Genetic approaches identify differential roles for α4β2* nicotinic receptors in acute models of antinociception in mice

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ABBREVIATIONS: nAChR, Acetylcholine nicotinic receptor; CNS, central nervous system; %MPE, percentage of maximum possible effect; WT, Wild-type; CL, confidence limit; s.c., subcutaneous injection; ED50, effective dose 50%; ACh, Acetylcholine; BTX, Bungarotoxin; *, denotes other undetermined nicotinic subunits.
Abstract – The effects of nicotine on the tail-flick and hot-plate tests were determined in order to identify
nicotinic receptor subtypes responsible for spinally- and supraspinally-mediated nicotine analgesia in knock-in mice
expressing hypersensitive α4 nicotinic receptors (L9'S) and in seven inbred mouse strains (C57BL/6, DBA/2, A/2,
CBA/2, BALB/cByJ, C3H/HeJ and 129/SvEv) and two F1 hybrids (B6CBAF1 and B6D2F1). L9'S heterozygotes
were ~6-fold more sensitive to the antinociceptive effects of nicotine than the WT controls in the hot-plate test, but
not in the tail-flick assay. Large differences in the effects of nicotine were also observed with both tests for the
seven mouse strains. A/J and 129 mice were 6-8 fold more sensitive than CBA and BALB mice. In addition,
B6CBAF1 hybrid mice were even less sensitive than CBA. Nicotinic binding sites were measured in three spinal
cord regions and the hindbrain of the inbred strains. Significant differences in cytisine-sensitive, high affinity [125I]-
epibatidine binding site levels (α4β2* subtypes), but not in [125I]-α-bungarotoxin binding (α7* subtypes) were
observed. Significant negative correlations between cytisine-sensitive [125I]epibatidine binding and nicotine ED50
for both tests were noted. Our results indicate that α4β2* nAChRs are important in mediating nicotine analgesia in
supraspinal responses, while also showing that α4β2* nAChRs and at least one other nAChR subtype appear to
modulate spinal actions.
Introduction

Findings that nicotine is an antinociceptive agent in animals (Aceto et al., 1986), and that cigarette smoking and nicotine reduce pain in humans (Lane et al., 1995; Jammer et al., 1998) suggest that nicotinic compounds might be useful in the treatment of pain. Furthermore, the observation that the nicotinic agonist epibatidine is 100 times more potent than morphine as an analgesic in the tail-flick and hot-plate tests (Spande et al., 1992) has spurred renewed interest in the development of nicotinic agents as analgesics (Flores and Hargreaves, 1998). Analgesic drugs currently used produce serious side effects upon long-term use, safer and more efficacious analgesic agents are needed. It is generally agreed that central pathways modulate the analgesic actions of nicotine (Sahley and Berntson, 1979); in particular, the spinal cord is a major site of action for tail-flick nociception given that intrathecal administration of nicotinic agonists produces antinociception in this test (Aceto et al., 1986; Christensen and Smith, 1990). In addition, Caggiula et al. (1995) suggested that nicotine’s effects on the tail-flick and hot-plate tests involve at least partially separate pathways. Unfortunately, only limited progress has been made towards identifying the neuronal nicotinic acetylcholine receptor (nAChR) subtypes expressed in spinal cord that modulate pain, perhaps because mRNAs for virtually all of the nAChR subtypes have been identified in dorsal root ganglion neurons of adult (Haberberger et al., 2004) and neonatal mice (Cordero-Erausquin et al., 2004). This complexity might explain why studies using pharmacological approaches to identify the nAChR subtypes that modulate the antinociceptive effects of nicotinic agonists (Damaj et al., 1999) and antagonists (Iwamoto and Marion, 1993) are not in complete agreement.

Studies using nAChR subunit null mutant (gene knock-out) mice have suggested roles for specific nAChRs subtypes in nicotine-induced antinociception. Mice lacking either the α4 or β2 nicotinic receptor subunit showed reduced sensitivity to nicotine-induced antinociception in the hot-plate and tail-flick acute pain tests (Marubio et al. 1999). Both α4 and β2 null mutant mice failed to respond to nicotine on the hot-plate test at doses that were fully efficacious in WT mice, suggesting a prominent role for α4β2-nAChR. In contrast, both α4 and β2 gene deletion resulted in only modest (2-3 fold) reductions in agonist potency with the tail-flick test.

