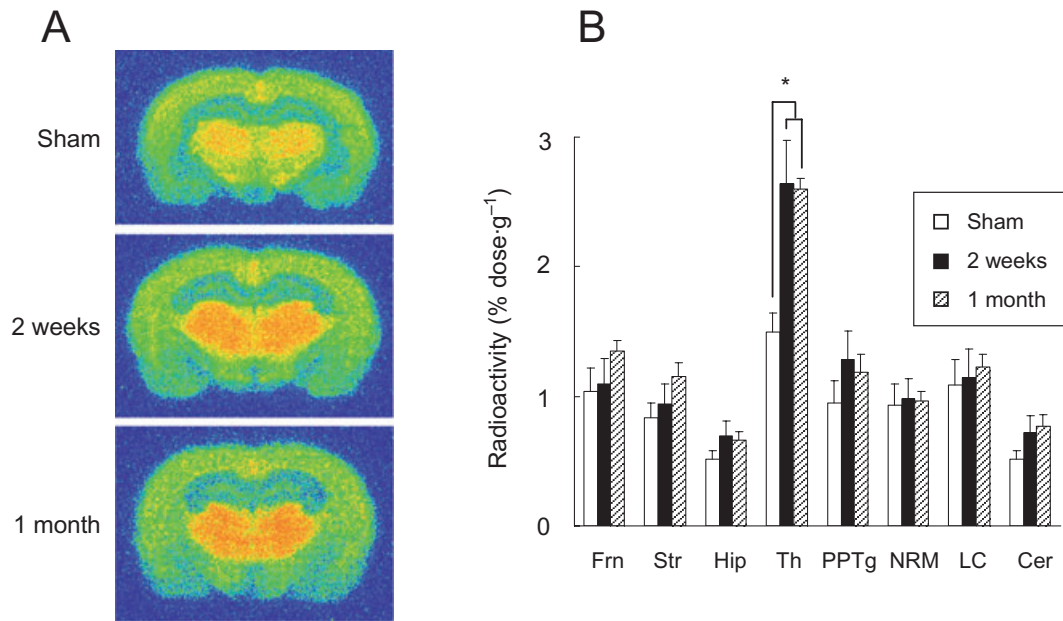
We compared [<sup>125</sup>I]5IA accumulation between the PSL model rats and the sham-operated rats using an *ex vivo* autoradiographic method. Representative autoradiograms are shown in Figure 2A. The thalamic signals of the PSL groups were stronger than those of the sham-operated group, while the cortical and hippocampal signals were similar among the three groups. There was no difference between the ligated and contralateral sides of the PSL model rats. A significant increase (170%) in the accumulation of [<sup>125</sup>I]5IA was observed only in the thalamus in both PSL groups and no detectable change was seen in other regions (Figure 2B).

### Measurement of the density of nACh receptors in vitro

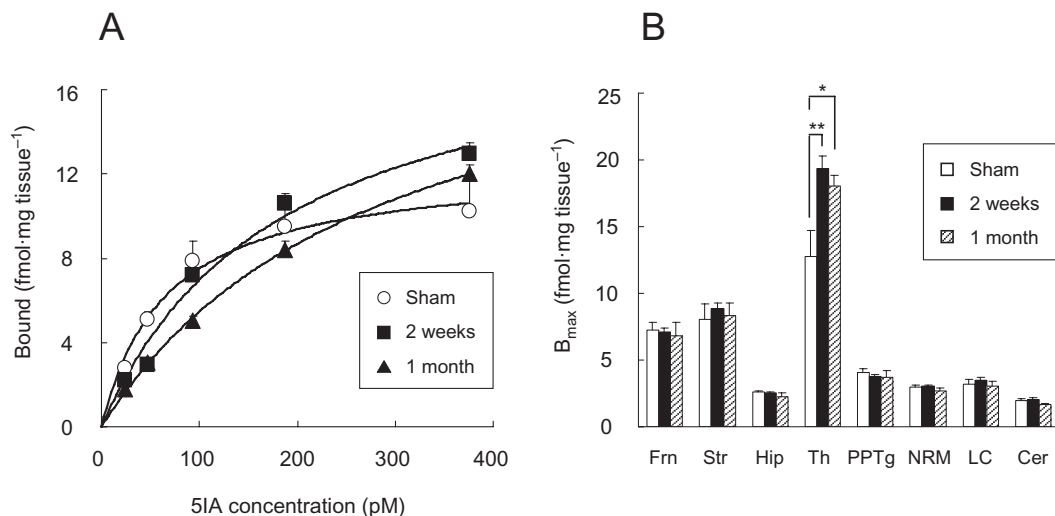
We performed an autoradiographic saturation assay using [<sup>125</sup>I]5IA as a radioligand. The saturation binding curves are



