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Characterization of pharmacological and behavioral differences to nicotine in C57Bl/6 and DBA/2 mice

Kia J. Jackson, Carrie L. Walters, Michael F. Miles, Billy R. Martin, and M. Imad Damaj

Department of Pharmacology and Toxicology, Medical Campus, Virginia Commonwealth University, Richmond, VA 23298-0613

Abstract

Approximately 50–70% of the risk for developing nicotine dependence is attributed to genetics; therefore, it is of great significance to characterize the genetic mechanisms involved in nicotine reinforcement and dependence in hopes of generating better smoking cessation therapies. The overall goal of these studies was to characterize behavioral and pharmacological responses to nicotine in C57Bl/6 (B6) and DBA/2 (D2) mice, two inbred strains commonly used for genetic studies on behavioral traits. B6 and D2 mice were subjected to a battery of behavioral tests to measure nicotine's acute effects, calcium-mediated antinociceptive responses, tolerance to chronic treatment with osmotic mini pumps, and following three days of nicotine withdrawal. In general, D2 mice were less sensitive than B6 mice to the acute effects of nicotine, but were more sensitive to blockade of nicotine-induced antinociceptive responses by a calcium/calmodulin-dependent protein kinase II (CaMKII) inhibitor. B6, but not D2 mice, developed tolerance to nicotine and nicotine conditioned place preference (CPP). While B6 and D2 mice both expressed some physical withdrawal signs, affective withdrawal signs were not evident in D2 mice. These results provide a thorough, simultaneous evaluation of the pharmacological and behavioral differences to experimenter-administered nicotine as measured in several behavioral tests of aspects that contribute to smoking behavior. The B6 and D2 strains show wide phenotypic differences in their responses to acute or chronic nicotine. These results suggest that these strains may be useful progenitors for future genetic studies on nicotine behaviors across batteries of mouse lines such as the BXD recombinant inbred panel.

Keywords

nicotinic acetylcholine receptors; ED₅₀; AD₅₀; conditioned place preference; conditioned place aversion; calcium/calmodulin-dependent protein kinase II

Introduction

Nicotine, a natural alkaloid of tobacco, is largely responsible for initiation and maintenance of tobacco dependence. Studies in humans suggest that approximately 50–70% of smoking behaviors are attributed to genetics (True et al, 1997). Despite the evidence suggesting strong

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*Address all correspondence to: Dr. Kia J. Jackson, Department of Pharmacology and Toxicology, Virginia Commonwealth University, Box 980613, Richmond, VA 23298-0613, Tel # (804) 828-8061, Fax # (804) 828-2117, jacksonkj@vcu.edu.

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genetic contributions to the etiology of nicotine dependence, studies are far from identifying the specific genetic basis of individual susceptibility to nicotine use and abuse. Because of extensive synteny between the mouse and human genomes (>80%), many studies utilize mouse models to assess the genetic variation of behavioral traits.

Indeed, the use of inbred mouse strains and genetically modified mice has proven very useful in examining genetic contributions to nicotine physiology and behavior (Hatchell and Collins, 1977; Picciotto et al., 2000). Numerous behavioral tests assessing acute responses to nicotine or nicotine consumption have been conducted on panels of inbred mouse strains to determine differences in nicotine responses across strains (Marks et al., 1985). These tests revealed that while variations in nAChR expression and sensitivity contribute to nicotine responses, there are clearly additional genetic factors that contribute to the behavioral effects of nicotine (Marks et al., 1983).

Behavioral genetic studies in mice frequently utilize inbred strains or various crosses derived from C57BL/6J (B6) and DBA2/J (D2) progenitor mouse strains. These strains are two of the most commonly used strains for examining behavioral effects of nicotine. While Collins et al. (1988) characterized some of nicotine's acute pharmacological effects and development of tolerance between these two strains, little information addressing addictive behaviors of nicotine is reported. Prior to proceeding with an extensive genetic analysis of nicotine dependence-related phenotypes across genetic panels, such as the recombinant inbred BXD lines, it is important to thoroughly characterize these phenotypes in the B6 and D2 progenitor strains. Thus, the purpose of the current study was to compare behavioral aspects of nicotine dependence, such as initial effects, tolerance, reward, and withdrawal in B6 and D2 mice using established nicotine dependence models. Furthermore, since nicotine-induced spinal antinociception was reported to be calcium/calmodulin-dependent protein kinase II (CaMKII)-dependent (Damaj, 2000), we determined if the two strains differ in their potency to post-receptor calcium-mediated effects.

Methods and Materials

Animals

Male B6 and D2 mice from Jackson Laboratories were housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care-approved animal care facility with food and water available *ad libitum*. 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 were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and in accordance with the National Institutes of Health Guide for Animal Care and Use.

Drugs

(–)-Nicotine hydrogen tartrate salt and mecamylamine hydrochloride were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Nicotine was dissolved in physiological saline (0.9% sodium chloride) and injected subcutaneously at a volume of 10 ml/kg body weight. 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) was purchased from Calbiochem (San Diego, CA) and was prepared in 25% dimethylsulfoxide (DMSO) solution. All doses are expressed as the free base of the drug.

