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Title Page

In vivo relationship between thalamic nicotinic acetylcholine receptor occupancy rates and antiallodynic effects in a rat model of neuropathic pain: Persistent agonist binding inhibits the expression of antiallodynic effects

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Running title:

NACHRs occupancy and antiallodynic effect

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Abstract

We have recently clarified that nicotinic acetylcholine receptors (nAChRs) expressed in the thalamus play an important role in antiallodynic effects produced by the nAChR agonist, 5-iodo-3-(2(*S*)-azetidynylmethoxy)pyridine (5IA). The present study aimed to reveal the *in vivo* relationship between thalamic nAChR occupancy rates and antiallodynic effects using 5IA and [¹²⁵I]5IA.

We partially ligated the sciatic nerve of a rat to induce neuropathic pain. Antiallodynic effects were evaluated at 15, 30, 60, and 90 min after intracerebroventricular (i.c.v.) administration of multiple doses (1–100 nmol) of 5IA by the von Frey filament test. Receptor occupancy rates were measured by autoradiography at 15 and 90 min after administration. Antiallodynic effects of repetitive treatment of 5IA (5 and 50 nmol) were also examined.

A significant and dose-dependent antiallodynic effect was observed 15 min after administration. It showed a good correlation with receptor occupancy rates ($r = 0.97$), indicating the binding of 5IA to nAChRs expressed in the thalamus involved in the antiallodynic effect. Five, 50, and 100 nmol of 5IA occupied the thalamic nAChRs until 90 min after administration, while the antiallodynic effect diminished. Five nanomoles of 5IA (which occupied 40% of thalamic nAChRs) showed a significant antiallodynic effect (percentage of the maximal

possible effect (%MPE): 35 ± 7) after the second administration, while 50 nmol of 5IA (which occupied 80% of thalamic nAChRs) did not (%MPE: 7 ± 1). These findings suggest that not clearance of 5IA but desensitization of nAChRs caused by persistent binding of 5IA is responsible for the disappearance of antiallodynic effects.

Text

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are a family of ligand-gated ion channels that regulate neurotransmission in the central and peripheral nervous systems. The central nAChRs are pentameric complexes consisting of various α ($\alpha 2$ – $\alpha 10$) and β ($\beta 2$ – $\beta 4$) subunits, with the heteromeric $\alpha 4\beta 2$ isoform being the most abundant nAChR subtype in the mammalian brain (Dwoskin et al., 2009). Indeed, it has been suggested that at least 90% of neuronal nAChRs in the brain are $\alpha 4\beta 2$ nAChRs (Flores et al., 1992; Lindstrom et al., 1995). The homomeric $\alpha 7$ isoform is the second most abundant nAChR subtype in the brain. These receptors are implicated not only in various brain functions, such as cognition, memory and learning, but also in many diseases, e.g., Alzheimer's disease (Court et al., 2000; Paterson and Nordberg, 2000). Thus, clarifying how nAChRs are associated with physiological or pathological function has been of great interest.

5- $[^{123/125}\text{I}]$ iodo-3-(2(*S*)-azetidinylmethoxy)pyridine ($[^{123/125}\text{I}]5\text{IA}$) is one of the tools that can make such researches possible. This compound is a radiolabeled form of 5IA, which is a derivative of A-85380, iodinated at the 5-position of the pyridine ring. $[^{123/125}\text{I}]5\text{IA}$ is a nAChR imaging probe with high selectivity and specificity for $\alpha 4\beta 2$ nAChRs not only in rodents but also in

humans (Mamede et al., 2004; Ogawa et al., 2009), and it has a relatively good safety profile (Brasic et al., 2009; Ueda et al., 2004). In fact, some methodologies have been developed for the noninvasive imaging and quantification of nAChR density *in vivo* (Fujita et al., 2003; Mamede et al., 2004; Staley et al., 2005). By using these techniques, changes in the nAChR density of living humans have been found, e.g., decrease in Alzheimer's disease and Parkinson's disease (Mitsis et al., 2009b; Oishi et al., 2007); age-related decline (Mitsis et al., 2009a); and upregulation and recovery after smoking cessation (Mamede et al., 2007; Mukhin et al., 2008).

