The Antinociceptive Effects of Nicotinic Partial Agonists Varenicline and Sazetidine-A in Murine Acute and Tonic Pain Models

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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 subcutaneous 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 mediated, however, by different nicotinic acetylcholine receptor (nAChR) subtypes. Sazetidine-A effects were mediated by β2* nAChR subtypes, whereas varenicline actions were attributed to α3β4 nAChRs. Moreover, low inactive doses of varenicline blocked nicotine’s actions in 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*-nAChR 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 heteropentomers or homopentomers (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), medulla (Iwamoto and Marion, 1999), nucleus raphe magnus (Iwamoto, 1991), thalamus, pedunculopontine tegmental nucleus (Iwamoto, 1989), and 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 the thalamus, dorsal raphe nucleus, and spinal cord (Gillberg et al., 1988; Cucchiaro et al., 2005). Although 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 models (such as tail-flick and hot-plate tests), tonic or persistent models (such as the formalin test), and chronic pain models (Bitner et al., 1998; Damaj et al., 1998, 2007; Flores et al., 1999; Marubio et al., 1999). We reported previously that the α5 nicotinic subunit, which coassembles with α4β2* and α3β4* nAChRs in the CNS (Mao et al., 2007), mediates nicotine-induced anti-
nociception in both the hot-plate and tail-flick tests (Jackson et al., 2010). Although nicotinic full agonists were reported to be effective in these acute pain tests, partial agonists such as 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 both were reported to be possess antinociceptive activity in tonic pain models such as the formalin test in rodents (Cucchiari 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 (Cucchiari et al., 2008; Gao et al., 2010). 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, Pfizer, New York, NY), is a potent partial agonist for the α4β2* nAChRs with 40 to 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 (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 (Xiao et al., 2006). It is a full agonist on the high-affinity α2(2)β2(3) nAChRs, whereas it had a very low efficacy on the low-affinity α4(3)β3β2(2) nAChRs in expressed oocytes (Zwart et al., 2008).

The present study was designed to characterize the antinociceptive effects of varenicline and sazetidine-A in acute thermal pain tests (hot plate and tail-flick tests) and the formalin test after acute administration in mice. The formalin test is commonly used as a model for tonic pain (Tjølsen 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 caused by an acute activation of nociceptors in the periphery, whereas the second phase is caused by the ensuing inflammatory response or a central sensitization. We first studied the activity and potency of varenicline and sazetidine-A in the above-mentioned pain tests and examined the role of the main nAChR subtypes, β2*-containing receptors and α3β4* and α7 subtypes, in mediating their antinociceptive responses.

Materials and Methods

Animals

Male ICR mice obtained from Harlan (Indianapolis, IN) and male C57BL/6 mice from The Jackson Laboratory (Bar Harbor, ME) were used throughout the study. Mice null for the α5 subunit (The Jackson Laboratory) and β2 subunit (Institut Pasteur, Paris, France) 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-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 AM). Mice were 8 to 10 weeks of age and weighed approximately 20 to 25 g at the start of the experiments. All experiments were performed during the light cycle (between 7:00 AM and 7:00 PM), 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 (Institute of Laboratory Animal Resources, 1996).

Drugs

(-)-Nicotine hydrochloride salt and mecamylamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Methyllycaconitine (MLA) citrate and dihydro-β-erythroidine (DHβE) were purchased from Sigma (St. Louis, MO). All drugs were dissolved in physiological saline (0.9% sodium chloride) and injected subcutaneously at a total volume of 1 ml/100 g of 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-6H,6,10-methano-6-epine] and sazetidine-A [6-[2-(2-isopropyl-5-methylhexyl)-3-pyridinyl-5-hexyn-1-ol] were supplied by the National Institute of Drug Abuse Drug Supply Program (Bethesda, MD). All doses are expressed as the free base of the drug.

Intrathecal Injections

Intrathecal injections were performed free-hand between the L5 and L6 lumbar spaces in unanesthetized male mice according to the method of Hylden and Wilcox (1980). The injection was performed by 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- to 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 percentage of maximum possible effect (%MPE), where %MPE = [(test value - control value)/cutoff (10 s) - control value] × 100. Groups of six to eight animals were used for each dose and each treatment. The mice were tested 5, 15, and 30 min after subcutaneous injections of nicotinic partial agonists for the dose-response evaluation. Antagonism studies were carried out by pretreating the mice with either saline or nicotinic agonists 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 was a rectangular heated surface surrounded by Plexiglas and maintained at 55°C. The device was connected to a manually operated timer that recorded the amount of time the mouse spent 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) of 8 to 12 s was assessed with a saline injection. To avoid tissue damage,
the hot plate automatically disengaged after 40 s. Groups of 8 to 12 mice were used for each dose and treatment condition. Antinociceptive response was calculated as %MPE, where %MPE = [(test value – control)/(cut-off time (40 s) – control) × 100]. The reaction time was scored when the animal jumped or licked its paws. Eight mice per dose were injected subcutaneously 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 subcutaneous 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 5 min 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° angle behind the cage to allow an unobstructed view of the paws. Mice were allowed to acclimate for 15 min in the test cage before injection. Either nicotinic analogs or control solution were injected subcutaneously 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) postformalin 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**