Acute nicotine assessment

Naïve mice were injected subcutaneously (s.c.), unless otherwise noted, with various doses of nicotine, and tested at different time points after injection. Antinociception using the tail-flick

and hot-plate tests, changes in body temperature, changes in locomotor activity, and plus maze performance were measured.

Spinal antinociception—Spinal antinociception was assessed by the tail-flick method of D'Amour and Smith (1941). Mice were lightly restrained while a radiant heat source was directed onto the upper portion of the tail. A control response (2–4 s) was determined for each mouse before treatment, and test latency was determined after drug administration. The apparatus has an automatic cut-off of 10 s to minimize tissue damage.

Supraspinal antinociception—Supraspinal antinociception was assessed using the hotplate test. Mice were placed into a 10-cm wide glass cylinder on a hot-plate (Thermojust Apparatus, Columbus, OH) as a measure of supraspinal 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). A control response (8–12 s) was determined for each mouse before treatment, and test latency was determined after drug administration. The timer has an automatic cut-off of 40 seconds to avoid tissue damage. Antinociceptive response for the tail-flick and hot plate tests was calculated as percentage of maximum possible effect (%MPE), where $\%MPE = [(test - control) / (10 (40 for the hot-plate) - control)] \times 100$.

Body temperature—Rectal temperature was measured by a thermistor probe (inserted 24 mm) and digital thermometer (YSI Inc., Yellow Springs, OH). Readings were taken just before and at 30 min after nicotine injection. The difference in rectal temperature before and after treatment was calculated for each mouse. The ambient temperature of the laboratory varied from 21–24°C from day to day.

Locomotor activity—Mice were placed into individual photocell activity cages (28 × 16.5 cm; Omnitech, Columbus, OH) 5 min after nicotine administration. Interruptions of the photocell beams (two banks of eight cells each) were then recorded for the next 10 min. Data are expressed as number of photocell interruptions.

Elevated plus-maze—An elevated plus-maze, prepared with gray Plexiglas, consisted of two open arms (23 × 6.0 cm) and two enclosed arms (23 × 6 × 15 cm in wall height) that extended from a central platform (5.5 × 5.5 cm). It was mounted on a base raised 60 cm above the floor. Fluorescent lights (350 lux intensity) located in the ceiling of the room provided the only source of light to the apparatus. The animals were placed in the center of the maze, and the time spent in the open arms was automatically recorded by a photocell beams system. The test lasted 5 min, and the apparatus was thoroughly cleaned after removal of each animal using a solution made up of 50% water and 50% Windex®. Results were expressed as percentage of time spent in open arms.

Blockage of nicotine-induced antinociception by intrathecal CaMKII injection—Mice received intrathecal (i.t.) injections of vehicle or a CaMKII inhibitor (KN-62) at various doses 5 minutes prior to i.t. injections of nicotine (20 µg/animal). Antinociceptive measurement using the tail flick test was initiated 5 min after nicotine injection. Mice were tested and AD₅₀ values were determined from dose response curves.

Intrathecal injections—Injections were performed free-hand between the L5 and L6 lumbar space in unanesthetized male mice according to the method of Hylden and Wilcox (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. In protocols where two sequential injections were required in an animal, the flicking motion of the tail could be elicited with the subsequent injection.

CaMKII phosphorylation assay

CaMKII activity was measured using a modified assay kit (Upstate Biotechnology, Lake Placid, NY). Mice were sacrificed by cervical dislocation and the spinal column was isolated and divided in thoracic, cervical and lumbar regions. The lumbar segment of spinal cord was removed from the spinal column by gentle flushing with ice cold, isotonic saline. Lumbar spinal cord tissues were homogenized using a microcentrifuge pestle in a calcium-free buffer that contains 20 mM HEPES (pH = 7.4), 2.6 mM EGTA, 80 mM beta-glycerolphosphate, 20 mM magnesium acetate, 0.1 μ M okadaic acid, 0.1 μ M calyculin, 0.1 mM DTT, 50 mM sodium-fluoride, 1 mM sodium-orthovanadate and 0.01 mg/ml CLAPS (0.1 mg/ml each of Pepstatin A, Chymostatin, Aprotinin, Leupeptin, Trypsin-Chymotrypsin Inhibitor). Homogenates were normalized for protein concentration. Samples were centrifuged in order to separate the membrane and the cytosol containing-kinase. Supernatant is retained (cytosolic fraction). The pellet is resuspended in homogenization buffer plus 1% NP-40 (IGEPAL) and allowed to incubate on ice 1 hour. The tubes are spun again and supernatant is retained (Membrane fraction). Standard phosphorylation reaction solutions contains 15 μ g extract protein, 100 μ M CaM Kinase II-specific substrate peptide (Autocamtide-2), 0.25 μ M protein kinase inhibitors (0.25 μ each of PKA & PKC inhibitor peptides), 75 mM Mg acetate, 500 μ M ATP, 20 mM HEPES, 25 mM beta-glycerolphosphate, 1 mM Na-orthovanadate, 1 mM DTT, 1 μ Ci of [32 P] ATP, 5 μ M CaCl₂ and 5 μ g calmodulin for the measurement of calcium-dependent activity. In aliquots used for calcium-independent activity, 5 mM EGTA was added and CaCl₂ and calmodulin were omitted. Standard reactions were performed in triplicate in a shaking water bath at 30°C for 10 min along with background controls lacking substrate. Activity was quantified by spotting half the reaction on phosphocellulose paper squares. Squares were washed in 0.75 % phosphoric acid (5 times) followed by a brief acetone rinse before analysis by scintillation counting. CaMKII activity was expressed in pmol phosphate/min/ μ g and determined using the following calculations: [(count-specific binding minus background) $\times$ (correcting factor)]/[(specific radioactivity) $\times$ time (10 min)].

