Characterization of Spontaneous and Precipitated Nicotine Withdrawal in the Mouse

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ABSTRACT

The nicotine withdrawal syndrome was validated and characterized in the mouse using both somatic and affective measures after infusion with nicotine daily via subcutaneous minipumps. The influence of dose, duration of infusion, and repeated withdrawal as well as the contribution of genetic factors were investigated. We then characterized the contribution of nicotinic receptor and site mechanisms to withdrawal signs using various nicotinic antagonists. Our results showed that spontaneous nicotine withdrawal increased the number of somatic signs, decreased the time spent in open arms of the plus-maze test, and induced hyperalgesia. The effect was dose-dependent in all measures with no significant changes at the lowest dose of nicotine (6 mg/kg/day). Withdrawal signs were prominent shortly after pump removal and remained prominent through day 3 or 4. The results with the different antagonists (mecamylamine, dihydro-β-erythroidine, and methyllycaconitine) suggest the involvement of several nicotinic subtypes such as α3β4*, α4β2*, and α7 in nicotine withdrawal. Increasing the duration of nicotine exposure (from 7 to 60 days) and the total nicotine exposure (increasing doses of infusing) augmented the severity of nicotine withdrawal signs. The withdrawal severity of nicotine differs between C57/BL and 129/SvEv inbred mice with nicotine withdrawal in C57 being more severe than in the 129 strain. In summary, our present results suggest that withdrawal from nicotine can be modulated by genetic factors, daily nicotine intake, duration of nicotine exposure, and withdrawal history. The present study demonstrates that our mouse nicotine withdrawal model will be useful for studying the pharmacological, biochemical, and genetic mechanisms involved in nicotine dependence.

Abundant clinical and experimental data revealed that nicotine produces tolerance and leads to physical dependence. Withdrawal from chronic use of nicotine results in an abstinence syndrome, which includes increased nicotine craving, anxiety and pain sensitivity, restlessness, appetite, and decreased cognitive capabilities. Onset is within approximately 8 h after the last cigarette; the symptoms peak within the first few days, then subside over the next few weeks. Although considerable variability exists, the severity of the symptoms is directly related to the level of nicotine dependence (for review, see Benowitz, 1992). This withdrawal syndrome is considered one of the major causes of the high relapse rate among people undergoing smoking cessation. The evaluation of nicotine withdrawal has been attempted using various models, such as operant schedules of reinforcement (Carroll et al., 1989; Corrigall et al., 1989), place preference (Costall et al., 1990; Suzuki et al., 1996), brain-stimulation reward threshold (Epping-Jordan et al., 1998), and auditory startle (Helton et al., 1993; Acri, 1994). Moreover, several groups reported that rats that have been chronically treated with nicotine for 7 days or more showed several withdrawal somatic signs after mecamylamine or dihydro-β-erythroidine injections such as shakes, tremors, wet dog shakes, teeth chatters, eye blinks, and abdominal constrictions (Malin et al., 1992; Hildebrand et al., 1997; Epping-Jordan et al., 1998; Bancroft and Levin, 2000) (however, also see Stolerman et al., 1973; Corrigall et al., 1989; Helton et al., 1993; Suzuki et al., 1996). Such complexity and variability in the response of rodents to nicotine using these models may be due to differences in the dose of nicotine, sex or age of the test animal, route or duration of administration, time of evaluation, or the behavioral task employed. The various models of physical dependence described above relied exclusively on the rat as a test animal. Unfortunately, only one published report described nicotine withdrawal in male mice (Isola et al., 1999), where nicotine was given to animals in s.c. injections every 4 h for 14 days. The observed nicotine abstinence (somatic signs such as jumping, rearing, shakes, abdominal constrictions, chewing, facial tremor, and scratching) after cessation of nicotine injections was mild and pro-

ABBREVIATIONS: MLA, methyllycaconitine; ANOVA, analysis of variance; nAChR, nicotinic acetylcholine receptor.
tracted. However, challenge with mecamylamine did not precipitate a robust abstinence.

Understanding the underlying mechanisms of nicotine withdrawal syndrome will potentially produce a substantial improvement in the pharmacotherapy of smoking cessation. In that regard, animal models of physical dependence on nicotine are potentially useful for investigating nicotine dependence. In particular, a mouse model offers several possibilities for exploring the underlying mechanisms of nicotine dependence and abstinence by using the transgenic and genetic mouse models available along with the pharmacological and biochemical approaches.