Moreover, 5IA acts as a nAChR agonist, and a neuroprotective effect and an analgesic effect of 5IA have been reported (Ueda et al., 2008; Ueda et al., 2010). The biodistribution and metabolism of [$^{123/125}$ I]5IA are both similar to those of 5IA because [$^{123/125}$ I]5IA is a radiolabeled analogue of 5IA. Accordingly, pharmacokinetics, receptor occupancy, and binding potential (an index of nAChR density) of 5IA can be easily measured using [$^{123/125}$ I]5IA. Studies using 5IA have the advantage of pharmacodynamic effects being directly compared to the pharmacokinetic profile of the compound.

Recently, we demonstrated an upregulation of thalamic nAChRs in a model of chronic pain. Moreover, we found that intrathalamic administration of 5IA attenuated tactile allodynia in a dose-dependent manner and that the

blockade of thalamic nAChRs reduced the antiallodynic effect of 5IA administered by an intracerebroventricular (i.c.v.) route (Ueda et al., 2010). These findings indicate that thalamic nAChRs are potentially where the antinociceptive action of 5IA is produced. Therefore, we focused on nAChRs in the thalamus and aimed to reveal the *in vivo* relationship between thalamic nAChR occupancy rates and antiallodynic effects in a rat model of neuropathic pain. Since it has been reported that A-85380 acts on both peripheral and central nAChRs, and shows antiallodynic effects after i.p. administration (Rueter et al., 2003), we adopted i.c.v. administration instead of systemic administration in order to evaluate the antiallodynic effect associated only with thalamic nAChRs. The similarity between 5IA and [$^{123/125}$ I]5IA has a great advantage that enables us to evaluate the relationship between pharmacological effects and receptor occupancy directly and with great precision. In the present study, we measured the occupancy rates of thalamic nAChRs by 5IA and compared the results with antiallodynic effects.

MATERIALS AND METHODS

Animals

Animal experiments were conducted in accordance with our institutional

guidelines. 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 animals were kept at a constant ambient temperature under a 12-hr light/dark cycle with free access to food and water.

Reagents

Iodine-125 radionuclide was purchased from PerkinElmer, Inc (Waltham, MA). (–)-Cytisine was purchased from Sigma-Aldrich, Co (St. Louis, MO). 5IA and [125 I]5IA were synthesized according to a previously published method (Saji et al., 2002). The specific radioactivity of [125 I]5IA was determined from the UV absorbance at 254 nm as more than 55 GBq/ μ mol (the limit with detection of this method). Drugs were administered to animals as physiological saline solution. All other chemicals used were of reagent grade.

Surgical operation

Neuropathic pain was established by a partial sciatic nerve ligation (PSL) according to a previously published method (Seltzer et al., 1990; Ueda et al., 2010). Under sodium pentobarbital anesthesia (50 mg/kg, intraperitoneally

[i.p.]), 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.

After recovery from PSL surgery, the rats were implanted with a stainless steel guide cannula (outside diameter [o.d.] 0.7 mm, inside diameter [i.d.] 0.38 mm) under sodium pentobarbital (50 mg/kg, i.p.) anesthesia for i.c.v. 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). The stereotaxic coordinates were guided by referring to the Atlas of Paxinos and Watson (Paxinos and Watson, 2005). The guide cannula was held firmly in place by dental acrylic cement. After surgery, the rats were individually returned to their cages and left to recover for 5 days or more until the following experiments.

von Frey filament test

Just before and 2 weeks after PSL, tactile sensitivity was measured using a calibrated von Frey filament ranging from 0.04–26 g (North Coast Medical, Morgan Hill, CA) as described previously (Ueda et al., 2010). Briefly, the

animals were individually placed on an elevated wire mesh floor for testing. After a habituation period of 15–30 min, a 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 a slight bend. One trial involved 10 applications of filaments every 1 to 2 sec. The threshold was determined to be the filament of the lowest stiffness, at which the rat responded (quick paw flick) in one or more of the trials. Rats that showed a lower threshold than preoperatively were considered to be demonstrating allodynia, and they were used in the following studies.