Administration of chronic nicotine

Mice were implanted with Alzet osmotic mini pumps [model 2002 (14 days) or model 2004 (28 days) Durect Corporation, Cupertino, CA] filled with (–)-nicotine or saline solution. The concentration of nicotine was adjusted according to animal weight and the mini pump flow rate, resulting in 36 mg/kg/day for 14 days. The mini pumps were surgically implanted s.c. under sterile conditions with sodium pentobarbital anesthesia (45 mg/ml, i.p.). An incision was made in the back of the animal, and a pump was inserted. The wound was closed with wound clips, and the animal was allowed to recover before being returned to its home cage. Based on plasma levels of nicotine in the mouse reported in the literature by Matta et al. (2007), the nicotine dose used for chronic studies corresponds to approximately 2 mg/kg/hr, which yields plasma levels of 0.1 μ M, a dose within the plasma range of human smokers.

Tolerance Studies

Mice were infused with subcutaneously implanted osmotic mini pumps containing saline or nicotine for 14 days. On day 15, mice were challenged with different doses of nicotine and tested after 5 minutes for antinociception (tail-flick and hot-plate tests), and after 30 minutes for hypothermia. Dose response curves and ED₅₀ values were determined for all tests.

Withdrawal Studies

Minipumps were removed on the evening of day 14. Beginning on day 15, somatic signs, hyperalgesia response (tail-flick and plantar stimulation tests), plus maze performance, and spontaneous activity were measured for 3 consecutive days. Hyperalgesia response was measured using the tail-flick (as described above), and using the plantar stimulation test as a measure of the supraspinal hyperalgesia response. The plus maze test was conducted as described above and spontaneous activity was assessed for 30 min as previously described (see “Locomotor Activity”).

Somatic signs—Animals were placed in individual, Plexiglas containers [28.5 cm (*L*)×18 cm (*W*)×13 cm (*H*)] and observed for 20 min for occurrences of paw tremors, backing and head shakes. For each animal, the total score for this assay was the sum of these individual behaviors.

Hyperalgesia using plantar stimulation—Subjects were placed in clear Plexiglas compartments [13 cm (*L*)×6.5 cm (*W*)×25.5 cm (*H*)]. A radiant heat source was applied to the rear right paw, and paw withdrawal latency was recorded (three to four measurements per animal). The apparatus has an automatic cut-off of 20 s to minimize tissue damage.

Nicotine CPP

Nicotine CPP was conducted using an unbiased design as previously described by Kota et al., (2007). In brief, mice were handled for three days prior to initiation of CPP testing. The CPP apparatus consists of a three-chambered box with a white compartment, a black compartment, and a center grey compartment. The black and white compartments also have different floor textures to help the mice further differentiate between the two environments. On day 1, mice were placed in the grey center compartment for a 5 min habituation period, followed by a 15 min test period to determine baseline responses. A pre-preference score was recorded and used to randomly pair each mouse with either the black or white compartment. Drug-paired sides were randomized so that an even number of mice received drug on the black and white side. Over the next 3 days, mice were conditioned for 20 min with the saline group receiving saline on both sides of the boxes and drug groups receiving nicotine (0.1, 0.3 0.5, 0.7, 1, or 1.5 mg/kg) on one side of the box and saline on the opposite side. Animals in the drug group received drug each day. On the test day, no injections were given. Time spent on each side was recorded, and data were expressed as time spent on the drug-paired side minus time spent on the saline-paired side. A positive number indicated a preference for the drug-paired side, whereas a negative number indicated an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

Nicotine CPA

The CPA protocol was conducted over the course of four days in a biased fashion as described in Jackson et al. (2008). The apparatus is the same as that used for CPP testing. In brief, mice were implanted with 28 day mini pumps 14 days prior to initiation of CPA testing to induce tolerance. Infusion continued throughout the duration of testing. Day 1 of CPA testing was the pre-preference day where mice were placed in the grey center compartment for a 5 min habituation period, followed by a 15 min test period to determine baseline responses. The pre-preference score was used to pair each mouse with mecamylamine (3.5. mg/kg) to its initially preferred compartment. On days 2 and 3 of CPA testing, all mice received injections of saline in the morning and were immediately confined to their non-preferred compartment for 30 min. No less than four hours later, mice received an injection of mecamylamine and were immediately confined to their preferred compartment for 30 min. Day 4 was a test free drug day, and the procedure was the same as day 1. A post-preference score was recorded for each mouse. Aversion was counted as mice spending less time in their initially preferred

compartment on test day when compared to time spent in the same compartment prior to drug conditioning.