The present study used two genetic approaches to investigate which nAChR subtypes modulate the actions of nicotine on the hot-plate and tail-flick tests. The first approach took advantage of knock-in mice that express mutated α4*- nAChRs (Labarca et al., 2001). These mice express α4 subunits with a leucine to serine substitution in the 9’ position (L9’S) of the pore-lining M2 region, which renders mice hypersensitive to nicotine in functional and behavioral tests (Labarca et al, 2001; Fonck et al., 2003). The second approach took advantage of the findings that
outbred and inbred mouse strains differ in sensitivity to the effects of nicotine (Seale et al., 1998) and epibatidine on thermal reactivity tests (Flores et al., 1999), locomotor activity (Collins et al., 1988; Marks et al., 1989), hypothermia (Tritto et al., 2001) and seizures (Miner and Collins, 1989). Mouse strains also differ in brain [3H]-nicotine (α4β2*-nAChRs) (Marks et al., 1989) and [125I]-α-bungarotoxin (α7-nAChRs) binding (Stitzel et al., 1998). Moreover, strain variability in [3H]-nicotine binding shows a strong correlation with variations in nicotine-induced locomotor activity and changes in body temperature, while variability in [125I]-α-bungarotoxin ([125I]-α-BTX) binding correlates with the severity of nicotine-induced seizures. Further nAChR subtype specificity can be achieved by measuring high affinity (K_D ≈ 20 pM) radiolabeled-epibatidine binding, which can be divided into two major components based on the sensitivity to inhibition by cytisine. The fraction sensitive to cytisine inhibition is primarily comprised of the α4β2 subtype whereas cytisine-resistant sites are thought to represent α3β2*, α3β4* and α6β2* receptors (Marks et al., 2006). The observation that strain variability in responses to nicotine is significantly correlated with differences in binding of radioactive nicotinic ligands, prompted us to study the effects of nicotine on the hot-plate and tail-flick tests of seven inbred mouse strains known to be differentially sensitive to nicotine for other measures (Marks et al. 1989) as well as for two F1 hybrids. We then measured cytisine-sensitive and cytisine-resistant [125I]-epibatidine binding and [125I]-α-BTX binding in hindbrain and cervical, thoracic and lumbar spinal cord of these mouse strains.
Materials and Methods

Animals. Generation of the L9’S strain, by replacing the native α4 subunit of nAChR with a mutated form, was previously described (Labarca et al., 2001). Only heterozygous L9’S mice with an intact neomycin resistance cassette were used because L9’S homozygous are neonatal lethal, therefore the L9’S designation used throughout the paper refers to these heterozygotes. Male mice from the following inbred strains were used: C57BL/6J, DBA/2Jbg, A/J, CBA/J, C3H/HeJ, BALB/cByJ, 129/SvEv, B6CBAF1 and B6D2F1. DBA/2Jbg mice were obtained from the breeding colony at the Institute for Behavioral Genetics (Boulder, CO). The 129/SvEv strain was obtained from Taconic (Germantown, NY). The remaining strains were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the mouse colony at the Institute for Behavioral Genetics for at least two weeks prior to testing. Mice were housed in groups of three to four per cage and had free access to food and water. All animals were 60-90 days of age at the time of testing. The study was reviewed and approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University and the Animal Care and Utilization Committee of the University of Colorado.

Drugs. Mecamylamine hydrochloride was a gift from Merck, Sharp and Dohme & Co. (West Point, PA). Morphine sulphate and (-)-nicotine were obtained from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in physiological saline (0.9% sodium chloride), and all doses are expressed as the free base of the drug.

Antinociceptive tests.

1. Tail-flick test. Mice were lightly restrained by hand while a radiant heat light source was shone onto the upper portion of the tail. Latency to remove the tail from the heat source was recorded for each animal. A control response (2-4 s latency) was determined for each mouse before treatment, and test latency was determined after drug administration. In order to minimize tissue damage, a maximum latency of 10 sec was imposed. Antinociceptive response was calculated as percent of maximum possible effect (% MPE), where %MPE = [(test value – baseline)/(cut-off time (10 s) – control value)] X 100 where baseline represents the value before nicotine. Groups of eight to twelve animals were used for each dose and for each treatment. Mice were tested 5 min after a subcutaneous (s.c.) injection of nicotine.
2. **Hot-plate Test.** Mice were tested 2 h before and 5 min after a subcutaneous injection of nicotine. The animals were placed on a 55°C platform (Harvard Apparatus, Holliston, MA) and were observed until they started to show pain avoidance behavior such as jumping or licking of the paws. Animals that did not respond to the noxious heat stimulus after 40 s were removed from the plate. Latency to pain avoidance measured in seconds was used to calculate a percent of maximal possible response (%MPE) with the following equation: \([\text{test value} - \text{baseline}] / (\text{cut-off time (40 s)} - \text{baseline}) \times 100\). Baseline latency that lasted eight to twelve seconds was assessed with a saline injection.

In order to assess specificity of the nicotine-induced analgesia in L9'S, animals were injected with mecamylamine (1 mg/kg, s.c.), 5 min before receiving a single nicotine injection in the hot-plate and the tail-flick tests. For comparison purposes, the specificity of nociceptive responses in L9'S mice was also evaluated by determining the potency of morphine in the hot-plate and the tail-flick tests. Animals were tested 20 min after a s.c. injection of morphine.

