THE ANTINOCICEPTIVE EFFECTS OF NICOTINIC PARTIAL AGONISTS VARENICLINE AND SAZETIDINE-A IN MURINE ACUTE AND TONIC PAIN MODELS

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ABBREVIATIONS: nAChR(s), nicotinic acetylcholine receptor(s); s.c., subcutaneous injection; subunits; DHβE, dihydro-beta-erythroidine; i.p., intraperitoneal injection; WT, wildtype; KO, knockout; MEC, mecamylamine; MLA, methyllycaconitine; CNS, central nervous system.

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Abstract

Nicotinic agonists display a wide range profile of antinociceptive activity in acute, tonic, and chronic pain models. However, their effectiveness is limited by their unacceptable side effects. We investigated the antinociceptive effects of two new α4β2* nicotinic partial agonists, varenicline and sazetidine-A in acute thermal and tonic pain mouse models. Both drugs failed to induce significant effects in the tail-flick and hot-plate tests after s.c. administration. However, they blocked nicotine’s effects in these tests at very low doses. In contrast to acute pain tests, varenicline and sazetidine-A dose-dependently induced an analgesic effect in the mouse formalin test after systemic administration. Their antinociceptive effects were however mediated by different nAChRs subtypes. Sazetidine-A effects were mediated by β2* nAChRs subtypes while varenicline actions were attributed to α3β4 nAChRs. Moreover, low inactive doses of varenicline blocked nicotine’s actions in the phase II of the formalin test. Overall, our results suggest that the antagonistic actions of varenicline at low doses are mediated by β2*-nAChRs, and at higher doses, as an agonist by α3β4*-nAChRs. In contrast, both actions of sazetidine-A are mediated by β2*-nAChRs subtypes. These results suggest that nicotinic partial agonists possess analgesic effects in a rodent tonic pain model and may provide a potential treatment for the treatment of chronic pain disorders.
Introduction

The current therapies for chronic pain have limited efficacy and are associated with dose-limiting side effects (Rau et al., 1993; Perkins et al., 1994). Compounds that act at nAChRs in the CNS and periphery have been reported to show antinociceptive activity in several rodent acute and chronic pain models (Decker et al., 2001). nAChRs are ligand-gated ion channels composed of α and β subunits that assemble to form hetero or homo-pentomers (Corringer et al., 2000) which are widely distributed in the peripheral and central nervous system. These nAChRs are expressed in the CNS, including many areas contributing to pain such as the midbrain (Mattila et al., 1968), the medulla (Iwamoto and Marion, 1993), the nucleus raphe magnus (Iwamato, 1991), the thalamus, the pedunculopontine tegmental nucleus (Iwamoto, 1989) and the spinal cord (Aceto et al., 1986; Christensen and Smith, 1990; Khan et al., 1997; Damaj et al., 1998). The most common CNS subtype α4β2* (asterisk indicates assembly with other nAChR subunits) is found in thalamus, dorsal raphe nucleus and spinal cord (Cucchiaro et al., 2005). While α3β4* is the major subtype expressed in peripheral ganglia (Mao et al., 2006), it is also expressed in the medial habenula, cerebellum and spinal cord (Turner and Kellar, 2005). Over the last several years, α4β2* nicotinic full agonists were reported to display a wide range profile of antinociceptive activity in acute (such as tail-flick and hot-plate tests), tonic or persistent (such as the formalin test) and chronic pain models (Bitner et al., 1998; Damaj et al., 1998; and Marubio et al., 1999; Flores et al., 1999; Damaj et al., 2007). Recently, we reported that the α5 nicotinic subunit, which co-assembles with α4β2* and α3β4* nAChRs in the CNS (Mao et al., 2007), mediate nicotine-induced antinociception in both the hot-plate and tail-flick tests (Jackson et al., 2010). While nicotinic full agonists were reported to be effective in these acute pain tests, partial agonists like cytisine (Damaj et al., 1998) and varenicline (Carroll et al., 2008) were not. In fact, varenicline blocked nicotine’s effects in these pain models (Carroll et al., 2008). In contrast, varenicline and sazetidine-A were both reported to be possess antinociceptive activity in tonic pain models such as the formalin test in rodents (Cucchiaro et al., 2008; Gao et al., 2010). However, despite the in vitro binding data indicating that
varenicline and sazetidine-A have higher affinity than nicotine to α4β2* nAChRs (more than 10-fold), they possessed lower potencies and efficacies than nicotine in the formalin test (Damaj et al., 1999). It is unclear whether these in vivo discrepancies are the result of differences in receptor efficacy and/or desensitization at various nAChR subtypes.