Statistical Analysis

Statistical analyses of behavioral studies were performed using two-way ANOVA (with strain and treatment as between subject factors for CPP and CPA assessments, or day and treatment as between subject factors for withdrawal studies) and one-way ANOVA for within strain differences in acute nicotine and tolerance tests. A Tukey's post hoc test when appropriate. *p* values <0.05 were considered to be statistically significant. ED₅₀ and AD₅₀ values with 95% confidence limits (CL) for acute and tolerance tests were calculated by unweighted least-squares linear regression as described by Tallarida and Murray (1987). If confidence limit values did not overlap, then the shift in the dose-response curve was considered significant.

Results

Nicotine potency in B6 and D2 mice after acute administration

Results of the acute nicotine assessment in D2 and B6 mice are shown in figure 1. Basal tail flick and hot plate latencies did not differ between the two mouse strains (Tail-flick: B6 vs. D2: 1.6 ± 0.3 s vs. 1.7 ± 0.1 s; Hot-plate: B6 vs. D2: 6.7 ± 0.3 s vs. 8.5 ± 0.6 s). Dose-response relationships were established for nicotine in B6 and D2 mice by measuring antinociception at the time of maximal effect (5 min) (Fig. 1). Nicotine produced a dose-responsive increase in the tail-flick latency (Fig. 1A) in the B6 and D2 mice with an ED₅₀ ($\pm$ CL) of 1.2 (0.9–1.45) and 2.0 (1.6–2.3) mg/kg, respectively (Table I). Nicotine was nearly 2-times more potent in B6 than D2 mice after s.c. administration in the tail-flick test. However, difference in nicotine potency was not observed in the hotplate test (Fig. 1B, Table I).

Nicotine decreased body temperature (Fig. 1C) in a dose-related manner, and the ED₅₀ values (Table 1) at the time of maximal effect (30 min after injection) showed that it was 1.8- times less potent in the D2 than B6 mice in inducing hypothermia (Table 1). While nicotine significantly decreased locomotor activity in both strains, there was no difference in nicotine's potency observed between mouse strains (Fig. 1D; Table 1). The largest observed difference was in the plus maze test for anxiety-related behavior, where with increasing doses of nicotine, B6 mice spent significantly more time on the open arms, while D2 mice spent significantly less time on the open arms (Fig. 1E). These results suggest that acute nicotine had anxiogenic like-effects in D2 mice, but anxiolytic like-effects in B6 mice (Table 1). There were no significant differences between D2 and B6 strains under baseline conditions (Fig. 1E).

Involvement of spinal CaMKII in nicotine-induced antinociception in B6 and D2 mice

Previous research from our laboratory indicates that nicotine binding to nAChRs causes an influx of calcium that reaches sufficient levels to activate the calcium-dependent protein, CaMKII; thus, neuronal calcium, acting via CaMKII, appears to mediate nicotine-induced antinociception at the spinal level (Damaj, 2000). Therefore, we assessed the involvement of CaMKII in nicotine-induced antinociception in the B6 and D2 mouse strains by using KN62, a CaMKII inhibitor. Similar to results in the acute tail flick assessment, i.t. injection of nicotine revealed that nicotine was more potent in B6 mice than in D2 mice in the tail flick test (data not shown); thus, mice were treated i.t. with nicotine doses of 15 and 25 μ g/animal respectively, 5 min after i.t. injection of various doses of KN62. Results of the assessment are shown in Figure 2. KN62 dose-dependently blocked the nicotine-induced antinociceptive tail flick response; however, results indicate that KN62 blockade was more potent in D2 mice compared to B6 mice. AD₅₀ ($\pm$ CL) values were 0.08 (0.03–0.11) for D2 mice and 0.5 (0.5–0.62) for B6 mice, thus confirming a significant difference in potency between the two strains.

To determine if the observed differences in potency were due to differences in baseline CaMKII activity, we measured basal levels of membrane spinal (lumbar region) CaMKII calcium-dependent and calcium-independent activity in B6 and D2 mice. There was no significant difference in baseline membrane spinal CaMKII calcium-dependent or independent activity between the two mouse strains (Table 2), suggesting that the differences in potency of KN62 blockade are not attributed to significant differences in basal membrane spinal CaMKII levels.