**Binding Experiments**

1. **Tissue Preparation.** Mice were killed by cervical dislocation. Their brains were removed and placed on ice and the hindbrains (pons medulla) were dissected. The spinal column was isolated and divided into thoracic, cervical and lumbar regions. Each segment of the spinal cord was removed from the spinal column by gentle flushing with ice cold, isotonic saline. The dissected tissue was subsequently placed in hypotonic buffer (14 mM NaCl, 0.2 mM KCl, 0.2 mM CaCl₂, 0.1 mM MgSO₄, 2 mM HEPES, pH=7.5). Samples were homogenized using a glass-teflon tissue grinder. Homogenized samples were centrifuged at 10 000 x g for 20 min and the supernatant was discarded. The pellet was re-suspended in hypotonic buffer and centrifuged again at 10 000 x g for 20 min. This procedure was repeated two more times. The final washed pellet was re-suspended in dilute buffer to a concentration of 1 - 2 mg protein/ml.

2. **[^125_I]-Epibatidine binding.**[^125_I]-Epibatidine (Specific Activity = 2200 Ci/mmol, NEN) binding was conducted essentially as described previously (Marks et al., 1998). Samples were incubated for 3 h in 30 µl of buffer (135 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 0.02% bovine serum albumin at 20 °C, 25 mM HEPES, pH = 7.5) using a final[^125_I]-epibatidine concentration of 400 pM. Cytisine resistant binding was initially measured by constructing inhibition curves for cytisine (cytisine concentrations from 1 x 10⁻⁹ M to 5 x 10⁻⁵ M) for two mice of each strain. Subsequently, cytisine-resistant sites were calculated from data obtained for binding in the
presence of 0 nM, 50 nM and 150 nM cytisine. Blanks were established using 0.1 mM nicotine. Blanks represented less than 5% if specific binding. Following the incubation, samples were filtered using an Inotech harvester and two layers of glass fiber filter (top filter MFS GC, bottom filter Gelman A/E both of which had been soaked in 0.1% polyethylenimine). Samples were washed six times with ice cold buffer without serum albumin. Samples were counted using a Packard Tri-Carb liquid scintillation spectrometer at 45% efficiency after the addition of 1.5 ml of Budget Solve scintillation cocktail (RBI).

3. [125I]-α-BTX Binding. [125I]-α-BTX (specific activity = 200 Ci/mmol, NEN) binding was essentially measured as described previously (Marks et al., 1986). Samples were incubated for 3 hr in 100 µl of buffer (135 mM NaCl, 2 mM KCl, 2 mM CaCl₂ 1 mM MgSO₄, 0.02% bovine serum albumin at 20 °C 25 mM HEPES, pH = 7.5) using a final [125I]-α-BTX concentration of 1 nM. Blanks were determined by including 1 mM nicotine in the incubations. Blanks represented approximately 50% of specific binding at this concentration of [125I]-α-BTX. Although maximal binding is not measured at this concentration, use of higher ligand concentrations resulted in very unfavorable signal/noise ratios. Following the incubation, samples were filtered using an Inotech harvester and two layers of glass fiber filter (top filter MFS GC, bottom filter Gelman A/E both of which had been soaked in 0.1% polyethylenimine). Samples were washed six times with ice-cold buffer without serum albumin. Samples were counted using a Packard gamma counter at 80% efficiency.

4. Protein. Protein was measured with the method of Lowry et al., (1951), using bovine serum albumin as the standard.

Statistical analysis. Statistical analysis of all behavioral studies was performed using either a t-test or analysis of variance (ANOVA) with a Dunnett’s t-test post hoc test when appropriate. All differences were considered significant at p < 0.05. ED₅₀ values with a 95% confidence level (CL) for behavioral data were calculated by unweighted least-squares linear regression as described by Tallarida and Murray (1987). One-way ANOVA followed by Dunnett’s post hoc t-test was used to evaluate differences in binding in each tissue for each binding site. Relationships among the parameters were assessed by linear regression.
Results

Antinociceptive Effects of Nicotine in the α4L9’S mice

Antinociceptive effects of nicotine on L9’S heterozygous and wild-type mice in the tail-flick and hot-plate tests are shown in Figures 1A and 1B. The latency response of non-injected control mice to a painful stimulus did not differ significantly between WT and L9’S animals in either test, indicating that the endogenous activation of the α4 nAChR subunit, even with a mutation conferring hypersensitivity, is not essential to the perception of acute thermal nociception.

The tail-flick test revealed no obvious hypersensitivity to nicotine in the L9’S mice (Figure 1A) after s.c. injection of various doses of nicotine. However, it should be noted that doses higher than 0.5 mg/kg were not tested in L9’S mice because animals displayed tremors, shaking and stereotypy when higher doses of nicotine were administered (Fonck et al, 2003). These behavioral responses in L9’S mice are thought to precede seizures, which are likely initiated in subcortical structures such as the hippocampus, thalamus or medial habenula and are not thought to be directly involved in antinociceptive responses. WT mice exhibited a dose-dependent antinociception following nicotine administration at doses greater than 0.5 mg/kg. In the hot-plate test (Figure 1C), WT mice showed a dose-dependent antinociceptive response with an ED50 (± CL) = 0.92 (0.7-1.1) mg/kg. In L9’S mice, the nicotine dose-response relation was shifted to the left with an ED50 value of 0.17 (0.1-0.2) mg/kg (Figure 1C). Thus, a 6-fold shift in the dose-response relation for nicotine-induced antinociception was observed between L9’S and WT mice.