We therefore investigated the nAChR receptor mechanisms of the antinociceptive effects of these two α4β2* nicotinic partial agonists in acute and persistent pain models. Varenicline, an approved antismoking drug (Chantix ®), is a potent partial agonist for the α4β2* nAChRs with 40-60% of the agonist efficacy of nicotine (Rollema et al., 2007). In addition, it is a full agonist at α3β4 nAChRs and the homopentameric α7 receptors (Luetje et al., 2006; Mihalak et al., 2006; Rollema et al., 2007, 2009). Sazetidine-A is a newly developed and highly selective α4β2* partial agonist with very low affinities for all other nAChR subtypes (Kellar et al., 2006, 2008). Interestingly, it is a full agonist on the high affinity α4(2)β2(3) nAChRs, whereas it had a very low efficacy on the low affinity α4(3)β2(2) nAChRs in expressed oocytes (Sher et al., 2008).

The present study was designed to characterize the antinociceptive effect of varenicline and sazetidine-A in the acute thermal pain tests (Hot-plate and tail-flick tests) as well in the formalin test after acute administration in mice. The formalin test is commonly used as a model tonic pain (Tjolsen et al., 1992; Abbott et al., 1995; Watson et al., 1997) and subcutaneous formalin injection into one hind paw in the conscious mouse produces biphasic nociceptive behaviors characterized by a brief initial phase (first phase) and a prolonged later phase (second phase), each consisting of elevation, licking, flinching and even biting of the injected hind paw. Traditionally, the first phase of formalin test has been viewed as being due to an acute activation of nociceptors in the periphery while the second phase is due to the ensuing inflammatory response or to a central sensitization. We first studied their activity and potency in the above mentioned pain tests and examined the role of the main nAChRs subtypes, β2*-containing receptors, α3β4* and α7 subtypes, in mediating their antinociceptive responses.
Materials and Methods

Animals

Male ICR mice obtained from Harlan Laboratories (Indianapolis, IN) and male C57BL/6 mice from Jackson Laboratories (Bar Harbor, ME) were used throughout the study. Mice null for the α5 (Jackson Laboratories) and β2 (Institut Pasteur, Paris, France) subunits and their wild-type littermates were bred in an animal care facility at Virginia Commonwealth University. For all experiments, mice were backcrossed at least 8 to 10 generations. Mutant and wild types were obtained from crossing heterozygote mice. This breeding scheme controlled for any irregularities that might occur with crossing solely mutant animals. Mice were housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-approved animal care facility. They were housed in groups of six and had free access to food and water. The rooms were on a 12-h light/dark cycle (lights on at 7:00 a.m.). Mice were 8–10 weeks of age and weighed approximately 20–25 g at the start of all the experiments. All experiments were performed during the light cycle (between 7:00 a.m. and 7:00 p.m.) and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out in accordance with the National Institute of Health guide for the Care and Use of Laboratory animals.

Drugs

(−)-Nicotine hydrogen tartrate salt, mecamylamine hydrochloride was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Methyllycaconitine citrate (MLA), dihydro-β-erythroidine (DHβE) were purchased from RBI (Natick, MA). All drugs were dissolved in physiological saline (0.9% sodium chloride) and injected subcutaneously at a total volume of 1ml/100 g body weight unless noted otherwise. α-conotoxin AuIB was synthesized as described previously (Luo et al., 1998) and was given intrathecally. Varenicline (7,8,9,10-tetrahydro-6,10-methano-6H-pyrazino (2,3-h)(3) benzazepine) and sazetidine-A (6-[5-[(2S)-2-Azetidinylmethoxy]-3-pyridinyl]-5-hexyn-1-ol) were supplied by the National Institute of Drug
Abuse (NIDA Drug Supply Program, Bethesda, MD). All doses are expressed as the free base of the drug.

Intrathecal injections

Intrathecal (i.t.) injections were performed free-hand between the L5 and L6 lumbar space in unanesthetized male mice according to the method of Hylden and Wilcox (1980). The injection was performed using a 30-gauge needle attached to a glass microsyringe. The injection volume in all cases was 5 μl. The accurate placement of the needle was evidenced by a quick “flick” of the mouse's tail. Thus, the accurate placement of all injections could be assured by watching the tail motion of the mouse.