Nicotine tolerance in B6 and D2 mice after chronic administration

Tolerance to nicotine's various pharmacological actions was seen in B6 mice infused with nicotine for 14 days (24 mg/kg/day). Dose-response curves for nicotine's effects in chronic nicotine and saline-treated animals are presented in Figure 3. B6 mice exhibited tolerance to the antinociceptive and hypothermic effects of nicotine after chronic exposure. There was a significant shift to the right in nicotine-treated B6 mice for both the tail-flick (Fig. 3A) and hot-plate tests 5 min after nicotine injection (Fig. 3B), indicating a decreased response to nicotine. There was also a significant shift in nicotine-dependent mice in the hypothermia assessment, 30 min after nicotine injection (Fig. 3C). $ED_{50} \pm CL$ values did not overlap in any behavioral test between saline and nicotine treated groups, thus confirming the development of significant tolerance in B6 mice (Table 3).

In contrast, D2 mice did not exhibit significant tolerance to nicotine's antinociceptive or hypothermic effects after 14 days of chronic nicotine exposure (Fig. 4). There was no significant shift in nicotine dose-response curves in the tail-flick (Fig. 4A) or hot-plate tests (Fig. 4B). There was also no difference in the hypothermia response between nicotine and saline treated D2 mice (Fig. 4C). $ED_{50} \pm CL$ values overlapped between saline and nicotine treated D2 mice, indicating no significant difference between the treatment groups (Table 4).

Nicotine CPP in B6 and D2 mice

The ability of various nicotine doses to produce CPP in B6 and D2 mice is presented in Figure 5. There were significant main effects of treatment ($F_{(6,107)} = 3.99$, $p < 0.05$) and strain ($F_{(1,107)} = 47.9$, $p < 0.0001$), but no significant interaction for the CPP assessment. Compared to saline controls, B6 mice conditioned with the intermediate doses of 0.3, 0.5 and 0.7 mg/kg nicotine showed significant CPP. There was no significant preference seen with the lower dose of 0.1 mg/kg, and the response disappeared at the higher dose of 1.0 mg/kg of nicotine. These results indicate that nicotine produced CPP within a narrow dose range in B6 mice. In D2 mice, however, nicotine failed to induce significant preference at any dose tested. A higher dose of nicotine in D2 mice revealed an aversive response to nicotine; thus, doses higher than 1.5 mg/kg were not tested.

Nicotine withdrawal in B6 and D2 mice

Withdrawal assessment after chronic exposure to nicotine revealed the presence of significant somatic signs in both B6 (Fig. 6A) and D2 (Fig. 7A) mice after 1, 2, and 3 days withdrawal from chronic nicotine. The plus maze assessment revealed a significant decrease in the time spent on the open arms by B6 mice on days 2 and 3 after nicotine withdrawal ($F_{(5,41)} = 3.03$, $p < 0.05$), indicating the presence of anxiety-related behavior (Fig. 6B). Interestingly, D2 saline treated mice had significantly lower baseline levels in the plus maze on days 1 and 2 compared to saline treated B6 mice, and nicotine-dependent D2 mice did not exhibit anxiety-related behavior on any days tested (Fig. 7B).

Although both strains expressed significant hyperalgesia responses in both the tail flick and plantar stimulation tests at some point during the 3-day withdrawal assessment, there were time-dependent differences in the expression of the responses between strains. The tail-flick hyperalgesia response was not present on day 1 after nicotine withdrawal in B6 mice, but was

observed on days 2 and 3 ($F_{(5,41)} = 2.92$, $p < 0.05$) (Fig. 6C). Assessment in D2 mice revealed a significant withdrawal-induced hyperalgesia response on day 1 for D2 mice ($F_{(5,35)} = 4.99$, $p < 0.05$) that diminished by day 2 after withdrawal (Fig. 7C). The plantar stimulation hyperalgesia test revealed a significant hyperalgesia response on day 1 in B6 mice ($F_{(5,41)} = 2.93$, $p < 0.05$) that diminished by day 2 after withdrawal (Fig. 6D), while in D2 mice, the plantar stimulation hyperalgesia response was present on days 1 and 2 after withdrawal ($F_{(5,35)} = 19.46$, $p < 0.0001$), but reduced by day 3 (Fig. 7D). The spontaneous activity assessment revealed an increase in locomotor activity 1 day after nicotine withdrawal in B6 mice ($F_{(5,41)} = 3.03$, $p < 0.05$) (Fig. 6E), while no effect was observed on any day in D2 mice (Fig. 7E).

Nicotine CPA in B6 and D2 mice

We extended our observation of affective withdrawal signs by measuring nicotine withdrawal-associated aversion using the CPA procedure. Figure 8 showed that mecamylamine (3.5 mg/kg) precipitated aversion in B6, but not in D2 nicotine-dependent mice. There was a significant main effect of treatment ($F_{(1,41)} = 7.159$, $p < 0.05$) and a significant interaction ($F_{(1,41)} = 5.086$, $p < 0.05$), suggesting that the observed effects depend on the animal genotype. The dose of mecamylamine used did not precipitate aversion in saline mice, and there were no differences in aversion score between saline treated D2 or B6 mice.