The nicotinic receptor antagonist mecamylamine (1 mg/kg) effectively blocked nicotine-induced analgesia described in these experiments. Mecamylamine blocked the nicotine-induced decrease in hot-plate nociception (data not shown) in both L9’S and WT animals. Mecamylamine also blocked the nicotine-induced delay in tail-flick responses in WT mice.

Antinociceptive effects of morphine in the hot-plate and tail-flick tests are shown in Figure 1B and 1D. L9’S and WT mice had similar dose-dependent antinociceptive responses to morphine in the hot-plate test (Figure 1D), yielding ED50 values of 6.8 (5.8-7.9) and 5.9 (4.8-7.1) mg/kg, respectively. Similar potency for morphine was also observed for L9’S and WT mice in the tail-flick (Figure 1B) test, with ED50 values of 0.90 (0.5-1.6) and 0.85 (0.4-1.8) mg/kg, respectively.

Antinociceptive Effects of Nicotine in seven inbred mouse strains

a. Tail-flick test. No differences among the strains in basal latencies for the tail-flick test were noted following saline injection (latencies range: 1.7-2.4 s).
The effects of nicotine administration on the tail-flick response of the seven inbred strains of mice are shown in Figure 2A and 2B and the ED$_{50}$ values calculated from these curves are shown in Figure 2C. Antinociceptive effects of nicotine differed significantly among the inbred strains. The dose-response curves for nicotine-induced antinociception for mice of the A/J and 129SvEv strains were similar and yielded similar ED$_{50}$ values (ED$_{50}$ (95% CL): 0.48 (0.34-0.67) and 0.62 (0.05-0.70) mg/kg, respectively). Dose-response curves of nicotine-induced antinociception for C57BL/6 and DBA/2 were also similar, but these mice were less sensitive to nicotine than either the A or 129 mice (ED$_{50}$ (95% CL): 1.07 (0.97-1.22) for C57BL/6 and 1.20 (0.94-1.60) mg/kg for DBA). C3H (ED$_{50}$ (95% CL): 1.97 (1.4-2.6)) and BALB (ED$_{50}$ (95% CL): 3.2 (2.6-3.8)) required significantly higher nicotine doses. Antinociceptive effects of nicotine in CBA mice were also small. Even at the highest dose of nicotine tested (2 mg/kg, s.c.), the effect (16%MPE) was not significantly different from saline control. Higher doses of nicotine (> 2 mg/kg) were not tested because of the increased risk of triggering seizures. The rank order of the strains for sensitivity to the antinociceptive effects of nicotine measured by tail-flick was: 129 ≥ A/J > C57BL/6 = DBA/2 > C3H = BALB = CBA. Mice of the 129 strain were at least 4-fold more sensitive than those of the CBA strain and 6-fold more sensitive than those of the BALB strain.

Antinociceptive effects were also measured with two F1 hybrids. The B6D2F1 (F1 cross of C57BL/6 and DBA/2) were more sensitive to nicotine [ED$_{50}$ (95% CL)= 0.68 (0.2-1.1) mg/kg] than the parental strains, while B6CBAF1 (F1 cross of C57BL/6 and CBA) were significantly less sensitive than either parent (1%MPE after a dose of 3.5 mg/kg).

b. Hot-plate test. Significant strain differences in baseline responsiveness in the hot-plate test were observed (latencies range: 5.5-9.7 s) [F(6,90)=5.8, p<0.05]. These differences were not unexpected since strain differences in baseline responsiveness have previously been noted for this test in inbred mouse strains (Mogil et al., 1999).

Dose-response curves for the antinociceptive effects of nicotine measured with the hot-plate test are shown in Figure 2D and 2E and ED$_{50}$ values calculated from these curves are shown in Figure 2F. Similar to the results of the tail-flick test, the potency of nicotine as an antinociceptive agent differed significantly among the strains. In general, ED$_{50}$ values were lower for the hot-plate test than for the tail-flick test. A/J mice were the most sensitive [ED$_{50}$ (95% CL)= 0.2 (0.16-0.24) mg/kg]. 129SvEv mice were also quite sensitive [ED$_{50}$ (95% CL)= 0.45 (0.41-0.49) mg/kg]. DBA/2 and C57BL/6 mice displayed similar responsiveness to nicotine (ED$_{50}$ (95% CL): 0.85 (0.5-1.2) and (0.92 (0.73-1.2) mg/kg, respectively), while C3H mice were slightly less sensitive [ED$_{50}$ (95% CL)=...
1.05(0.8-1.3) mg/kg]. The BALB [ED50(95%CL)= 1.3(0.97-1.8) mg/kg] and CBA [ED50(95%CL)= 1.55(1.2-1.85) mg/kg] mice required higher nicotine doses for antinociception. In contrast to the tail-flick test, nicotine-mediated antinociception was measurable in CBA mice in the hot-plate test, but the ED50 value for CBA was the highest of the seven strains tested [ED50(95%CL)= 1.55(1.2-1.85) mg/kg]. Thus, the rank order for sensitivity to nicotine-induced antinociception for the hot-plate test was: A/J > 129 > DBA/2 ≈ C57BL/6 > C3H > BALB > CBA. The ED50 value for CBA mice was approximately 7.5 fold higher than that for A/J mice.