Antinociceptive tests

Tail-Flick Test.

The antinociceptive effect of drugs was assessed by the tail-flick method of D’Amour and Smith (1941), as modified by Dewey et al. (1970). A control response (2–4 s latency) was determined for each mouse before treatment, and test latency was determined after drug administration. To minimize tissue damage, a maximum latency of 10 s was imposed. Antinociceptive response was calculated as percent maximum possible effect (%MPE), where %MPE = [(test value-control value)/(cut-off (10 s) – control value)] × 100. Groups of 6 to 8 animals were used for each dose and for each treatment. The mice were tested 5, 15, and 30 min after s.c. injections of nicotinic partial agonists for the dose-response evaluation. Antagonism studies were carried out by pretreating the mice with either saline or nicotinic antagonists 15 min before nicotinic agonists. The animals were tested 5 min after administration of the agonist.

Hot-Plate Test.

Mice were placed into a 10-cm wide glass cylinder on a hot-plate (Thermojust Apparatus, Columbus, OH) as a measure of antinociception. The hot plate is a rectangular heated surface surrounded by plexiglass and maintained at 55°C. The device is connected to a manually operated timer that records the amount of time the mouse spends on the heated surface before showing signs of nociception (e.g. jumping, paw licks). Two control latencies at least 10
min apart were determined for each mouse. The normal latency (reaction time) was 8 to 12 s was assessed with a saline injection. To avoid tissue damage, the hot-plate will automatically disengage after 40 seconds. Groups of eight to twelve mice were used for each dose and treatment condition. Antinociceptive response was calculated as percentage of maximum possible effect (% MPE), where \[ \% \text{MPE} = \left( \frac{\text{test value} - \text{control}}{\text{cut-off time (40 s)} - \text{control}} \times 100 \right) \]. The reaction time was scored when the animal jumped or licked its paws. Eight mice per dose were injected s.c. with the partial agonists and tested at various times thereafter to establish a time course. The mice were tested 5, 15 and 30 min subsequent to s.c. injection of nicotinic partial agonists for the dose–response evaluation. The antagonism studies were carried out by pretreating the mice with either saline or various antagonists 15 min before the injection of the nicotine. All animals were tested five minutes after the final injection of the nicotine.

**Formalin Test.**

The Formalin test was carried out in an open Plexiglas cage, with a mirror placed at a 45° degree angle behind the cage to allow an unobstructed view of the paws. Mice were allowed to acclimate for 15 min in the test cage prior to injection. Either nicotinic analogs or control solution were injected s.c. at varying time points before the formalin injection. Each animal was injected with 20 μl of 2.5% Formalin in the intraplantar region of the right hindpaw. Each mouse was then immediately placed in a Plexiglas box. Up to two mice at one time were observed from 0 to 5 min (phase 1) and 20 to 45 min (phase 2) post-Formalin injection. The period between the two phases of nociceptive responding is generally considered to be a phase of weak activity. The amount of time spent licking the injected paw was recorded with a digital stopwatch.

**Motor coordination**

In order to measure motor coordination, we used Rotarod (IITC Inc. Life Science). The animals are placed on textured drums (1¼ inch diameter) to avoid slipping. When an animal drops onto the individual sensing platforms, test results are recorded. Five mice tested at a rate of 4 rpm. Naive mice were trained until they could remain on the rotarod for 5 min. Animals that
failed to meet this criterion within three trials were discarded. Fifteen minutes after the injection of vehicle or drugs, mice were placed on the rotarod for 3 min. If a mouse fell from the rotarod during this time period, it was scored as motor impaired. Percent impairment was calculated as follows: \( \% \text{ impairment} = \left[ \frac{180 - \text{test time}}{180} \times 100 \right] \). Mice were pretreated with saline, varenicline (0.5, 1, and 3 mg/kg) and sazetidine-A (0.1, 0.5, and 1.5 mg/kg) s.c. 15 min before the test.

### 2.6. Statistical analysis

Data was expressed as mean ± SEM of licking time. Statistical analysis was done by ANOVA followed by post hoc, Tukey test. *P<0.05 is considered to be statistically significant. ED50 (effective dose 50%) and AD50 (antagonist dose 50%) values with 95% confidence limits (± CLs) for behavioral data were calculated by unweighted least-squares linear regression as described by Tallarida and Murray (1987).