To measure motor coordination, we used a rotarod (ITC Inc. Life Science, Woodland Hills, CA). The animals were placed on textured drums (1¼-inch diameter) to avoid slipping. When an animal Science, Woodland Hills, CA). The animals were placed on textured

Motor Coordination

To measure motor coordination, we used a rotarod (ITC Inc. Life Science, Woodland Hills, CA). The animals were placed on textured drums (1¼-inch diameter) to avoid slipping. When an animal dropped onto the individual sensing platforms, test results were 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. Percentage of impairment was calculated as: % impairment = [(180 – test time)/(180 × 100)]. 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) subcutaneously 15 min before the test.

**Statistical Analysis**

Data were expressed as mean ± S.E.M. of licking time. Statistical analysis was done by analysis of variance followed by post hoc Tukey test. p < 0.05 was considered to be statistically significant. ED50 (effective dose 50%) and AD50 (antagonist dose 50%) values with 95% confidence limits 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 subcutaneous 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 (Fig. 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 doses 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 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 shown in Fig. 1B, both varenicline and sazetidine-A dose-dependently blocked nicotine-induced antinociception with an AD50 of 0.0002 (0.0001–0.0005) and 0.00085 (0.00065–0.0011) mg/kg, respectively when given subcutaneously 15 min before nicotine in the tail-flick test ($F_{4,25} = 9.707, p < 0.05$; $F_{4,25}$
dine-A in both phases as determined by ED_{50} values of 0.70 (early phase, ing as indicated by an overall significant effect of treatment 15 min after injection of the drugs. In both phases (Fig. 2A), the antinociceptive effect of varenicline (3 mg/kg s.c.). As predicted, 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 antinocicep-
tion by intrathecal α-conotoxin AuIB was dose-dependent, with an AD_{50} of 167 (108–260) pmol/mouse (Fig. 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 only the second phase, whereas the higher dose of 700 pmol/mouse totally reversed 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 by using the β2 KO mice. As shown in Fig. 3C, varenicline-induced antinociception in both phase I and II was preserved. No difference in the baselines of nociceptive behaviors was observed between the β2 KO and WT mice in the vehicle-treated group (Fig. 3C).

It is noteworthy that low inactive doses of veranicline (0.04 and 0.2 mg/kg) blocked nicotine’s effects in the formalin test (Fig. 4A) only in the second phase (F_{2,20} = 32.405; p < 0.0001). This effect of low doses of varenicline in phase II seems to be mediated by β2-containing nAChR subtypes, because nicotine’s effects in this phase were totally eliminated in β2 KO mice (Fig. 5).

Role of Various nAChR Subtypes in Sazetidine-A-Induced Antinociception in the Formalin Test. Similar to varenicline, we examined the role of β2*, α3β4*, and α7 nAChR subtypes in mediating the antinociceptive effect of sazetidine-A (3 mg/kg s.c.). As predicted, mecamylamine (2 mg/kg s.c.), a noncompetitive and nonselective nicotinic antagonist, blocked sazetidine-A’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). We were surprised to find that intrathecal pretreatment with conotoxin AulB (70 pmol/mouse), an α3β4 nicotinic antagonist, completely blocked varenicline’s effects in both phase I and II (Fig. 3, A and B). 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). Likewise, 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 antinocicep-
tion by intrathecal α-conotoxin AuIB was dose-dependent, with an AD_{50} of 167 (108–260) pmol/mouse (Fig. 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 only the second phase, whereas the higher dose of 700 pmol/mouse totally reversed varenicline-induced antinociception in both phases of the formalin test (Fig. 3B).

Effects of Varenicline and Sazetidine-A on Mouse Locomotor Coordination. Various doses of varenicline and sazetidine-A were tested in the rotarod test after subcutaneous injection. As expected, nicotine induced significant impairment of the animals’ motor coordination in a dose-related manner when tested 5 min after injection (Fig. 7). In contrast, varenicline and sazetidine-A failed to significantly alter the animals’ motor coordination 15 min after subcutaneous 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 compared with the vehicle group (Fig. 5).