Antinociceptive effects were also measured with two F1 hybrids. As was the case with the tail-flick test, the B6D2F1 (F1 cross of C57BL/6 and DBA/2) were slightly more sensitive to nicotine [ED50(95%CL)= 0.68(0.2-1.1) mg/kg] than the parental strains, while B6CBAF1 (F1 cross of C57BL/6 and CBA) were significantly less sensitive than either parent (40%MPE after a dose of 3.5 mg/kg).

Receptor Determinations in seven inbred mouse strains

The density of three nicotinic binding sites was measured to explore possible relationships between nAChR levels and sensitivity to nicotine in the mouse strains studied. Binding was measured in hindbrain and the cervical, thoracic and lumbar regions of spinal cord. The three binding sites measured were: [125I]-α-BTX (measuring α7-nAChR), cytisine-sensitive [125I]-epibatidine (measuring β2*-nAChR, primarily α4β2*-nAChR) and cytisine-resistant [125I]-epibatidine (measuring a mixed population of β2*-nAChR (non α4β2*-nAChR) and β4*-nAChR) (Marks et al., 2006).

a. Cytisine-Sensitive [125I]-Epibatidine binding. The density of high-affinity [125I]-epibatidine binding sites sensitive to inhibition by cytisine was measured using a [125I]-epibatidine concentration of 400 pM. This concentration is saturating, thereby providing a measurement of maximal binding site density. Results for these measurements are shown in Figure 3.

Significant differences in binding site density among the seven strains were observed (hindbrain, F(6,44)=2.34, p<0.05; thoracic spinal cord, F(6,44)=2.84, p<0.05; and lumbar spinal cord, F(6,44)=2.37, p<0.05; however, cervical spinal cord was not significant, F(96,44)=2.01, P>0.05)). In every sample prepared, tissue prepared from A/J mice had the highest densities. Binding for A/J mice was significantly higher that that for CBA and C3H mice in every region. Relative binding site densities measured for tissue obtained from DBA/2 and C57BL/6 mice were similar to each other and varied among the regions. Binding for these two strains was relatively high in hindbrain, cervical and thoracic spinal cord, similar in magnitude to that of the A/J mice.
Cytisine-sensitive $[^{125}\text{I}]{\text{epibatidine}}$ measured in hindbrain and cervical spinal cord for the B6CBAF1 mice was intermediate between that of the two parental strains, while that measured in thoracic spinal cord resembled that of the CBA parent (lower than midpoint) and that in the lumbar spinal cord was lower than that for either parent. A similar pattern was also observed for the B6D2F1 mice, although binding in both thoracic and lumbar spinal cord was lower than that of either parent.

\textit{b. Cytisine-Resistant $[^{125}\text{I}]{\text{Epibatidine Binding.}}$} The density of high affinity $[^{125}\text{I}]{\text{epibatidine}}$ binding sites was calculated from binding using a saturating concentration of 400 pM $[^{125}\text{I}]{\text{epibatidine}}$ in the presence of 0 nM, 50 nM and 150 nM cytisine (Marks et al., 1998). Results for these measurements are shown in Figure 4.

The cytisine-resistant component of the high affinity $[^{125}\text{I}]{\text{epibatidine}}$ binding represented a relatively small fraction of total high affinity binding (<20% in hindbrain, <15% in the spinal cord). While no significant differences in the number of these binding sites among the strains were detected in either hindbrain, cervical spinal cord, or lumbar spinal cord, significant differences among the strains were noted for thoracic [\(F(6,44)=2.82,\ p<0.05\)] with samples prepared from A/J mice exhibited higher binding than those prepared from both DBA/2, BALB, C3H and 129 mice.

\textit{c. $[^{125}\text{I}]{\text{α-BTX binding.}}$} The density of $[^{125}\text{I}]{\text{α-BTX}}$ binding sites was measured using a ligand concentration of 1 nM, which is near the \(K_D\). Therefore, these measurements do not represent maximal site densities. Results for $[^{125}\text{I}]{\text{α-BTX}}$ binding in the four regions of all inbred strains are shown in Figure 5.

No significant differences in $[^{125}\text{I}]{\text{α-BTX}}$ binding were observed for hindbrain or any area of the spinal cord.