In the present study, we validated and characterized an animal model of physical dependence to nicotine in mice. We first investigated whether our model met validity criteria, including reversibility of abstinence signs by nicotine replacement and the precipitation of these signs by antagonist challenge. We then characterized the contribution of nicotinic receptor to these signs with the use of various nicotinic agonists and antagonists. We also assessed the influence of dose and duration of nicotine exposure, as well as the effects of repeated precipitated withdrawal by mecamylamine, on the severity of nicotine withdrawal signs. Finally, we performed an initial evaluation of the potential influence of genotypic factors in this nicotine physical dependence model.

Materials and Methods

Animals

Male ICR mice (20–25 g) obtained from Harlan (Indianapolis, IN) were used throughout the study. Male C57/BL/6J and 129/SvEv inbred mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in groups of six and had free access to food and water. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care approved facility, and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs

Mecamylamine hydrochloride was supplied as a gift from Merck Research Labs (West Point, PA). (-)-Nicotine was obtained from Aldrich Chemical Co. (Milwaukee, WI) and converted to the ditartrate salt as described by Aceto et al. (1979). Metanicotine oxalate was synthesized as described by Acheson et al. (1980). Dihydro-β-erythroidine, hexamethonium, and MLA citrate were purchased from Sigma/RBI (Natick, MA). All drugs were dissolved in physiological saline (0.9% sodium chloride). All doses are expressed as the free base of the drug.

Dependence Induction

Mice were implanted with Alzet osmotic minipumps [model 2001 (7 days); model 2002 (14 days); model 2004 (28 days); Alza Corporation, Palo Alto, CA] filled with either (-)-nicotine or sterile physiological saline solutions. For most experiments, the concentration of nicotine solution was adjusted according to animal weight, resulting in 24 mg/kg/day for 14 days. The minipumps were surgically implanted s.c. under sterile conditions with pentobarbital anesthesia (50 mg/kg, i.p.). An incision was made in the back of the animals, 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. Animals were tested 14 days later. For spontaneous withdrawal studies, minipumps were removed at day 15, and withdrawal data were collected at 24-h intervals for 7 days following withdrawal of nicotine. For precipitated withdrawal studies, in the morning of day 15 of nicotine infusion, different groups of mice were injected s.c. with various nicotinic antagonists: mecamylamine (1–3 mg/kg), dihydro-β-erythroidine (1.5–3 mg/kg), MLA (7.5 mg/kg), and hexamethonium (1 mg/kg), and withdrawal signs were measured immediately. The doses of different antagonists were chosen based on their potency in totally blocking nicotine’s acute effects or their binding affinity to specific nicotinic receptor subtypes. For the experiments using multiple exposure to mecamylamine, mice were implanted with Alzet osmotic minipumps [model 2004 (28 days); Alza Corporation] filled with either (-)-nicotine (24 mg/kg/day) or sterile physiological saline solutions for 28 days. At days 7, 14, 21, and 28, mice were injected with mecamylamine (2 mg/kg, s.c.) and assessed for withdrawal as described below. For experiments requiring 2 months of nicotine exposure, mice were first implanted with 28-day Alzet osmotic minipumps. At day 28, the empty minipumps were removed under anesthesia and new 28-day minipumps were implanted. Testing the animals was performed at the end of this almost 2-month treatment regimen.

Dependence Assessment

Twenty-four hours after minipump removal or immediately after antagonist injection, mice were observed for somatic signs of withdrawal for 20 min. Mice were then evaluated in the plus-maze test. Five minutes later, animals were evaluated for hyperalgesia. Experimenters will be blind to the treatment in all experiments.

Somatic Signs. Mice were observed for 20 min in clear activity cages for typical somatic withdrawal behaviors and any unique behavior. Typical withdrawal signs that were tallied included head shakes, paw tremors, repositioning, scratching, backing, piloerection, and Straub tail. Somatic signs were calculated as the mean ± S.E.M. number of signs displayed by mice during the 20-min observation period.

Hyperalgesia. Hyperalgesia was assessed using two thermal pain models: tail-flick and hot-plate tests. After the latency in seconds to the tail-flick response was recorded, mice were then placed into a 10-cm wide glass cylinder on a hot plate (Thermojust Apparatus, Richmond, VA) maintained at 55°C. The normal latency (reaction time for jumping or paw-licking) was recorded.

Elevated Plus-Maze. An elevated plus-maze, prepared with gray Plexiglas, consisted of two open arms (23 × 6 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. Results were expressed as percentage of time spent in open arms.