**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 (% MPE). Each point represents the mean  $\pm$  SEM of the ligated paws of five to six animals per group. \* $P < 0.05$  versus vehicle at the same time point. (B) Effect of mecamylamine (MEC,  $5 \text{ mg}\cdot\text{kg}^{-1}$ , s.c., 30 min prior) on the anti-allodynic effect induced by 5IA (i.c.v.). Data are presented as a percentage of the maximum possible effect. Each point represents the mean  $\pm$  SEM of the ligated paws of five to six animals per group. \*\* $P < 0.01$  versus vehicle, # $P < 0.05$ , ## $P < 0.01$  versus MEC alone, and † $P < 0.05$ , †† $P < 0.01$  versus 5IA alone at the same time point. 5IA, 5-iodo-3-(2(S)-azetidylmethoxy)pyridine; MPE, maximal possible effect.



**Figure 2** (A) Representative autoradiograms of brain regions from sham-operated and PSL rats given  $[^{125}\text{I}]5\text{IA}$ , i.v. (B) Changes in the regional accumulation of  $[^{125}\text{I}]5\text{IA}$  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  $\pm$  SEM of four to seven animals per group.  $*P < 0.05$  versus sham. PSL, partial sciatic nerve ligation; 5IA, 5-iodo-3-(2(S)-azetidylmethoxy)pyridine; Frn, frontal cortex; Str, striatum; Hip, hippocampus; Th, thalamus; PPTg, pedunclopontine tegmental nucleus; NRM, nucleus raphe magnus; LC, locus coeruleus; Cer, cerebellum.