\textit{Relationship between nicotinic binding sites and antinociception.}$^\text{1}$

Since both the potency of nicotine’s antinociceptive effects and the density of nicotinic binding sites have been measured in each of the seven inbred strains, linear regression analyses were conducted to compare the various measures. Scattergrams comparing the \(ED_{50}\) values for nicotine-induced antinociception and density of cytisine-sensitive $[^{125}\text{I}]{\text{epibatidine}}$ binding sites, cytisine-resistant $[^{125}\text{I}]{\text{epibatidine}}$ binding sites and $[^{125}\text{I}]{\text{α-Bgt}}$ binding sites in hindbrain are shown in Figure 6. \(ED_{50}\) values for the two thermal pain tests for the seven inbred mouse strains were very highly correlated (Panel 6A, \(r = 0.92\)). Highly significant (\(P <0.01\)) correlations between \(ED_{50}\) for tail-flick (Panel 6E, \(r=-0.89\)) and hot-plate (Panel 6B, \(r=-0.85\)) and the density of cytisine-sensitive $[^{125}\text{I}]{\text{epibatidine}}$ binding sites were also observed. No significant correlations between the other two binding sites and the pain tests or among the three binding sites noted. Correlations between the density of cytisine-sensitive $[^{125}\text{I}]{\text{epibatidine}}$
binding sites in cervical, thoracic or lumbar spinal cord and the ED_{50} values for the hot-plate tests were not so robust 

\( r = -0.62, p = 0.06 \) as those for binding sites in hindbrain.
Discussion

Nicotinic agonists elicit antinociceptive responses in several acute pain tests, including tail-flick and hot-plate tests. Responses to the hot-plate are thought to be centrally mediated, whereas the tail-flick is considered a spinal reflex (Caggiula et al., 1995). This study takes advantage of the rare opportunity to study a behavioral test by exploiting both specific targeted mutations in a candidate molecule, and inbred strains that differ in many more genes. The studies reported here further tested the postulate that activation of $\alpha_4 \beta_2$ nAChRs evoke the antinociceptive effects of nicotine on the hot-plate and tail-flick tests.

Previous data based on gene knockouts (Marubio et al., 1999) and on antisense-mediated knockdown (Bitner et al., 2000) indicate that $\alpha_4$ and $\beta_2$ receptors are necessary for a major antinoceptive component as measured in the hot plate test; but the present finding that L9'S mice, which express a hypersensitive $\alpha_4$ receptor, are much more sensitive to the effects of nicotine in the hot-plate test than are the controls, show that nicotine activation of $\alpha_4$ receptors mice is sufficient to produce such antinociception. Thus, the combination of necessity and sufficiency does provide compelling indications that, among the set of nAChR receptor subtypes, $\alpha_4 \beta_2$ nAChRs dominate nicotine’s actions on the hot-plate test.

Marubio et al. (1999) observed that $\alpha_4$ and $\beta_2$ null mutant mice were 2- to 3-fold less sensitive to nicotine than WT mice on the tail-flick test and concluded that the effects of nicotine on this test are modulated by the $\alpha_4 \beta_2$ nAChR subtype plus another, lower affinity nAChR. We did not detect an increase in sensitivity to nicotine’s analgesic effects on the tail-flick test in L9’S mice. However, L9’S could not be tested with the full range of nicotine doses. The highest dose that was tested (0.5 mg/kg) produced no measurable change in response in either WT or mutant mice. The higher doses (1.0 and 2.0 mg/kg), which elicited effects in WT mice, were not used in L9’S because doses that exceed 0.5 mg/kg elicit tremors, shaking, stereotypy and convulsions in these mice (Fonck et al., 2003). Thus, the data obtained with the L9’S mutant mice support the assertion that $\alpha_4 \beta_2$ nAChRs play a more dominant role in regulating nicotine’s effects on the hot-plate test than for the tail-flick response.

Studies with both knock-out and knock-in mice provide valuable insights into the role of $\alpha_4$-nAChRs in pain modulation, but adaptive changes elicited by these mutations could affect the nociceptive phenotype. For example, the number of dopamine neurons in the substantia nigra is reduced by ~35% in adult L9’S mice (Orb et al., 2004) and $\alpha_4$ null mutant mice exhibit alterations in arborization of the dendrites of dopamine neurons (Parish et al., 2005). These concerns prompted us to evaluate the effects of nicotine in seven inbred mouse strains to evaluate the variation in nicotine-induced antinociception.
Inbred mouse strains also vary substantially in sensitivity to nicotine, as measured by a battery of behavioral tests and the seven inbred strains selected for the current study represent a wide range of sensitivity to nicotine (Marks et al., 1989). The seven strains tested also differed markedly in sensitivity to the effects of nicotine in both the hot-plate and tail-flick tests. Indeed, the 7.5-fold difference in sensitivity to nicotine’s effects on the hot-plate test between the most sensitive (A/J) and the least sensitive (CBA) inbred strains was as great as the difference between the L9’S and their WT littermates. These results are consistent with the finding that inbred mouse strains exhibit a 4-fold variation in sensitivity to epibatidine-induced antinociception for the tail-immersion withdrawal thermal assay, with the A/J strain being the most sensitive (Flores et al., 1999).