**Discussion**

In the present study, we examined the antinociceptive effects of two nicotinic partial agonists with differing ac-
nicotine to 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- to 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 (Fig. 1C). These results support the idea that varenicline and sazetidine-A block nicotine’s effects by either competing with nicotine and/or desensitizing the $\alpha 4\beta 2^*$ nAChRs for several reasons. Nicotine-induced antinociception in the tail-flick and hot-plate tests were largely mediated by $\alpha 4\beta 2^*$ nAChRs in spinal and supraspinal sites (see Introduction). In addition, the potency of varenicline and sazetidine-A in blocking nicotine’s effects was in line with their binding affinity to $\alpha 4\beta 2^*$ nAChRs to low concentrations of varenicline effectively blocks subsequent activation by acetylcholine in the low nanomolar range, similar to binding $K_i$ values for sazetidine-A and varenicline were 0.4 and 0.2 nM, respectively) (Xiao et al., 2006; Rollema et al., 2007). Finally, in vitro experiments have demonstrated that exposure of $\alpha 4\beta 2^*$ nAChRs to low concentrations of varenicline effectively blocks subsequent activation by acetylcholine in the low nanomolar range, similar to binding $K_i$ 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 $\beta 2^*$ nAChR 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 $\alpha 4\beta 2^*$ nAChR subtype distributions or forms mediating nicotine’s effects in the two pain tests could explain sazetidine-A’s effects. It has been recognized that $\alpha 4\beta 2^*$ nAChR subtypes are present in two main forms: the high-affinity $\alpha 4(2/\beta 2/3)$ or the low-affinity $\alpha 4(3/\beta 2/2)$. It is conceivable that nicotine responses in the tail-flick engage both low- and high-affinity $\alpha 4\beta 2^*$ nAChR subtypes, whereas the effects in the hot-plate test were me-

![Fig. 5](Image)

**Fig. 5.** Effects of nicotine in $\beta 2$ KO mice using the formalin test. Antinociceptive effects of nicotine in the $\beta 2$ WT and KO mice are shown. Mice received a dose of 1.5 mg/kg s.c. nicotine and 5 min later were tested in the formalin test. Data are expressed as mean ± S.E.M. of licking time. Each group represents the mean ± S.E. of 8 to 12 mice.

![Fig. 6](Image)

**Fig. 6.** Nicotinic receptor 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 is shown. Mice were pretreated with various nicotinic antagonists (MEC, 2 mg/kg s.c.; DHβE, 2 mg/kg s.c.; MLA, 10 mg/kg, s.c.; $\alpha$-conotoxin AuIB, 700 pmol/mouse i.t.) 15 min (5 min for $\alpha$-conotoxin AuIB) before a dose of 1.5 mg/kg sazetidine-A. Fifteen minutes later, mice were injected with formalin (2.5% intraplantary; 20 $\mu$L) and then observed for pain behaviors. B, antinociceptive effects of sazetidine-A in the $\beta 2$ WT and KO mice are shown. 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 are expressed as mean ± S.E.M. of licking time. Each group represents the mean ± S.E. of 8 to 12 mice. *p < 0.05 versus vehicle.
Acute administration of relatively high doses of varenicline and sazetidine-A elicited antinociceptive actions in both phase I and II of the formalin test without impairing the motor coordination of the animals (Fig. 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 possible species differences in drug pharmacokinetics and/or dynamics. Although both partial agonists were active in the formalin test, their antinociceptive effects were mediated by different nAChR 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 acting on α4β2(3) high-affinity subtypes, because it was reported to be a full agonist on these high-affinity subtypes, whereas it had a very low efficacy on the low-affinity α4(3)β2(2) nAChRs (Zwart et al., 2008).

In summary, these studies demonstrated the efficacy of the 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 chronic pain disorders.

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Authorship Contributions
Participated in research design: AlSharari and Damaj.
Conducted experiments: AlSharari.
Contributed new reagents or analytic tools: Carroll and McIntosh.
Performed data analysis: AlSharari and Damaj.
Wrote or contributed to the writing of the manuscript: AlSharari, Carroll, McIntosh, and Damaj.

References

Fig. 7. Effects of nicotinic partial agonists on motor coordination. Dose response curves of nicotine, varenicline, and sazetidine-A in the rotarod test after subcutaneous administration in mice are shown. 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 ± S.E. of 8 to 12 mice.
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