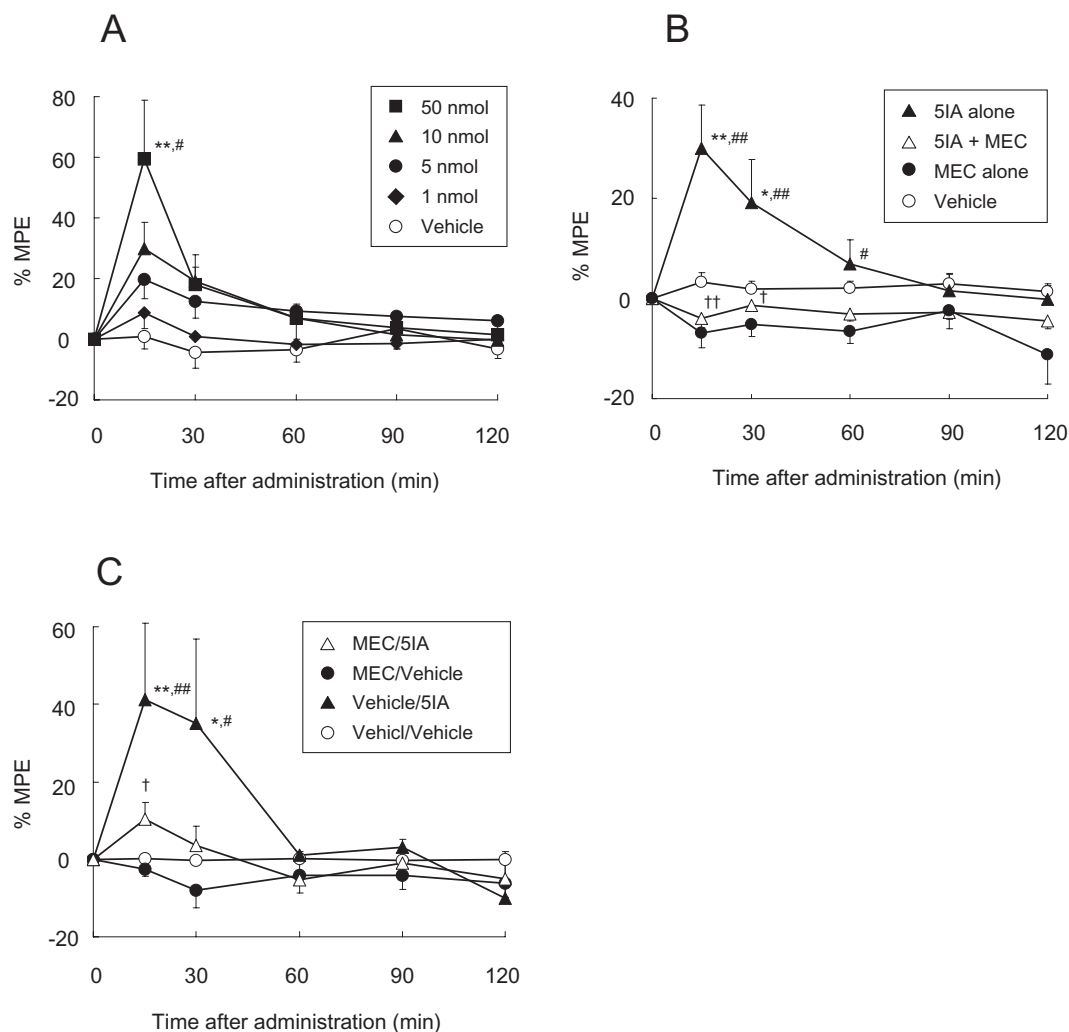


**Figure 3** (A) Saturation curves of  $[^{125}\text{I}]5\text{IA}$  binding to sham-operated and PSL rat thalamus determined *ex vivo*. Each point represents the mean  $\pm$  SEM of five to seven animals per group. The  $B_{\text{max}}$  values estimated by nonlinear regression analysis of these mean 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 brain regions of rats with 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  $\pm$  SEM of five to seven animals per group.  $*P < 0.05$ ,  $**P < 0.01$  versus sham. PSL, partial sciatic nerve ligation; 5IA, 5-iodo-3-(2(S)-azetidylmethoxy)pyridine; Frn, frontal cortex; Str, striatum; Hip, hippocampus; Th, thalamus; PPTg, pedunclopontine tegmental nucleus; NRM, nucleus raphe magnus; LC, locus coeruleus; Cer, cerebellum.

presented in Figure 3A. As with the *ex vivo* autoradiography, both PSL groups showed a significant increase in  $B_{\text{max}}$  value that occurred in the thalamus only (Figure 3B). The percentage increase in  $B_{\text{max}}$  values in the thalamus (150%) was similar to those observed in the *ex vivo* autoradiographic findings.

#### Anti-allodynic effect of 5IA given intra-VPL

Based on previous reports (Kupers and Gybels, 1993; Derbyshire *et al.*, 1997; Gybels, 2001), we predicted that the VPL was involved in the expression of anti-nociceptive effects and investigated the association between nACh receptors expressed in the VPL and the anti-allodynic effect of 5IA.



**Figure 4** (A) Effect of 5IA injected intra-VPL on neuropathic tactile allodynia. Data are presented as a percentage of the maximum possible effect (% MPE). Each point represents the mean  $\pm$  SEM of the ligated paws of five to six animals per group. \* $P < 0.05$ , \*\* $P < 0.01$  versus vehicle, and # $P < 0.05$  versus 1 nmol of 5IA at the same time point. (B) Effect of mecamlamine (MEC, 10 nmol in 0.5  $\mu$ L) administered concurrently with 5IA in an intra-VPL manner on the anti-allodynic effect produced by 5IA (10 nmol in 0.5  $\mu$ L, intra-VPL). Data are presented as a percentage of the maximum possible effect. Each point represents the mean  $\pm$  SEM of the ligated paws of five to six animals per group. \* $P < 0.05$ , \*\* $P < 0.01$  versus vehicle, # $P < 0.05$ , ## $P < 0.01$  versus MEC alone, and † $P < 0.05$ , †† $P < 0.01$  versus 5IA alone at the same time point. (C) Effect of mecamlamine (MEC, 10 nmol in 0.5  $\mu$ L, intra-VPL, 5 min prior) on the anti-allodynic effect produced by 5IA (10 nmol in 5  $\mu$ L, i.c.v.). Data are presented as a percentage of the maximum possible effect. Each point represents the mean  $\pm$  SEM of the ligated paws of four to seven animals per group. \* $P < 0.05$ , \*\* $P < 0.01$  versus vehicle/vehicle, # $P < 0.05$ , ## $P < 0.01$  versus MEC/vehicle, and † $P < 0.05$  versus vehicle/5IA at the same time point. 5IA, 5-iodo-3-(2(S)-azetidylmethoxy)pyridine; MPE, maximal possible effect; VPL, ventral posterolateral thalamic nucleus.