The rank orders of ED50 values for the strains tested on the hot-plate (A/J > 129 > DBA/2 ≈ C57BL/6 > C3H > BALB > CBA) and tail-flick (129≥A/J>C57BL/6=DBA/2>C3H>BALB=CBA) are very similar (correlation coefficient for ED50 values = 0.94), but not identical, inasmuch as the ED50 values for the hot-plate test tend to be lower than those for the tail-flick test. One possible explanation for these findings is that α4β2* nAChRs dominate the effects of nicotine on the hot-plate test, while an additional, lower affinity nAChR contributes to the response in the tail-flick test. Indeed, α7 selective antagonists (α-BTX and methyllycaconitine) and partial agonists (DMXB, 4-OH-DMXB) blocked the antinociceptive effects of choline (an α7 agonist) on the tail-flick test (Damaj et al., 2000). These results suggest that the α7 nAChR may be the lower affinity receptor that also modulates the tail-flick test.

Inbred strain differences in drug sensitivity could be attributed to pharmacodynamic or pharmacokinetic differences. Nicotine metabolism and elimination do not differ among several of these inbred strains (Petersen et al., 1984). Therefore, it is reasonable to assume that the pharmacogenetic variability observed in our studies is due to differences in nicotinic receptors or any of the various downstream mechanisms engaged by these receptors. In particular, although the L9’S mice have a subset of α4* receptors that bear a mutation conferring a difference in ACh sensitivity, it seems possible that overall ACh sensitivity can also be governed by straightforward differences in the number of receptors. Inbred mouse strains differ in the number of brain nAChRs, as measured by high affinity [3H]-nicotine (α4β2*) and [125I]-α-BTX (α7) binding (Marks et al., 1989). Indeed, differences in binding site density are significantly correlated with differences in effects of nicotine on maze activities and hypothermia (Marks et al., 1989). These results prompted us to examine the potential associations between nicotine-induced antinociception and brain stem/spinal cord nAChR numbers.

We measured nAChR binding in the spinal cord and the hindbrain, using [125I]-epibatidine and [125I]-α-BTX as ligands. The high affinity epibatidine binding that is sensitive to cytisine inhibition measures α4β2*...
nAChRs (Marks et al., 2006). Strain differences in cytisine-sensitive $[^{125}\text{I}]$-epibatidine binding sites were seen in hindbrain as well as cervical, thoracic and lumbar regions of spinal cord. Regression analysis indicated a highly significant inverse correlation between cytisine-sensitive $[^{125}\text{I}]$-epibatidine binding and ED$_{50}$ values for both tail-flick ($r=-0.89$) and hot-plate ($r=-0.85$) tests. This result and the fact that similar, but less robust correlations were found between binding site densities in spinal cord and ED$_{50}$ values ($r=-0.65$) indicate that mice expressing higher $\alpha_4\beta_2^*$-nAChR levels are more responsive to nicotine-mediated antinociception. These correlations between nicotinic analgesia and $\alpha_4\beta_2$-nAChR binding sites are similar to those obtained for other nicotine-mediated behavioral responses such as hypolocomotion and hypothermia (Marks et al., 1989). It is also likely that nicotine-evoked responses in these complex behavioral assays depend on downstream events not directly related to receptor levels. Such a possibility is reinforced by the results with the F1 hybrids, where the B6CBAF1 showed striking overdominance toward resistance to nicotine, while the B6D2F1 showed modest dominance toward sensitivity. Such complex inheritance patterns for nicotine-mediated behavioral responses have been observed for both nicotine-induced locomotor (Marks et al., 1986) and hypothermic (Marks et al., 1985) responses.

nAChRs are known to mediate release of several neurotransmitters and neuropeptides such as ACh, serotonin and norepinephrine (Wonnacott et al., 1996), thus differences in these parameters could contribute to the strain difference observed. Alternatively activation of the endogenous opiate system may occur downstream from $\alpha_4\beta_2^*$ nAChRs. Many studies have addressed the question of whether nicotinic agonists elicit their antinociceptive effects via interaction with endogenous opiate systems. There is substantial evidence that nicotinic agonists increase release of endogenous opiates and increase their synthesis in the brain (Houdi et al., 1998). The findings that pretreatment with opiate antagonists inhibits the antinociceptive effects of nicotine in rats (Biala et al., 2005) and mice (Berrendero et al., 2002), and the observation that preproenkephalin knock-out mice exhibit decreased sensitivity to nicotine’s effects on tail-immersion and hot-plate tests (Berrendero et al., 2005), suggest that nicotinic agonists exert some of their analgesic effects via endogenous opiate release. However, we did not find any change in sensitivity to the effects of morphine on either the hot-plate or tail-flick tests in L9'S mutant mice. Similarly, $\alpha_4$ knock-out mice did not show a detectable change in sensitivity to the effects of morphine on the hot-plate and tail-flick tests (Marubio et al., 1999). These findings argue that opiate analgesia does not require direct activation of $\alpha_4^*$-nicotinic receptors.