Statistical Analysis

Withdrawal scores are expressed as the mean ± S.E.M. of 6 to 12 subjects. Statistical analysis of all behavioral studies was performed with mixed-factor ANOVA. Two-way, repeated-measure ANOVAs were used to analyze response latency, overall and individual somatic signs, time in the open arm with two levels of the between-treatments groups (nicotine or saline), and antagonist dose as the within-subjects factor or time after nicotine cessation. Significant overall ANOVAs are followed by post hoc comparisons when appropriate (Fisher’s protected least significant test). P values of less than 0.05 were considered to be significant.

Results

Spontaneous Nicotine Withdrawal after Different Doses of Nicotine. Spontaneous withdrawal from chronic nicotine administration significantly induced different measures of withdrawal signs after infusion of increasing doses of nicotine...
nicotine for 14 days, as illustrated in Fig. 1. Spontaneous nicotine withdrawal increased the number of somatic signs (Fig. 1A) and decreased the time spent in open arms of the plus-maze test (Fig. 1B). At a dose of 24 mg/kg/day, paw tremors (with a mean of 7 ± 3) and head shakes (with a mean of 5 ± 2) were the most prevalent somatic signs observed. In addition, an increase in “backing” signs was also noted (3 ± 0.5). The effect was dose-dependent in both measures with no significant changes at the lowest dose of 6 mg/kg/day of nicotine. A similar pattern was observed when mice were tested for hyperalgesia in the tail-flick and hot-plate assays. A “ceiling” withdrawal was however observed at higher infused dose of nicotine. The effect on the time spent in the open arm of the plus-maze decreased after withdrawal in a dose-related manner with no significant change in the number of crossings between arms (data not shown).

The increase in somatic signs (Fig. 2A) and the time spent in open arms (Fig. 2B) were reversed by pretreatment with nicotine in a dose-dependent manner. A low dose (0.1 mg/kg) of nicotine partially reversed withdrawal signs in both measures. Nicotine at 0.5 mg/kg totally reversed withdrawal signs as well as a dose of 2 mg/kg of metanicotine, a nicotinic agonist with preferential affinity at α4β2* neuronal nicotinic receptors. A similar reversal was also observed in the tail-flick and hot-plate assays (data not shown).

**Time Course of Spontaneous Nicotine Withdrawal.** To establish the duration of nicotine withdrawal signs, mice were infused with nicotine (24 mg/kg/day) for 14 days. Following removal of the pumps on day 15, the animals were examined for somatic signs (Fig. 3A) as well as in the elevated plus-maze (Fig. 3B) for the next several days. As noted in Figs. 1 and 2, somatic signs were prominent shortly after pump removal, and the signs remained prominent through day 4. There was also a significant attenuation in the time spent in the open arm of the plus-maze after pump removal. Although there is an appearance of decreased time in the open arms over several days, it was only significant at days 2 and 3.

**Precipitated Nicotine Withdrawal after Challenge with Various Nicotinic Antagonists.** As shown in Fig. 4, nicotine-dependent mice injected s.c. with mecamylamine displayed far more somatic withdrawal signs than were displayed in the control group. Significant increase in paw tremor, head shakes, and backing was observed after

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**Fig. 1.** Spontaneous withdrawal following termination of chronic nicotine infusion using minipumps. Minipumps were removed from animals at day 15, and withdrawal data were collected 24 h later. Somatic withdrawal signs (A), percentage of time spent in open arms (B), hot-plate time (C), and tail-flick time (D) latencies were measured after cessation of 6, 24, and 48 mg/kg/day doses of chronic nicotine. Sal, saline; Nic, nicotine. Each point represents the mean (seconds) ± S.E. of 8 to 12 mice. * P < 0.05 compared with saline correspondent zero time point.
mecamylamine injection in a dose-dependent manner. Injection of dihydro-β-erythroidine, a competitive nicotinic antagonist, produced a different withdrawal profile of somatic signs. At doses of 1.5 and 3 mg/kg, it failed to significantly elevate the signs seen after mecamylamine injection. The only significant sign seen was an increase in the appearance of writhings at the highest dose tested. MLA, an α7 antagonist, at a dose of 7.5 mg/kg produced a significant elevation only in paw tremors. However, when total signs were considered, it produced a mild but statistically significant increase in somatic signs. Finally, hexamethonium, a peripheral nicotinic antagonist, elicited a profile similar to that of MLA by only increasing paw tremors. However, it failed to produce a significant increase in total signs. In another measure of withdrawal, mecamylamine dose-dependently induced hyperalgesia in both tail-flick and hot-plate assays (Fig. 5, A and B). Hyperalgesia was only observed in the hot-plate but not the tail-flick test after challenge with MLA. Hexamethonium and dihydro-β-erythroidine failed to engender any significant hyperalgesia in both tests. When tested in the elevated plus-maze, dihydro-β-erythroidine was the only antagonist that produced a significant decrease in time spent in the open arms.