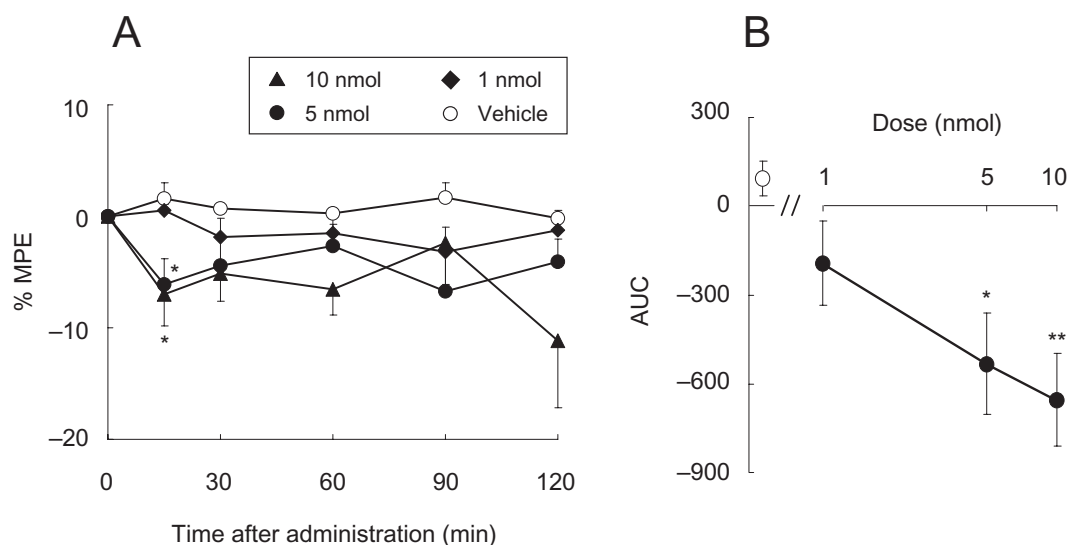
Figure 4A shows the anti-allodynic effects that occurred in the ligated paws of rats that received 5IA intra-VPL. Two-way ANOVA demonstrated significant main effects of treatment ( $F_{4,115} = 3.97$ ,  $P = 0.014$ ) and time ( $F_{4,115} = 13.6$ ,  $P < 0.001$ ) and a significant interaction between 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. 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 mecamlamine (10 nmol each) intra-VPL and performed the von Frey filament test. Two-way ANOVA demonstrated signifi-

cant 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 ( $F_{12,85} = 4.05$ ,  $P < 0.001$ ). Mecamlamine antagonized the 5IA-induced anti-allodynic effect completely, and furthermore, the % MPE values of mecamlamine-treated rats tended to decrease compared with those of the vehicle-treated rats, although the difference was not significant (Figure 4B).

#### *Involvement of nACh receptors in the VPL in the anti-allodynic effect induced by i.c.v. 5IA administration*

To explore to what extent nACh receptors in the VPL were involved in the anti-allodynic effects arising from central



**Figure 5** (A) Effect of mecamlamine injected intra-VPL on neuropathic tactile allodynia. Data are presented as a percentage of the maximum possible effect (% MPE). Each point represents the mean  $\pm$  SEM of the ligated paws of five to six animals per group. \* $P < 0.05$  versus vehicle at the same time point. (B) Dose-response curve of mecamlamine-induced hyperalgesia. The open symbol represents the AUC of the vehicle-treated group, and the closed symbols represent that of each concentration of the mecamlamine-treated group. \* $P < 0.05$ , \*\* $P < 0.01$  versus vehicle. AUC, area under the curves; MPE, maximal possible effect; VPL, ventral posterolateral thalamic nucleus.

nACh receptor activation, we evaluated the effect of intra-VPL administered mecamlamine on the anti-allodynic 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. This result was consistent with the anti-allodynic effect produced by i.c.v. administered 5IA (Figure 1). Pretreatment with intra-VPL mecamlamine (10 nmol) significantly inhibited the effect of i.c.v. administered 5IA (10 nmol). The % MPE values for rats treated with mecamlamine alone (MEC/Vehicle group) also tended to decrease compared with those of the vehicle treated rats, although the difference was not significant.

#### Hyperalgesic effect of intra-VPL administered mecamlamine

To examine the effects of mecamlamine on tactile allodynia in more detail, we injected multiple doses of mecamlamine 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 demonstrated the dose-dependent hyperalgesic effects of mecamlamine (Figure 5B).

#### Effects of 5IA and mecamlamine on motor function

We conducted an inclined plane test to evaluate the effects of 5IA and mecamlamine on general motor functions. The

**Table 1** Effects of 5IA on motor functions

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

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

5IA, 5-iodo-3-(2(S)-azetidylmethoxy)pyridine; VPL, ventral posterolateral thalamic nucleus.

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 mecamlamine).

## 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 anti-nociceptive effects in the region where changes occurred. First, we evaluated the anti-allodynic effect of 5IA after i.c.v. administration and found that the anti-allodynic effect was dose-dependent. Because the effect was completely antagonized by mecamlamine, the anti-allodynic 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 exhibited anti-allodynic effects without affecting motor functions.