Discussion

The present research aimed to study and compare behavioral responses after acute and chronic nicotine, such as initial effects, tolerance, reward, and withdrawal in B6 and D2 mice, the progenitor strains for the BXD recombinant inbred mouse line. Our results show that these two strain strains show wide phenotypic differences in their responses to acute or chronic nicotine. D2 mice were less sensitive than B6 mice to the acute effects of nicotine in some behavioral evaluations, but these effects were not uniform across all tests. Since nicotine-induced spinal antinociception was reported to be CaMKII-dependent (Damaj, 2000), we determined if the two strains differ in their potency to post-receptor calcium-mediated effects. Although in the tail flick response, D2 mice were less sensitive to nicotine's acute effects than B6 mice, blockade of this response by the CaMKII inhibitor, KN62, was more potent in D2 mice than in B6 mice. B6, but not D2 mice, developed tolerance to nicotine, suggesting that D2 mice are less sensitive to nicotine's chronic effects. While B6 mice found nicotine rewarding in the CPP model, this effect was not observed at any dose tested in D2 mice. Results with withdrawal studies revealed that although B6 and D2 mice both expressed some physical withdrawal signs, affective withdrawal signs were not evident in D2 mice.

Although results were not uniform across tests for the acute assessment, in general, D2 mice were less sensitive to nicotine's acute effects than B6 mice. While no difference was observed in the hot plate and hypomotility assessments, B6 mice were more sensitive to nicotine's initial effects in the tail flick, plus maze, and hypothermia evaluations. Our results are similar to those reported by Marks et al (1989), where they found that D2 mice were less sensitive than B6 mice to nicotine-induced changes in body temperature, respiratory rate and heart rate. B6, but not D2 mice, also developed tolerance to nicotine as measured by the tail flick, hot plate, and hypothermia tests. Similarly, Marks et al., (1991) reported that while B6 mice developed significant tolerance to nicotine-induced hypothermia at the lowest infusion dose (12 mg/kg/day, i.v.), D2 mice did not develop measurable tolerance until the highest infusion dose was used (72 mg/kg/day, i.v.).

While D2 mice were less sensitive than B6 mice to nicotine-induced antinociception in the tail flick response, blockade of this response using KN62, a CaMKII inhibitor, was more potent in D2 mice than in B6 mice. These results indicate that D2 mice are more sensitive to a calcium-mediated mechanism, which acts via CaMKII, to mediate spinal nicotine-induced

antinociception. One explanation for these results could be differences in the basal level of CaMKII; however, our measurements of basal membrane spinal CaMKII activity in both strains revealed no significant difference in basal levels, thus ruling out this possibility. It is also feasible that nAChR binding or calcium permeability is altered between strains, leading to a reduction in calcium influx in D2 mice. Such an event may lead to differences in CaMKII activation. Previous results indicate that the $\alpha 4\beta 2^*$ nAChR subtype partially mediates spinal nicotine analgesic responses, yet binding levels of $\alpha 4\beta 2^*$, $\alpha 3\beta 4^*$ and $\alpha 7$ in the spinal cord of B6 and D2 mice were not significantly different (Damaj et al., 2007). Interestingly, D2 mice have a point mutation on the CHRNA4 locus that is not found in B6 mice, resulting in altered responses to nicotine (Dobelis et al., 2002); thus, although binding properties are unchanged, with the current experiments, it cannot be ruled out that this mutation plays a role in the reduced sensitivity to the acute nicotine-induced spinal antinociceptive response in D2 mice, as well as the enhanced sensitivity to calcium-mediated mechanisms involved in antinociception. Further, the observed potency differences between the two strains may reflect differences in downstream signaling conduction events. Additional studies assessing receptor permeability and downstream mechanisms would be necessary to evaluate these effects.

Nicotine induced significant rewarding properties in B6 mice, as measured by the CPP model. Results show an inverted U-shaped curve response in B6 mice in response to nicotine. Conversely, D2 mice did not find nicotine rewarding at any dose tested. These results are consistent with previous results from our laboratory, which conducted a CPP assessment using various mouse strains (Grabus et al., 2006). To expand on these results, in the current evaluation, we tested a wider range of nicotine doses and measured locomotor activity during the CPP test to determine if differences in this measure attributed to the observed differences in nicotine preference. Results revealed that differences in nicotine's rewarding actions in CPP were not due to differential sensitivity of the drug on locomotor activity between the two strains. The difference between the two strains may reflect a significant difference in the rate of learning, and additional conditioning sessions may be necessary for D2 strains to acquire nicotine CPP; however, the two strains showed no difference in learning a nicotine discrimination cue (Stolerman et al., 1999). It is also conceivable that the strain-dependent effect of nicotine on CPP could be dependent on a specific experimental parameter (conditioning time, number of sessions). Additional nicotine CPP studies in the D2 mouse strain are necessary to confirm the accuracy of this possibility.