### Results

#### Effects of varenicline and sazetidine-A in the tail-flick and hot-plate tests.

Various doses of varenicline and sazetidine-A were tested in the tail-flick and hot-plate tests after s.c. injection the drugs. As expected, nicotine-induced significant antinociceptive effects in both tests at a dose of 2.5 mg/kg when tested 5 min after injection (Figure 1A). In contrast, neither varenicline nor sazetidine-A showed significant antinociceptive effects at any of the doses tested. Figure 1A shows the lack of antinociceptive activity 15 min after administration of the highest dose of varenicline (3 mg/kg) and sazetidine-A (2 mg/kg). A similar lack of effect was observed at lower doses of the drug and at different pretreatment times (Data not shown).

Varenicline and sazetidine-A were then evaluated for their ability to antagonize a 2.5 mg/kg dose of nicotine in the tail-flick and hot-plate procedures. As showed in Fig. 1B, both varenicline and sazetidine-A dose-dependently blocked nicotine-induced antinociception with
an AD\textsubscript{50} of 0.0002 (0.0001-0.0005) and 0.00085 (0.00065-0.0011) mg/kg, respectively when given s.c. 15 min before nicotine in the tail-flick test [F (4,25)= 9.707, p< 0.05; F(4,25)= 7.609, p< 0.05 respectively]. In contrast, only sazetidine-A blocked nicotine-induced antinociception in the hot-plate test with an AD\textsubscript{50} of 0.0055 (0.003-0.009) mg/kg [F (4,25)= 7.831, p<0.05] (Figure 1C). Varenicline at the highest dose of 0.1 mg/kg, failed to significantly reduce nicotine-induced antinociception in the hot-plate test. There were no observed significant effects of sazetidine-A and varenicline in the hot-plate and tail-flick tests at any of the doses used on their own (data not shown).

**Effects of varenicline and sazetidine-A in the formalin test**

The effect of systemic (s.c.) varenicline and sazetidine-A treatment on both phase 1 (0-5 min) and phase 2 (20-45 min) of the formalin test was investigated 15 min after injection of the drugs. In both phases (Figure 2A), varenicline dose-dependently attenuated nocifensive responding as indicated by an overall significant effect of treatment [early phase: F (3,20) =13.53, p < 0.05; late phase: F(3,20) = 16.51, p < 0.05]. However, varenicline was less potent than sazetidine-A in both phases as determined by ED\textsubscript{50} values of 0.70 (0.49-1.0) and 1.9 (1.5-2.4) mg/kg for phase 1, and 0.3 (0.24-0.38) and 1.1 (0.78-1.5) mg/kg for phase 2, respectively. Both drugs were more potent in reducing the nociceptive behavior in phase II compared to phase I. (Figure 2A and B).

**Role of various nAChRs subtypes in varenicline-induced antinociception in the formalin test**

Using antagonists for various nAChR subtypes, we examined the role of β2*, α3β4* and α7 nAChRs subtypes in mediating the antinociceptive effect of varenicline (3 mg/kg s.c.). As predicted, mecamylamine (2 mg/kg, s.c.), a non-competitive and non-selective nicotinic antagonist, blocked varenicline’s effects in both phase I and II (Fig. 3A). In contrast, DHβE (2 mg/kg, s.c.), a β2-containing selective antagonist, failed to block varenicline’s actions in the formalin test. A similar lack of antagonism was also seen with MLA (10 mg/kg, s.c.), a relatively selective α7 antagonist (Fig. 3A). Surprisingly, i.t pretreatment with CnTx AulB (700
pmol/mouse), an α3β4 nicotinic antagonist, completely blocked varenicline’s effects in both phase I and II (Fig. 3A and 3B). The dose of α-conotoxin AuIB used in this assessment did not significantly alter nociceptive behavior in the formalin test on its own (Fig. 3B). Similarly, mecamylamine, MLA and DHβE failed to have significant effects in the formalin test on their own (Data not shown). Furthermore, the blockade of varenicline-induced antinociception by i.t. α-conotoxin AuIB was dose-dependent, with an AD₅₀ of 167 (108-260) pmol/mouse (Figure 3B) in phase II. The lower dose of α-conotoxin AuIB (70 pmol/mouse, i.t.) did not significantly block the antinociceptive effect of varenicline (3 mg/kg s.c.) in both phases; however increasing the dose to (140 pmol/mouse) blocked the second phase only, while the higher dose of 700 pmol/mouse totally reverses varenicline-induced antinociception in both phases of the formalin test (Fig. 3B).