We found that bilateral up-regulation of thalamic nACh receptors occurred in the PSL model of neuropathic pain. This is the first report of up-regulation of thalamic nACh receptors under chronic painful conditions. Because the up-regulation of both muscarinic and cannabinoid CB<sub>1</sub> receptors have been reported to contribute to the increased analgesic efficacy of each agonist (Siegling *et al.*, 2001; Chen and Pan, 2003), up-regulated nACh receptors may also contribute to the potentiation of anti-allodynic 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 up-regulation must occur bilaterally. In the present study, we performed an autoradiographic saturation assay to determine the regional B<sub>max</sub> of nACh receptors. As no report concerning autoradiographic saturation assays using [<sup>125</sup>I]5IA has been published, it is important to validate the method used. Doura *et al.* (2008) performed quantitative autoradiography using [<sup>125</sup>I]5IA, but, in their study, they incubated brain slices with only a single concentration of [<sup>125</sup>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.* (2008), suggesting that our method was valid.

A previous PET study showed that the increase in cerebral blood flow in the VPL positively correlated with pain intensity, suggesting 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 (Kupers and Gybels, 1993; Gybels, 2001). These findings suggest that the VPL is involved in the expression of anti-nociceptive 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. As 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 anti-allodynic 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 anti-allodynic effect of i.c.v.-administered 5IA. Moreover, the intra-VPL injection of mecamylamine alone 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 anti-allodynic mechanism by which ACh activates nACh receptors expressed in the VPL may be present and antagonism of these receptors by mecamylamine causes the hyperalgesic effect. That is to say, the nACh receptors expressed in the VPL may participate in anti-allodynic effects produced not only by exogenous but also endogenous agonists.

The present findings do not negate the involvement of nACh receptors in anti-allodynic 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. The antinociception produced by nicotinic stimulation of the PPTg or the NRM depended upon muscarinic cholinergic, 5-hydroxytryptaminergic and adrenergic systems at the level of the lumbar spinal cord (Iwamoto and Marion, 1993). Curzon *et al.* (1998) 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. Indeed, we demonstrated that VPL blockade by mecamylamine before i.c.v. administration of 5IA decreased the anti-allodynic effect by up to approximately 70%, not 100%. Thus, the remainder of the anti-allodynic effect is possibly caused by the binding of 5IA to the NRM and/or the PPTg.

Recently, Mogg *et al.* (2004) have reported that 5IA can functionally activate the  $\alpha 4\beta 2$ -nACh and  $\alpha 6\beta 2$ -nACh receptors. However, Perry *et al.* have reported that the nACh receptors expressed in the thalamus are mainly of the  $\alpha 4\beta 2$  sub-type (Perry *et al.*, 2002) and that the  $\alpha 6$  subunit is present in less than 4% of the thalamic nACh receptors (Perry *et al.*, 2007). Therefore, the anti-allodynic effect we reported was probably mediated via the  $\alpha 4\beta 2$ -nACh receptors, at least when 5IA was administered into the VPL. Whereas many results have shown that  $\alpha 4$  subunit-containing nACh receptors play an important role in nACh receptor-mediated antinociception (Bitner *et al.*, 1998; Marubio *et al.*, 1999; Bitner *et al.*, 2000), the contribution of the  $\alpha 6\beta 2$ -nACh receptors to the anti-nociceptive effect is unknown. As A-186253 has been developed as a specific antagonist of the  $\alpha 4\beta 2$ -nACh receptors (Itier *et al.*, 2004), it may be possible to investigate  $\alpha 6\beta 2$ -nACh receptor-mediated antinociception using both 5IA and A-186253.

In summary, we have demonstrated for the first time that up-regulation of thalamic nACh receptors occurs in a model of chronic pain. Moreover, we found that intra-VPL administration of 5IA attenuated tactile allodynia, dose-dependently. This effect was completely antagonized by co-administered mecamylamine. The blockade of nACh receptors in the VPL by mecamylamine caused a decrease in the anti-allodynic effect of i.c.v.-administered 5IA. These findings indicate that the nACh receptors expressed in the VPL are a potential site of the anti-nociceptive action produced by 5IA. Furthermore, mecamylamine, given intra-VPL, induced a hyperalgesic effect. This effect is likely to be responsible for the mecamylamine antagonism of the intrinsic anti-allodynic mechanism induced by endogenous acetylcholine. These findings suggest that the nACh receptors expressed in the VPL play an important role in the anti-allodynic effects produced by exogenous and endogenous agonists.

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## Conflicts of interest

The authors state that they have no conflicts of interest.

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