The spontaneous withdrawal assessment revealed the presence of somatic withdrawal signs in both D2 and B6 mice. Interestingly, the plus maze assessment revealed anxiety-related behavior in nicotine-withdrawn B6 mice; however, this effect was not present in nicotine-withdrawn D2 mice. A similar observation was recently reported by Jonkman et al (2005) who reported that nicotine withdrawal was found mildly anxiogenic in B6, but not D2 mice, using the light-dark box test. It is noted in our plus maze withdrawal study that saline baseline scores were significantly lower in D2 than in B6 mice, whereas this difference in baseline scores was not observed in our acute nicotine plus maze measure; thus, the absence of an anxiety-related response in D2 mice could be attributed to a "floor effect". Because saline treated D2 mice exhibited minimal open arm time on days 1 and 2, it may be more difficult to detect increased anxiety in nicotine treated D2 mice. It is also possible that D2 mice did not express an anxiety-related response because tolerance to nicotine did not develop in this strain. Further assessment of affective measures in these two strains using the CPA model revealed that D2 mice do not develop withdrawal-induced aversion, as seen in B6 mice. Overall, these results would suggest that D2 mice are less sensitive to affective nicotine withdrawal signs than B6 mice.

These behavioral differences in the various aspects of nicotine dependence between the two strains are interesting and could reflect differences in nicotine pharmacokinetic and distribution factors and/or differences in the expression and function of the various nAChRs subtypes or

their post-receptor neurobiological signaling pathways. Recently, Siu and Tyndale (2007) assessed differences in nicotine pharmacokinetics and metabolism between B6 and D2 mice after acute administration of nicotine and found no difference in the rate of nicotine kinetics and elimination. Interestingly, cotinine, a major but inactive metabolite of nicotine, was found at much higher levels in D2 mice compared to B6 mice (Siu and Tyndale, 2007). Thus, nicotine kinetics and elimination are unlikely to account for nicotine potency after acute administration of the drug. The Siu and Tyndale study (2007), however, did not assess the pharmacokinetics after chronic nicotine exposure. Our three-day withdrawal assessment after chronic nicotine did reveal some differences in intensity in some withdrawal measures, which could be attributed to pharmacokinetic differences; however, in the plus maze test, the differences in intensity were in opposite directions, suggesting a minor role for pharmacokinetic differences.

Differences may also be attributed to variation in the expression and function of various nAChRs subtypes; however, earlier results by Marks et al. (1989; 1991) suggest little difference between the two inbred strains in the basal levels of $\alpha 4\beta 2^*$ and $\alpha 7^*$ nAChRs or in nicotine-induced upregulation of these subtypes after chronic nicotine infusion. Differences in basal levels of additional subtypes were not reported.

In summary, we have provided a thorough evaluation of the pharmacological and behavioral differences to nicotine as measured in several behavioral tests of aspects that contribute to nicotine addiction. While several previous studies have compared nicotine behavioral responses across B6, D2, and other inbred strains (Marks et al., 1983, 1985, 1989, 1991; Collins et al., 1988), the current study, in addition to nicotine's acute and chronic effects, provides characterization of withdrawal and reward, two important aspects of nicotine dependence, in inbred strains; thus, the current study is the most comprehensive simultaneous comparison of acute and chronic responses to nicotine in these two widely studied inbred strains. The results of the current study suggest that the B6 and D2 strains may be useful progenitors for future genetic studies on nicotine behaviors across batteries of mouse lines such as the recombinant inbred BXD panel.

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Figure 1. Acute pharmacological response to nicotine in D2 and B6 mice

Different groups of D2 (open circles) and B6 (closed circles) mice were treated with increasing doses of nicotine, and nicotine's effects on tail flick, hot plate, body temperature, locomotor activity, and plus maze were measured. Each point represents mean $\pm$ S.E.M. of 8–10 mice per group. * denotes $p < 0.05$ vs. lowest nicotine dose tested for each strain. MPE, maximum possible effect

Figure 2. Involvement of spinal CaMKII in nicotine-induced antinociception in D2 and B6 mice
 Different groups of D2 (open circles) and B6 (open squares) mice were treated with varying doses of the CaMKII inhibitor KN62 (i.t.), 5 minutes prior to injection of 25 or 15 $\mu\text{g/animal}$ of nicotine respectively. Tail flick assessment revealed significant dose-dependent blockade of nicotine-induced antinociception by KN62 in both mouse strains; however, the effect was more potent in D2 mice compared to B6 mice. Each point represents mean $\pm$ S.E.M. of 8–10 mice per group. * denotes $p < 0.05$ vs. lowest nicotine dose tested for each strain

Figure 3. Evaluation of nicotine tolerance in B6 mice after chronic administration

Different groups of B6 mice chronically treated with saline or nicotine for 14 days received challenge injections of various nicotine doses. A dose response curve shift to the right was noted in **A**, the tail flick, **B**, hot plate, and **C**, body temperature assessment for nicotine treated B6 mice, indicating the development of tolerance to nicotine. Each point represents the mean $\pm$ S.E.M. of 8–10 mice per group. * denotes $p < 0.05$ vs. lowest nicotine dose tested for each strain

Figure 4. Evaluation of nicotine tolerance in D2 mice after chronic administration

Different groups of D2 mice chronically treated with saline or nicotine for 14 days received challenge injections of various nicotine doses. There were no differences between saline and nicotine treated mice in the **A.** tail flick, **B.** hot plate, or **C.** body temperature assessment, indicating that D2 mice did not develop tolerance to nicotine. Each point represents the mean $\pm$ S.E.M. of 8–10 mice per group.