That the β2 subunit is not required for varenicline’s action was confirmed using the β2KO mice. As shown in Fig. 3C, varenicline-induced antinociception in both phase I and II was preserved. No difference in nociceptive behaviors baseline was observed between the β2 KO and WT mice in vehicle-treated group (Fig. 3C).

Interestingly, low inactive doses of veranicline (0.04 and 0.2 mg/kg) blocked nicotine’s effects in the formalin test (Fig. 4A) in the second phase only [F(2,22)=32.405, p<0.0001]. This effect of low doses of varenicline in phase II seems to be mediated by β2 containing nAChRs subtypes since nicotine’s effects in this phase was totally eliminated in β2 KO mice (Fig. 5).

**Role of various nAChRs subtypes in sazetidine-A -induced antinociception in the formalin test**

Similar to varenicline, we examined the role of β2*, α3β4* and α7 nAChRs subtypes in mediating the antinociceptive effect of sazetidine-A (1 mg/kg s.c.). As predicted, mecamylamine (2 mg/kg, s.c.) completely blocked sazetidine-A’s effects in both phase I and II (Fig. 6A). Similarly, DHβE (2 mg/kg, s.c.), also blocked sazetidine-A’s actions in the formalin test. In contrast, MLA (10 mg/kg, s.c.) and α-conotoxin AuIB (700 pmol/mouse, i.t.), failed to significantly block sazetidine-A’s effects in both phases (Fig. 6A).
The blockade of sazetidine-A’s effects in the formalin test being mediated through a $\beta_2^*$ nAChR was confirmed using the $\beta_2$ KO mice. As shown in Fig. 6B, sazetidine-A -induced antinociception in both phase I and II was lost in the $\beta_2$ KO mice compared to their WT littermates.

As observed with varenicline, a low inactive dose of sazetidine-A (0.2 mg/kg) blocked nicotine’s effects in the formalin test (Fig. 4B) in the second phase only.

**Effects of varenicline and sazetidine-A on the mouse locomotor coordination**

Various doses of varenicline and sazetidine-A were tested in the rotarod test after s.c. injection. As expected, nicotine-induced significant impairment of the animals’ motor coordination in a dose-related manner when tested 5 min after injection (Figure 7). In contrast, varenicline and sazetidine-A failed to significantly alter the animals’ motor coordination 15 min after s.c. injection of various doses of the drugs. Doses of varenicline (0.5, 1 and 3 mg/kg) and sazetidine-A (0.1, 0.5 and 1.5 mg/kg) that were found active in the formalin test, did not significantly induce locomotor incoordination as compared to vehicle group (Figure 5).

**Discussion**

In the present study, we examined the antinociceptive effects of two nicotinic partial agonists with differing activity profiles, varenicline and sazetidine-A, in acute and tonic mouse pain models. Contrary to nicotine, both of the nicotinic partial agonists tested lacked significant antinociceptive effect in the acute thermal models (the tail-flick and hot-plate tests) at the doses and times tested. In contrast, both drugs were active in the formalin test, a persistent and tonic pain model. These results suggest that efficacy of nicotinic agonists at nicotinic receptors, in particular $\beta_2^*$ nAChRs, plays an important role for their effects in acute thermal pain models and that both varenicline and sazetidine-A do not activate $\beta_2^*$-nAChRs sufficiently to produce measurable effects in the tail-flick and hot-plate tests. Interestingly however, both drugs blocked nicotine-induced antinociception in the tail-flick test at very low doses. Indeed, results shown in Figure 1B demonstrate that varenicline inhibits the effects of a 2.5 mg/kg dose of nicotine with
an ED$_{50}$ value of 0.0002 mg/kg (0.94 nmol/kg). Sazetidine-A was 2.5-times less potent than varenicline in blocking nicotine’s effects in the tail-flick test with an ED$_{50}$ value of 0.00085 mg/kg (2.56 nmol/kg). These two partial agonists were at least 50-100 fold more potent than mecamylamine in blocking nicotine’s effects in the tail-flick test (Damaj et al., 1995). However, only sazetidine-A blocked nicotine’s effects in the hot-plate test with a 11-fold lower potency than in the tail-flick test (Figure 1C). These results support the idea that varenicline and sazetidine-A block nicotine’s effects by either competing with nicotine and/or desensitizing the a4b2* nAChRs for several reasons. Nicotine-induced antinociception in the tail-flick and hot-plate tests are largely mediated by a4b2* nAChRs in spinal and supraspinal sites (see Introduction). In addition, the potency of varenicline and sazetidine-A in blocking nicotine’s effects is in line with their binding affinity to a4b2* nAChRs (Ki values for sazetidine-A and varenicline are 0.4 and 0.2 nM, respectively) (Xiao et al., 2006; Rollema et al., 2007). Finally, in vitro experiments that have demonstrated that exposure of a4b2* nAChRs to low concentrations of varenicline effectively blocks subsequent activation by ACh in the low nanomolar range, similar to binding Ki values (Mihalak et al., 2006; Rollema et al., 2010; Papke et al., 2011). Our results with nicotine-induced antinociception were similar to those recently reported with varenicline blocking the effects of nicotine-induced hypothermia and locomotor depression through a b2* nAChRs mechanism (Ortiz et al., 2012).