Effect of 5IA on tactile allodynia

For single 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. Various concentrations of 5IA (1, 3, 5, 10, 50, and 100 nmol dissolved in 5 μ L of saline) or vehicle were then infused at 5 μ L/rat with a constant rate of 10 μ L/min by using a microsyringe pump (EP-60, Eicom Corporation, Kyoto, Japan). Each group consisted of four to six animals. The injection cannula was retained in place for an additional 1 min to prevent a backflow of the drugs. Just before and 15, 30, 60, and 90 min after administration, tactile allodynia of the ligated paws 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 equation:

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

For repetitive administration, the injection cannula was inserted, and an initial injection (5 or 50 nmol of 5IA dissolved in 5 μ L of saline or vehicle) was performed using the method described above. A second injection of the same dose as that initially used was given 70 min later. Both groups consisted of four animals. Just before and 15, 30, 60, 85, 100, 130, and 160 min after the initial injection, tactile allodynia of the ligated paws was evaluated using the von Frey filament test, and %MPE values were calculated.

Autoradiographic study

For calculation of receptor occupancy, allodynia-expressing rats received an i.c.v. injection containing 370 kBq of [125 I]5IA with a vehicle or various concentrations of 5IA (1, 3, 5, 10, 50, and 100 nmol) concomitantly (n = 5–6 in each group). The injected volume was 5 μ L. Fifteen or 90 min later, they were decapitated. Their brains were quickly removed, frozen in hexane (–80°C), and cut into 20 μ m-thick coronal sections with a cryostat (JUNG CM3000, Leica

Microsystems GmbH, Wetzlar, Germany). Autoradiograms were obtained, and quantitative analyses were performed according to a previously reported method (Kudo et al., 2009; Ueda et al., 2010). The data were expressed as a percentage of injected doses per gram of tissue (%ID/g) based on the data derived from ^{125}I autoradiographic microscale standards (Amersham Biosciences, Buckinghamshire, UK). Regions of interest were drawn over the thalamus, and the accumulated total radioactivity (%ID/g_T) was determined. Nonspecifically accumulated radioactivity (%ID/g_{NS}) was also determined using rats that were administered (–)-cytisine (1 mg/kg, 200 μL) intravenously for 5 min prior to i.c.v. injection of [^{125}I]5IA. The binding of [^{125}I]5IA was completely blocked by administering (–)-cytisine via this route in our previous study (Saji et al., 2002).

For the time course study of [^{125}I]5IA accumulation in the thalamus, normal rats received an i.c.v. injection containing 370 kBq/5 μL of [^{125}I]5IA (n = 5–6 in each group). Five, 15, 30, 60, and 90 min later, they were decapitated, and the following procedures were performed in a similar manner as described above.

Data Analyses

Receptor occupancy was calculated as per previous reports (Liu et al., 2009; Miller et al., 2009) with a slight modification. The previous reports

regarded binding of the probes in the reference region as nonspecific binding. However, in lacking an nAChR reference region, we used (–)-cytisine overloading to quantify the nonspecific binding of [¹²⁵I]5IA. The specific accumulation (%ID/g_S) of [¹²⁵I]5IA in the thalamus was obtained by subtracting the nonspecific accumulation from the corresponding total accumulation (%ID/g_T – %ID/g_{NS}). The specific accumulation to nonspecific accumulation ratio (SNR) was determined by dividing the specific accumulation by the nonspecific accumulation (%ID/g_S / %ID/g_{NS}). The receptor occupancy rate was then calculated according to following equation:

$$\% \text{ Occupancy} = \frac{\text{SNR}_{\text{vehicle}} - \text{SNR}_{\text{5IA-loaded}}}{\text{SNR}_{\text{vehicle}}} \times 100$$

Statistical Analyses

Analyses of the data from the von Frey filament test were performed using two-way analysis of variance (ANOVA) with repeated measures. If there was a significant difference, *post hoc* one-way ANOVA followed by the Tukey-Kramer multiple comparison test was performed using each treatment combination as an independent group. Correlation coefficients were assessed with Spearman rank correlation coefficients. Differences were considered significant at $p < 0.05$.