**Influence of Duration of Nicotine Exposure on the Magnitude of Precipitated Nicotine Withdrawal.** Different measures of precipitated withdrawal signs after 7, 14, 30, and 60 days of nicotine exposure are shown in Table 1. Significant increases in the somatic signs of withdrawal were observed as early as 7 days of nicotine exposure and continued to the same degree even after 60 days of infusion. However, statistically significant changes in elevated plus-maze performance were only evident after 60 days of exposure of nicotine. Hyperalgesia in the hot-plate test was evident after 2 weeks of exposure and disappeared at a longer duration of infusion. In the tail-flick test, however, hyperalgesic withdrawal signs were maintained at 4 and 8 weeks of exposure with a significant increase in its intensity.

**Effects of Multiple Injections of Mecamylamine on the Magnitude of Precipitated Nicotine Withdrawal.** As reported in Table 2, the intensity of somatic withdrawal...
signs after multiple exposure of mecamylamine (2 mg/kg, s.c.) decreased significantly after the third injection of the antagonist. An increase in the elevated plus-maze performance was however observed after the third injection of the antagonist. No change in the degree of hyperalgesia was seen after multiple challenges with mecamylamine in the hot-plate test.

Effects of Genotype on Precipitated Nicotine Withdrawal Signs. The development of tolerance to nicotine after chronic exposure was reported in different outbred and inbred mouse strains. To determine whether nicotine withdrawal syndrome was not unique to outbred mice, we investigated the development of nicotine withdrawal in two inbred strains. Nicotine infused at 24 mg/kg/day for 14 days in C57 and 129 strains produced a very different withdrawal syndrome profile (Fig. 6). In contrast to 129 strain mice, C57 mice challenged with an s.c. dose of mecamylamine at 2 mg/kg produced a significant increase in somatic signs, time spent in the open arms, and hyperalgesia in both tests (Fig. 6A).

Discussion

The major findings of our study are: 1) that a nicotine withdrawal syndrome with both somatic and affective signs can be observed in the mouse whether by cessation of nicotine chronic exposure (spontaneous) or by acute challenge with various nicotinic antagonists (precipitated); 2) that nicotine withdrawal signs seem to be mediated by multiple neuronal nicotinic subtypes; and 3) that genotypic factors, withdrawal history, and dose and duration of nicotine exposure influence the development of nicotine withdrawal and its severity.

The observed nicotine abstinence in the mouse displayed a variety of withdrawal signs that were dose-dependent and attenuated by a single dose of nicotinic agonists including nicotine and metanicotine, a nicotinic agonist with preferential affinity to neuronal nicotinic receptors (Bencherif et al., 1996). Furthermore, it was precipitated by a single dose of mecamylamine, a noncompetitive nicotinic antagonist. These results show clearly that our mouse model meets important criteria of validity which we will be able to use in future investigations. In general, somatic signs observed in our results resemble those described in mice (Isola et al., 1999) and rats (Malin et al., 1992); however, we failed to observe signs such as teeth chattering/chews, jumping, writhes, and ptosis reported in these studies and others. Species (mice versus rats), the route and mode of administration [repeated injections (Isola et al., 1999) versus continuous infusion], the dose of nicotine, and other methodological approaches may underlie the differences between studies. For the first time, our results provide evidence that chronic exposure of nicotine to mice produces both somatic and affective signs (anxiety-like effect in the plus-maze test) after terminating nicotine treatment. The nicotine withdrawal in mice was mild but prolonged. The signs peaked at about 24 to 48 h after spontaneous withdrawal and lasted for almost 4 days similar to the time course described in rats and mice (Epping-Jordan et al., 1998; Isola et al., 1999).