The fact that sazetidine-A but not varenicline, blocked nicotine’s effects in the hot-plate test was surprising. It is possible that differences in a4b2* nAChR subtypes distributions or forms mediating nicotine’s effects in the two pain tests could explain sazetidine-A’s effects. Recently, it has been recognized that a4b2 nAChR subtypes are present in two main forms; the high affinity (a4)2(b2)3 or the low affinity (a4)3(b2)2. It is conceivable that nicotine responses in the tail-flick engages both low and high affinity a4b2* nAChR subtypes, while the effects in the hot-plate test is mainly mediated by the high affinity a4b2* nAChR form. Sazetidine-A might then mediate its functional antagonistic effects in the hot-plate test by acting on (a4)2(b2)3 high affinity subtypes, since it was reported to be a full agonist on these high affinity
subtypes, whereas it had a very low efficacy on the low affinity \( \alpha 4(3)\beta 2(2) \) nAChRs (Sher et al., 2008).

Acute administration of relatively high doses of varenicline and sazetidine-A elicited antinociception actions in both phase I and II of the formalin test without impairing the motor coordination of the animals (Figure 7). The potency of both drugs in the mouse formalin test was higher than the one reported in the rat formalin model (Cucchiaro et al., 2008; Gao et al., 2010), suggesting species differences possibly in drug pharmacokinetics and/or dynamics. While both partial agonists were active in the formalin test, their antinociceptive effects were mediated by different nAChRs subtypes as shown by the use of various nicotinic antagonists and nicotinic KO mice. Sazetidine-A elicits its nociceptive effects in the formalin test by activation of \( \beta 2^*-nAChRs \) in (Figure 6) as shown in the \( \beta 2 \) KO mice and after pretreatment with DH\( \beta E \), a selective \( \beta 2^*-nAChRs \) antagonist. Neither \( \alpha 7 \) nor \( \alpha 3\beta 4^* \) nAChR subtypes were shown to mediate the effects of sazetidine-A in the formalin test. In contrast, \( \beta 2^*-nAChRs \) subtypes do not mediate the effects of varenicline in the same test. Varenicline’s antinociceptive effects were not significantly reduced in the \( \beta 2 \) KO mice or after pretreatment with DH\( \beta E \) (Figure 3). A similar lack of involvement of \( \alpha 7 \) nAChR subtypes was also observed since MLA, a relatively selective \( \alpha 7 \) antagonist, failed to reduce varenicline’s effects in the formalin test. However, \( \alpha 3\beta 4^* \) nAChR subtypes in the spinal cord appear to play a predominant role in varenicline effects. Indeed, pretreatment with intrathecal \( \alpha \)-conotoxin AuIB, a selective \( \alpha 3\beta 4 \) antagonist (Luo et al., 1998), dose-dependently blocked varenicline-induced antinociception (Figure 3). This is in line with the recent study of Ortiz et al. (2012) which reported that agonist effects of varenicline in inducing hypothermia and hypomotility in the mouse are mediated by \( \alpha 3\beta 4^* \) nAChR subtypes. These results are consistent with \textit{in vitro} assays showing that varenicline is a full agonist at \( \alpha 3\beta 4^* \) nAChR subtypes (Mihalak et al., 2006; Rollema et al., 2010). While varenicline is also a full agonist at the \( \alpha 7 \) nAChRs (Mihalak et al., 2006) \textit{in vitro} functional assays, this receptor subtype does not play an important role in varenicline’s \textit{in vivo} effects since no loss of effectiveness of the drug was seen after pretreatment with the \( \alpha 7 \) antagonist MLA.
Interestingly, lower doses of varenicline (0.04 and 0.2 mg/kg) blocked the effects of nicotine in the phase II only of the formalin test (Figure 4), likely indicating an antagonist effect at β2*-nAChRs. This is consistent with the fact that nicotine’s effects in phase II but not phase I of the formalin were eliminated in the β2 KO mice (Figure 4). Overall, the actions of varenicline at low doses, as an antagonist of β2*-nAChRs, and at higher doses, as an agonist of α3β4*-nAChRs as seen in the formalin test, appear consistent with varenicline’s effects on body temperature, locomotion (Ortiz et al., 2012) and responding for food in mice (Cunningham and McMahon, 2011). In contrast, the agonist effects of sazetidine-A in the formalin test appears to be mediated largely by β2*-nAChRs subtypes. As observed with varenicline, low doses of sazetidine-A nicotine’s effects in the phase II of the formalin test. Taken together, the data of acute and persistent pain models suggest that both the actions of sazetidine-A at low doses, as an antagonist and at higher doses, as an agonist are mediated by β2*-nAChRs subtypes.