RESULTS

Antiallodynic effect of single administration of 5IA

Paw withdrawal thresholds decreased from 12.2 ± 1.3 g to 3.4 ± 0.4 g in ligated paws. Effects of i.c.v. administration of 5IA on paw withdrawal thresholds are shown in Fig 1. Two-way ANOVA demonstrated significant main effects of treatment ($F_{6, 118} = 4.55$, $P = 0.002$) and time ($F_{3, 118} = 24.2$, $P < 0.001$), and a significant interaction between treatment and time ($F_{18, 118} = 3.10$, $P < 0.001$). A significant and dose-dependent antiallodynic effect was observed 15 min and 30 min after administration (vehicle: 3 ± 6 , 50 nmol: 62 ± 17 , 100 nmol: 65 ± 15 at 15 min). On the other hand, the effects were disappeared 90 min after administration (vehicle: -2 ± 3 , 100 nmol: -1 ± 3) (Fig. 1).

Relationship between thalamic nAChR occupancy by 5IA and antiallodynic effect

The time course study revealed that the specific accumulation of [125 I]5IA in the thalamus increased in a time-dependent manner, and reached a plateau ($1.5 \pm 0.3\%$ ID/g) at 30 min after administration. Next, thalamic nAChR occupancy by 5IA was determined 15 min and 90 min after i.c.v. administration.

The results are shown in Table 1. Both nAChR occupancy rates and antiallodynic effect reached a plateau at 50 or 100 nmol of 5IA. Fig. 2 shows a relationship between nAChR occupancy and antiallodynic effect at 15 min after i.c.v. administration, revealing a high positive correlation between them ($r = 0.97$, $P < 0.05$). On the other hand, receptor occupancy rates did not decrease at 90 min after administration, although the antiallodynic effects were disappeared.

Antiallodynic effect of repetitive administered 5IA

We administered 5IA at 0 and 70 min, and performed the von Frey filament test. Two-way ANOVA demonstrated significant main effects of treatment ($F_{2, 63} = 14.5$, $P = 0.002$) and time ($F_{6, 63} = 7.48$, $P < 0.001$), and a significant interaction between treatment and time ($F_{12, 63} = 6.73$, $P < 0.001$). When administered 5 nmol of 5IA, which occupied about 40% of nAChRs in the thalamus, a significant antiallodynic effect was observed at 15 min (vehicle: -5 ± 5 , 5 nmol: 21 ± 2). The effect diminished completely at 60 min (vehicle: 0.1 ± 4 , 5 nmol: 1 ± 4). Fifteen minutes after the second administration at 70 min, a significant antiallodynic effect was observed again (vehicle: -1 ± 7 , 5 nmol: 35 ± 7).

When 50 nmol of 5IA was administered, which occupied approximately 80% of nAChRs in the thalamus, a significant antiallodynic effect was also

found at 15 min (50 nmol: 51 ± 5), and the effect decreased at 60 min (50 nmol: 2 ± 2). In contrast to the significant antiallodynic effects observed after both the initial and second administration of 5 nmol of 5IA, repetitive administration of 50 nmol of 5IA did not produce an antiallodynic effect at 85 min (15 min after the second administration) (50 nmol: 7 ± 1).

DISCUSSION

Numerous studies have shown the antinociceptive effects of nicotine in a variety of preclinical pain models (Iwamoto and Marion, 1993; Jain, 2004; Kiguchi et al., 2008). However, antinociception in animals has been typically found to be of limited duration and attenuated with repeated dosing (Decker et al., 2004). Epibatidine also has been found to have a relatively short duration of action, with a substantial decay in effects observed within the first 30 min after administration—although it is a very potent antinociceptive agent with broad-spectrum activity (Gilbert et al., 2001). Antinociceptive effects of other nAChR agonists (A-85380 and ABT-594) have also been found to diminish until 120 min after administration (Bannon et al., 1998; Rueter et al., 2003). Thus, the relatively short duration of action has been suggested as a common problem of nAChR agonists.