Figure 5. Nicotine CPP in B6 and D2 mice

The rewarding effects of nicotine were measured using the CPP model. A dose response curve for nicotine CPP was generated in B6 and D2 mice. The x-axis represents nicotine dose in mg/kg and the y-axis represents the preference score in s. Each point represents the mean $\pm$ S.E.M. of 8–10 mice per group. *denotes $p < 0.05$ vs. vehicle

Figure 6. Nicotine withdrawal assessment in B6 mice

Mini pumps (MP) were removed from mice treated with saline (circles) or nicotine (squares) for 14 days, and testing initiated 18–24 hours after removal. Responses were measured in **A.** somatic signs, **B.** plus maze, **C.** tail flick, **D.** plantar stimulation, and **E.** locomotor activity for three days following mini pump removal. Each point represents the mean $\pm$ S.E.M. of 8–10 mice per group. * denotes $p < 0.05$ vs. saline

Figure 7. Nicotine withdrawal assessment in D2 mice

Mini pumps (MP) were removed from mice treated with saline (circles) or nicotine (square) for 14 days, and testing initiated 18–24 hours after removal. Responses were measured in **A.** somatic signs, **B.** plus maze, **C.** tail flick, **D.** plantar stimulation, and **E.** locomotor activity for three days following mini pump removal. Each point represents the mean $\pm$ S.E.M. of 8–10 mice per group. * denotes $p < 0.05$ vs. saline

Figure 8. Nicotine CPA in B6 and D2 mice

CPA, an affective component of nicotine withdrawal, was measured in B6 and D2 mice.

Mecamylamine (mec, 3.5 mg/kg) precipitated aversion in B6 nicotine-treated mice, but not in D2 mice. The dose of mecamylamine utilized had no effect on saline treated mice. Each point represents the mean $\pm$ S.E.M. of 10–12 mice per group. * denotes $p < 0.05$ vs. saline B6 group. MP= mini pump.

Table I

Summary of the potency of nicotine's effects after acute administration in B6 and D2 mice. Potency is expressed as $ED_{50} \pm CL$ (mg/kg). **Each group** contained 8–10 mice.

Response	B6	D2
Tail flick	1.2 (0.9– 1.45) *	2.0 (1.6– 2.3)
Hot plate	1.0 (0.8– 1.3)	0.85 (0.6–1.0)
Hypothermia	1.1 (0.9– 1.5) *	1.9 (1.6– 2.3)
Hypomotility	0.50 (0.3– 0.8)	0.39 (0.2– 0.7)
Plus maze increase	0.15 (0.1– 0.19) *	-
Plus maze decrease	-	0.30 (0.22– 0.38)

* $P < 0.05$ denotes significance vs. D2 mice.

Table II

Basal levels of membrane spinal (lumbar region) CaMKII calcium-dependent and calcium-independent activity in C57 and DBA mice. Activity was expressed in pmol/min/ μ g protein. **Each group** contained 10–12 mice.

Strain	Dependent	Independent
C57	2.35 ± 0.12	0.32 ± 0.08
DBA	2.04 ± 0.1	0.28 ± 0.07

Table III

Summary of the potency of nicotine's effects in tolerant B6 mice in response to tail flick, hot plate, and body temperature. Potency is expressed as $ED_{50} \pm$ confidence limits (mg/kg). **Each group** contained 8–10 mice.

Response	Saline	Nicotine
Tail flick	1.0 (0.85–1.25)	4.8 (4.5–5.4) *
Hot plate	0.95 (0.81–1.2)	4.3 (4.05–4.6) *
Hypothermia	1.2 (0.9–1.6)	3.8 (3.2–4.3) *

* denotes $p < 0.05$ vs. saline.

Table IV

Summary of the potency of nicotine's effects in tolerant D2 mice in response to tail flick, hot plate, and body temperature. Potency is expressed as $ED_{50} \pm$ confidence limits (mg/kg). **Each group** contained 8–10 mice.

Response	Saline	Nicotine
Tail flick	1.6 (1.2–1.7)	1.6 (1.1–1.5)
Hot plate	0.75 (0.5–1)	0.74 (0.5–0.91)
Hypothermia	1.7 (1.5–2.1)	1.83 (1.5–2.2)