In more complex tests for cognition and attention in rats, low doses of sazetidine-A (as low as 0.01 mg/kg) reversed dizocilpine-induced impairments in performance (Rezvani et al., 2011) suggesting that it is the functional “antagonist” effect of sazetidine-A that may mediate changes in these behaviors. However, in mouse tests for anxiety and depression, relatively high doses of sazetidine-A similar in range to our formalin test results (0.5-1.5 mg/kg) (Turner et al., 2010) were showed to be active in these models supporting the a role for “agonist” effects of the drug in these behaviors.

In summary, these studies demonstrated the efficacy of nicotinic partial agonists sazetidine-A and varenicline in tonic but not acute pain models. The efficacy and potency of sazetidine-A in the formalin test suggest that its “agonist” effects at α4β2* nAChR subtypes may provide a potential treatment for the treatment of chronic pain disorders.

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References


FOOTNOTES

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**FIGURE LEGENDS**

**Figure 1. Effects of varenicline and sazetidine-A in the tail-flick and hot-plate tests.**

(A) Effects of varenicline (3 mg/kg, s.c.), sazetidine-A (2 mg/kg, s.c.) and nicotine (2.5 mg/kg, s.c.) in the tail-flick and hot-plate tests in mice. Mice were tested 5 and 15 min after nicotine and varenicline/ sazetidine-A, respectively. Each group represents the mean ± SE of 8-12 mice and *denotes p<0.05 vs. vehicle. The ability of varenicline and sazetidine-A to antagonize a 2.5 mg/kg dose of nicotine in the tail-flick (B) and hot-plate (C) tests was also determined. The two drugs were given s.c. 15 min before nicotine and mice were tested 5 min later. Each group represents the mean ± SE of 8-12 mice and *denotes p<0.05 vs. nicotine. (C) Var = varenicline; Saz = sazetidine-A; Nic = nicotine; Veh = vehicle.

**Figure 2. Effects of varenicline and sazetidine-A in the mouse formalin test.**

The effect of various doses of (A) varenicline (1, 2 and 3 mg/kg) and (B) sazetidine-A (0.1, 0.5, 1 and 1.5 mg/kg) after s.c. administration on formalin-induced pain behavior in the mouse. Mice were treated with s.c. varenicline and sazetidine-A 15 min prior to formalin (2.5%, 20 μl) injection into the plantar region of the right hind paw. The cumulative pain response of time of licking was measured during the period of 0-5 min (first phase) and 20-40 min (second phase). Data was expressed as mean ± SEM of licking time. Each group represents the mean ± SE of 8-12 mice. Var = varenicline; Saz = sazetidine-A; Nic = nicotine; Veh = vehicle.