In the present study, we demonstrated a dose-dependent antiallodynic effect of 5IA 15 min after i.c.v. administration. The effect disappeared 60 min or 90 min after administration. These results are consistent with our previous report. Since the maximum antiallodynic effect was observed 15 min after administration, we measured receptor occupancy rates at the same time point and compared them with each other. In result, a high positive correlation was observed between them in the thalamus (Fig. 2). This result is consistent with our previous finding that the nAChRs expressed in the thalamus mediated the antiallodynic effect (Ueda et al., 2010).

We then attempted to explore the reason why the antiallodynic effect of 5IA disappeared at 90 min after administration. The examination of temporal changes in thalamic [125 I]5IA accumulation revealed that, rather than decreasing, [125 I]5IA accumulation increased in a time-dependent manner, reaching a plateau at 30 min. Furthermore, the receptor occupancy rates at 90 min tended to be greater than those at 15 min (Table 1). A time-dependent clearance from the thalamus of 5IA not bound to nAChRs may be responsible for the increase in receptor occupancy rates. These results demonstrated that 5IA continues binding to nAChRs in the thalamus for at least 90 min after i.c.v. administration. Thus, the disappearance of antiallodynic effects was not due to the clearance of 5IA from the thalamus.

It is well known that nAChRs can undergo desensitization, a reversible reduction in response during sustained agonist application. Buisson et al. found that recovery from epibatidine-induced desensitization is very slow using a cell line stably expressed nAChRs. The authors suggested that epibatidine may have remained tightly bound to nAChRs in a non-activatable state because of its extremely high affinity (Buisson et al., 2000). In an *in vivo* experiment, the repetitive administration of epibatidine intrathecally within 30 min actually resulted a significant reduction of apparent antinociceptive effects (Khan et al., 1998). Thus, epibatidine may induce desensitization of nAChRs by binding to them persistently *in vivo*.

For this reason, we examined the possibility of desensitization of nAChRs caused by the persistent binding of 5IA. The similarity between 5IA and [¹²⁵I]5IA is an appropriate property to use for deciding on the two doses of 5IA: one is the dose used to produce a significant antiallodynic effect with full receptor occupancy; and the other is the dose used to produce a significant antiallodynic effect with leaving unoccupied nAChRs. Based on the results shown in Fig. 2, we decided on the following two dosages: a dose of 5 nmol, which had shown half-maximal occupancy rates; and a dose of 50 nmol, which had shown the maximal occupancy rates. When administered 5 nmol of 5IA, approximately 50% of nAChRs in the thalamus were occupied 90 min after the

initial administration. Therefore, 50% of the nAChRs in the thalamus probably existed still free at the second administration. With these unoccupied nAChRs, 5IA could bind them and produce an antiallodynic effect again after the second administration. Conversely, 50 nmol of 5IA already occupied 80% of the nAChRs, with almost all the receptors kept occupying and temporarily inactive at the second administration. There were probably few available nAChRs in the thalamus; and thus, the second 5IA administration could not produce a significant effect.

Many studies have shown that $\alpha 4$ -subunit-containing nAChRs, probably $\alpha 4\beta 2$ nAChRs, play an important role in nAChR-mediated antinociception (Bitner et al., 1998; Bitner et al., 2000; Marubio et al., 1999). Since it has been reported that $\alpha 4\beta 2$ nAChRs desensitize slowly (in terms of seconds) but are very prone to desensitization (Giniatullin et al., 2005), the relatively short duration of the antiallodynic effect produced by nAChR agonists was accounted for by the desensitization of $\alpha 4\beta 2$ nAChRs. This suggests that there may be therapeutic benefits of positive allosteric modulators (PAMs) of $\alpha 4\beta 2$ nAChRs, although the therapeutic potential of $\alpha 4\beta 2$ nAChRs has been amply documented through use of selective agonists (Decker et al., 2004; Jain, 2004; Rowbotham et al., 2009). PAMs would not be expected to induce cumulative receptor desensitization and should act in a manner consistent with the timing and

localization of endogenous cholinergic neurotransmission. For such reasons, the research efforts of pharmaceutical companies are now also focused on PAMs (Changeux, 2010; Taly et al., 2009).