In summary, the studies reported here provide a new dimension that complements the conclusions drawn from the $\alpha_4$ and $\beta_2$ knock-out studies (Marubio et al., 1999). Taken together, studies using knock-out, gain-of-
function and inbred mouse strain indicate that within the set of nAChR subtypes, activation of α4β2* nAChRs is both necessary and sufficient for nicotine-evoked antinociception in the hot-plate test. In contrast, the α4β2* nAChR and at least one other nAChR subtype regulates the effects of nicotine in the tail-flick test. The inbred strain studies also indicate a role for α4β2-nAChR, but also clearly illustrate that factors downstream from nAChR activation influence responses to nicotine. Future studies with inbred strains and their hybrids may provide a basis for further genetic analysis and could be useful for identifying the gene(s) that influence variability of nicotinic receptor mediated analgesic mechanisms.

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References


FOOTNOTES

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Legends for Figures

Fig. 1  Dose-response curves of nicotine and morphine-induced antinociception in L9’S heterozygote (dark grey circles) and WT (black circles) mice. Animals were tested in the hot-plate and the tail-flick apparatus as described in Materials and Methods. Animals were tested 5 min after a s.c. nicotine injection in A) the tail-flick and B) hot-plate tests. Each data point is the mean ± S.E. of n = 6-8 animals. A different set of animals was tested 20 min after a s.c. morphine injection in B) the tail-flick and D) hot-plate tests. Each data point is the mean ± S.E., n = 6 - 8.

Fig. 2  Antinociceptive effects of nicotine in seven inbred mice strains and two F1 hybrids measured using the tail-flick and hot-plate tests. Effects of nicotine were measured in inbreds (open circles) A/J, (black squares) C57BL/6, (light gray upright triangles) DBA, (dark gray diamonds) 129, (white squares) BALB, (dark grey inverted triangles) C3H and (dark grey circles) CBA and in hybrid (dark grey hexagons) B6CBAF1 and (light grey diamonds) B6D2F1 mice after s.c. administration of the indicated doses of the drug. Mice were tested 5 min after injection. Each point represents the mean ± S.E., n = 8 to 12 mice.

Dose-response curves for nicotine effects on tail-flick test are shown in Panels A and B and ED50 values calculated from these curves are shown in Panel C.

Dose-response curves for nicotine effects on hot-plate test are shown in Panels D and E and ED50 values calculated from these curves are shown in Panel F.

Fig. 3  Cytisine-sensitive, high affinity [125I]-epibatidine binding. The density of cytisine-sensitive high affinity [125I]-epibatidine binding sites in the hindbrain and cervical, thoracic and lumbar regions of the spinal cord was measured using 400 pM ligand in membranes prepared from each of the seven different inbred mouse strains and the two F1 hybrids as described in the Methods. Results are expressed in fmol/mg protein ± SE for preparations from five mice of each strain.

Fig. 4  Cytisine-resistant, high affinity [125I]-epibatidine binding. The density of cytisine-resistant high affinity [125I]-epibatidine binding sites in the hindbrain and cervical, thoracic and lumbar regions
of the spinal cord was measured using 400 pM ligand in membranes prepared from the seven
different inbred mouse strains and the two F1 hybrids as described in the Methods. Results are
expressed in fmol/mg protein ± SE for preparations from five mice of each strain.

**Fig. 5**

$^{125}$I-α-BTX binding. The density of $^{125}$I-α-BTX binding sites in the hindbrain and cervical,
thoracic and lumbar regions of the spinal cord was measured using 1 nM ligand in membranes
prepared from the seven different inbred and two hybrid mouse strains as described in the
Methods. Results are expressed in fmol/mg protein ± SE for preparations from five mice of each
strain.

**Fig. 6**

Correlations among ED50 values for hot-plate and tail-flick tests and nicotinic binding sites in
hindbrain. Each point represents the mean±SEM for the indicated value for the seven inbred
strains. Correlation coefficients for each panel are listed in the parentheses. Regression lines are
shown for correlations that regression lines are statistically significant (P < 0.05).
Figure 3

A. Hindbrain

B. Cervical Spinal Cord

C. Thoracic Spinal Cord

D. Lumbar Spinal Cord

Cytosine-Sensitive, High-Affinity [125I]Epibatidine Binding (fmol/mg protein)

A  BALB  C3H  CBA  B6CBAF1  C57  B6D2F1  DBA  129

A  BALB  C3H  CBA  B6CBAF1  C57  B6D2F1  DBA  129
Figure 4.

A  Hindbrain

B  Cervical Spinal Cord

C  Thoracic Spinal Cord

D  Lumbar Spinal Cord

Cytisine-Resistant, High-Affinity $[^{125}]$Epibatidine Binding (fmol/mg protein)

A  BALB  C3H  CBA  B6CBAF1  C57  B6D2F1  DBA  129

A  BALB  C3H  CBA  B6CBAF1  C57  B6D2F1  DBA  129