**Figure 3. Nicotinic receptors subtypes involved in varenicline-induced antinociception in the formalin test.**

(A) Blockade of the antinociceptive effect of varenicline in the formalin test by different nicotinic antagonists. Mice were pretreated with various nicotinic antagonists [Mecamylamine (2 mg/kg, s.c.); DHβE (2 mg/kg, s.c.); MLA (10 mg/kg, s.c.); α-conotoxin AuIB (700 pmol/mouse, intrathecally) 15 min (5 min for α-conotoxin AuIB) before an active dose of 3.
mg/kg of varenicline. Fifteen min later, mice were injected with formalin (2.5% intraplantary, 20 μl) and then observed for pain behaviors. (B) Effects of different doses of the α3β4* antagonist α-conotoxin AuIB on varenicline-induced antinociception in the formalin test. Mice were injected intrathecally with different doses of α-conotoxin AuIB (70, 140 and 700 pmol/mouse) and 5 min later they received a dose of 3 mg/kg of varenicline. Fifteen min later, mice were injected with formalin (2.5% intraplantary, 20 μl) and then observed for pain behaviors. (C) Antinociceptive effects of varenicline in the β2 WT and KO mice. Mice received a dose of 3 mg/kg of varenicline and 15 min later were tested in the formalin test. Data was expressed as mean ± SEM of licking time. Each group represents the mean ± SE of 8-12 mice and *denotes p<0.05 vs. vehicle. Meca = mecamylamine; Var = varenicline; Veh = vehicle; α-ctx AuIB = α-conotoxin AuIB.

**Figure 4. Blockade of nicotine-induced antinociception by varenicline and sazetidine-A in the formalin test.**

Effects of (A) varenicline and (B) sazetidine-A on the antinociceptive effect of nicotine in the formalin test. Mice were pretreated with varenicline (0.04 and 0.2 mg/kg, s.c.) or sazetidine-A (0.2 mg/kg, s.c.) 15 min before an active dose of nicotine (1.5 mg/kg, s.c.). Five min later, mice were injected with formalin (2.5% intraplantary, 20 μl) and then observed for pain behaviors. Data was expressed as mean ± SEM of licking time. Each group represents the mean ± SE of 8-12 mice and *denotes p<0.05 vs. vehicle. Var = varenicline; Saz = sazetidine-A; Veh = vehicle; Nic = nicotine.

**Figure 5. Effects of nicotine in β2 KO mice using the formalin Test.**

Antinociceptive effects of nicotine in the β2 WT and KO mice. Mice received a dose of 1.5 mg/kg of s.c. nicotine and 5 min later were tested in the formalin test. Data was expressed as mean ± SEM of licking time. Each group represents the mean ± SE of 8-12 mice and *denotes p<0.05 vs. respective vehicle. Nicotine = nicotine; Veh = vehicle.
Figure 6. Nicotinic receptors subtypes involved in sazetidine-A-induced antinociception in the formalin test.

(A) Blockade of the antinociceptive effect of sazetidine-A in the formalin test by different Effects of nicotinic antagonists. Mice were pretreated with various nicotinic antagonists (Mecamylamine (2 mg/kg, s.c.); DHβE (2 mg/kg, s.c.); MLA (10 mg/kg, s.c.); α-conotoxin AuIB (700 pmol/mouse, intrathecally) 15 min (5 min for α-conotoxin AuIB) before a dose of 1.5 mg/kg of sazetidine-A. Fifteen min later, mice were injected with formalin (2.5% intraplantary, 20 μl) and then observed for pain behaviors. (B) Antinociceptive effects of sazetidine-A in the β2 WT and KO mice. Mice received an active dose of sazetidine-A (1.5 mg/kg, s.c.) and 15 min later they were tested in the formalin test. Data was expressed as mean ± SEM of licking time. Each group represents the mean ± SE of 8-12 mice and *denotes p<0.05 vs. respective vehicle. Meca = mecamylamine; Saz = sazetidine-A; Veh = vehicle; α-ctx AuIB = α-conotoxin AuIB.

Figure 7. Effects of nicotinic partial agonists on motor coordination

Dose-response curves of nicotine, varenicline and sazetidine-A in the rotarod test after s.c. administration in mice. Mice were tested 5 and 15 min after nicotine and varenicline/sazetidine-A injections respectively for 5 min on the rotarod. Each point represents the mean ± SE of 8 to 12 mice.
Figure 5

The graph shows the Paw Licking duration (sec) for different conditions across Phase I and Phase II.

- **Veh** (open bars) and **Nic (1.5)** (hatched bars) are the conditions compared.
- **β2 WT** and **β2 KO** are the genotypes.

In Phase I:
- **β2 WT** has a lower Paw Licking duration compared to **β2 KO**.
- **Nic (1.5)** significantly reduces the Paw Licking duration compared to **Veh**.

In Phase II:
- **β2 WT** and **β2 KO** show a similar trend as in Phase I, with significant differences between **Veh** and **Nic (1.5)**.

**p<0.001** indicates statistical significance.