The involvement of $\alpha 7$ nAChRs in antinociceptive effects is controversial. Some studies have found $\alpha 7$ -nAChR ligands to be efficacious (Young et al., 2008) while others have not (Gao et al., 2010). Mukhin et al. reported that the $\alpha 7/\alpha 4\beta 2$ affinity ratio of 5IA, calculated from the K_i value, was 25,000, indicating a high selectivity of 5IA for $\alpha 4\beta 2$ nAChRs (Mukhin et al., 2000). Perry et al. have reported that nAChRs expressed in the thalamus are mainly of the $\alpha 4\beta 2$ subtype (Perry et al., 2002), and thus the antiallodynic effect observed when 5IA bound to thalamic nAChRs was probably mediated via $\alpha 4\beta 2$ nAChRs. However, 5IA might act on $\alpha 7$ and/or other nAChRs expressed in extrathalamic (central) or peripheral regions after the systemic administration of large doses in vivo. Relationships between receptor occupancy rates in these regions and antiallodynic effects remain uncertain.

In summary, we measured the thalamic nAChR occupancy rates by 5IA in the PSL model of neuropathic pain, and then compared them to antiallodynic effects. A significant and dose-dependent antiallodynic effect was observed 15 min after i.c.v. administration of 5IA. It was highly correlated with receptor occupancy rates, consistent with our previous finding that nAChRs expressed in

the thalamus mediate the antiallodynic effect. On the other hand, the antiallodynic effect was diminished in a short time, although thalamic nAChRs were kept occupying by 5IA 90 min after administration. A low dose of 5IA (which showed low receptor occupancy rates) exhibited a significant antiallodynic effect after the second administration, while a high dose of 5IA (which occupied almost all the thalamic nAChRs) showed no effect. These findings suggest that not clearance of 5IA but desensitization of nAChRs caused by persistent binding of 5IA is responsible for the disappearance of the antiallodynic effect.

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Table 1. Thalamic nAChR occupancy by 5IA after i.c.v. administration

dose (nmol)	%RO at 15 min	%RO at 90 min
1	14 ± 7.1	n.d.
3	22 ± 8.8	n.d.
5	41 ± 21	55 ± 1.4
10	63 ± 10	n.d.
50	79 ± 7.0	94 ± 1.3
100	81 ± 6.9	99 ± 0.5

Data represent the mean ± SEM for five to six animals per group.

%RO: % receptor occupancy; n.d.: not determined.

Figure 1

Effects of multiple doses of 5IA on neuropathic tactile allodynia. 5IA was administered i.c.v. at time 0. Data are presented as a percentage of maximum possible effect (% MPE). Each point represents the mean $\pm$ SEM of ligated paws of four to six animals per group. * $P < 0.05$, ** $P < 0.01$ vs. vehicle, # $P < 0.05$ vs. 1 nmol of 5IA, † $p < 0.05$ vs. 3 nmol of 5IA at same time point.

Figure 2

Correlation between antiallodynic effect and receptor occupancy at 15 min after i.c.v. administration of 5IA. The ordinate indicates the antiallodynic effect of multiple doses of 5IA and the abscissa indicates thalamic nAChR occupancy by the same doses of 5IA. The number in parentheses is the amount of 5IA administered. Each point represents the mean for four to six animals per group. The correlation coefficient (r) is 0.97, indicating a significant high correlation ($P < 0.05$). MPE, maximal possible effect.

Figure 3

Antiallodynic effect of 5IA on neuropathic tactile allodynia after repetitive i.c.v. administration. The arrow indicates the time point of second administration (70 min after first administration). Data are presented as a percentage of maximum possible effect (% MPE). Each point represents the mean $\pm$ SEM of the ligated paws of four animals per group. * $P < 0.05$, ** $P < 0.01$ vs. vehicle, # $P < 0.05$, ## $P < 0.01$ vs. 50 nmol of 5IA at the same time point.