Our results with the different antagonists suggest the involvement of several nicotinic subtypes in nicotine withdrawal. Mecamylamine, a noncompetitive nicotinic antagonist with a preferential effect on α3β4* receptor subtype (Papke et al., 2001), precipitated both somatic signs and hyperalgesia but not the plus-maze behavior after nicotine withdrawal. Anxiety-like effect in the plus-maze test and somatic signs were also precipitated with the competitive nicotinic antagonist dihydro-β-erythroidine, a relatively selective α4β2* blocker. The role of α7 in nicotine withdrawal is illustrated by the effects of MLA, an α7 agonist. Indeed, MLA precipitated hyperalgesia (but not plus-maze behavior) and mild but significant somatic signs. A report by Nomikos et al. (1999) showed that MLA injected in the ventral tegmental area precipitated withdrawal signs in nicotine-dependent rats, suggesting that α7 nicotinic receptors are involved in nicotine withdrawal.
Previous reports (Hildebrand et al., 1997; Watkins et al., 2000) suggest the involvement of peripheral nicotinic receptors in nicotine withdrawal. Systemic administration of hexamethonium, a peripheral nicotinic antagonist, precipitated somatic but not affective withdrawal signs in rats. Results from our mice studies confirm the involvement of peripheral nicotinic receptors in somatic withdrawal signs. We also observed a precipitated hyperalgesia in the tail-flick but not the hot-plate test, suggesting the possible contribution of peripheral nicotinic receptors in affective signs. It is important to note, however, that in addition to neuronal receptors, peripheral nAChRs were shown to mediate nicotine's antinociceptive effects in the tail-flick but not the hot-plate assay (Marubio et al., 1999). It is therefore possible that hexamethonium-precipitated hyperalgesia reflects the involvement of peripheral but not central nicotinic receptors. Our data complement the results of recent studies that addressed the issue of involvement of different subtypes of nAChRs. Taken together, our data suggest that the activity of many subtypes of nAChRs is modulated during nicotine dependence.

Our present study examined for the first time in a systematic way the influence of the duration of nicotine exposure and repeated withdrawal episodes. Increasing the duration of nicotine exposure (from 7 to 60 days) and increasing nicotine dose augmented the severity of affective (plus-maze behavior) and somatic signs of nicotine withdrawal. Our findings are consistent with previous clinical results that demonstrated that the levels of nicotine and its main metabolite cotinine in serum or saliva correlated positively with withdrawal severity (McNeill et al., 1986) or relapse (Piascik et al., 2000). Repeated precipitated withdrawals by mecamylamine during chronic nicotine exposure significantly increased the intensity of affective (anxiety-like behavior) but decreased the severity of somatic signs of nicotine withdrawal. Unfortunately, relating our data to human studies is difficult since repeated spontaneous withdrawals from chronic nicotine use was not performed in our study.

Our results with two different inbred mouse strains extend previous observations that genetic factors influence the development of tolerance to nicotine after chronic exposure (Marks et al., 1991). The two strains tested displayed a

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Fig. 4. Total and individual somatic withdrawal signs precipitated by various nicotinic antagonists during chronic nicotine infusion (24 mg/kg/day for 14 days) or saline. Mice received an acute s.c. injection of either saline or nicotinic antagonist, and withdrawal signs were measured for 20 min immediately afterward. S, saline; M1, mecamylamine 1 mg/kg; M3, mecamylamine 3 mg/kg; D1.5, dihydro-β-erythroidine 1.5 mg/kg; D3, dihydro-β-erythroidine 3 mg/kg; M7.5, MLA 7.5 mg/kg; H1, hexamethonium 1 mg/kg. Each point represents the mean ± S.E. of 8 to 12 mice. *, P < 0.05 compared with saline.
different nicotine withdrawal in both affective and somatic components. The withdrawal severity of nicotine differs between the two strains, with nicotine withdrawal in C57 being more severe than in the 129 strain. This difference suggests that two different neurochemical mechanisms may be activated by nicotine withdrawal. One of the strains we included in our study (129/SvEv) has recently been used rather extensively as the genetic background for the development of tar-

**TABLE 1**

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<th>Week 2</th>
<th>Week 4</th>
<th>Week 8</th>
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<td></td>
<td></td>
<td></td>
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<td>17 ± 2*</td>
<td>11 ± 3*</td>
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<tr>
<td><strong>Plus-maze (% open)</strong></td>
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<td></td>
<td></td>
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<td>29 ± 3</td>
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<td><strong>Tail-flick (seconds)</strong></td>
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*P < 0.05 compared with saline treatment.
same mice were injected with mecamylamine (2 mg/kg, s.c.) and assessed for withdrawal as described under Materials and Methods. Results are expressed the mean ± S.E.M. of 6 to 8 mice.

<table>
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<td>Hot-plate (seconds)</td>
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* P < 0.05 compared with saline treatment.

A

Fig. 6. Nicotine withdrawal signs precipitated by an acute dose of mecamylamine (2 mg/kg, s.c.) in C57/BL (A) and 129/SvEv (B) inbred mice during chronic nicotine infusion (24 mg/kg/day) or saline for 14 days. Mice received an acute s.c. injection of either saline or nicotinic antagonist, and withdrawal signs were measured immediately afterward. Elevated plus-maze performance was measured as percentage of time spent in open arms (% open) and hyperalgesia as time (seconds) spent in the tail-flick and the hot-plate tests. Each point represents the mean ± S.E.M. of 8 to 12 mice. * P < 0.05 compared with saline.

B

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References


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