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Characterization of spontaneous and precipitated nicotine withdrawal in the mouse

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ABBREVIATIONS : nAChR, Acetylcholine nicotinic receptor; CNS, Central nervous system; s.c., subcutaneous injection; methyllycaconitine, MLA;

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 sites mechanisms to withdrawal signs using various nicotinic antagonists. Our results showed that spontaneous nicotine withdrawal increased the number of somatic sign, 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 6 mg/kg/day of nicotine. 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 MLA) 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 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 exits, 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 (Acri, 1994; Helton et al., 1993). 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 (Hildebrand et al., 1997; Malin et al., 1992; Epping-Jordan et al., 1998; Bancroft and Levin, 2000) (see however (Corrigall et al., 1989; Helton et al., 1993; Stolerman et al., 1973; 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 hours 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 protracted. 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 if 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 (20-25g) obtained from Harlan Laboratories (Indianapolis, IN) were used throughout the study. Male C57BL/6J and 129/SvEv inbred mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in groups of six and had free access to food and water. Animals were housed in an AALAC 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, Sharp and Dohme & Co. (West Point, PA). (-)-Nicotine was obtained from Aldrich Chemical Company, Inc. (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 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 mini-pumps [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 it is returned to its home cage. Animals were tested 14 days later. For spontaneous withdrawal studies, mini-pumps were removed at day 15, and withdrawal data were collected at 24-h intervals for seven 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 mini-pumps [model 2004 (28 days); Alza Corporation, Palo Alto, CA)] filled with either (-)-nicotine (24 mg/kg/day) or sterile physiological saline solutions for 28 days. At day 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-days Alzet osmotic mini-pumps. At day 28, the empty mini-pumps were removed under anesthesia and new 28-days mini-pumps were implanted. Testing the animals was done at the end of this almost two-month treatment regimen.

Dependence assessment. 24 hours after mini-pumps 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 min later, animals were evaluated for hyperalgesia. Experimenters will be blind to the treatment in all experiments.

1. 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, retropulsion, writhing, scratching, backing, piloerection and straub tail. Somatic signs were calculated as the mean and SEM number of signs displayed by mice during the 20-min observation period.

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

3. Elevated plus maze: An elevated plus maze, prepared with gray Plexiglass, consisted of two open arms (23 x 6.0 cm) and two enclosed arms (23 x 6 x 15 cm in wall height) that

extended from a central platform (5.5 x 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 % time spent in open arms.

Statistical analysis. Withdrawal scores are expressed as the mean ± SEM of 6-12 subjects. Statistical analysis of all behavioral studies was performed with mixed-factor ANOVA. Two-way repeated-measures ANOVAs were used to analyse 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 ANOVA are followed by post hoc comparisons when appropriate (Fisher 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 for 14 days, as illustrated in Figure 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 were 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 hyperlagesia 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 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) was 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 $\alpha 4\beta 2^*$ 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

In order 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) and in the elevated plus maze (Fig. 3B) for the next several days. As noted in Figures 1 & 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. While 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 Figure 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, backing were observed after mecamylamine injection in a dose-dependent manner. Injection of dihydro-β-erythroidine, a competitive nicotinic antagonist, produced a different somatic signs withdrawal profile. 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, hexamthonium, 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 other measure of withdrawal, mecamylamine dose-dependently induced hyperlagesia in both tail-flick and hot-plate assays (Figure 5 A & B). Hyperlagesia 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 hyperlagesia 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 measure of precipitated withdrawal signs after 7, 14, 30 and 60 days of nicotine exposure are shown in Table 1. Significant increase in somatic signs 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 was only evident after 60 days of exposure of nicotine. Hyperlagesia in the hot-plate test was evident after 2 weeks of exposure and disappeared at longer duration of infusion. In the tail-flick test,

however, hyperalgesic withdrawal signs were maintained in the 4 and 8-weeks 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 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. In order to determine if 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 (Figure 6). In contrast to 129 mice, C57 mice challenged with a s.c. dose of mecamylamine at 2 mg/kg produced a significant increase in somatic signs, time spent in the open arms and hyperlagesia in both tests (Figure 6A).

Discussion

The major findings of our study are (i) 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), (ii) that nicotine withdrawal signs seem to be mediated by multiple neuronal nicotinic subtypes, and (iii) that genotypic factors, withdrawal history, 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, which were dose-dependent and were 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 non-competitive nicotinic antagonist. These results show clearly that our mouse model meet important criteria of validity which will enable us to use it for future investigations. In general, somatic signs observed in our results resemble that described in mice (Isola et al., 1999) and rats (Malin et al., 1992). However, we failed to observe signs such 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. Our studies, for the first time, 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 24-48 h after spontaneous withdrawal and lasted for almost 4 days similar to that described in mice and rats (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 non-competitive nicotinic antagonist with a preferential effect on $\alpha 3\beta 4^*$ receptor subtype (Papke et al., 2001), precipitated both somatic and hyperalgesia but not plus maze behavior of 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 antagonist. Indeed, MLA precipitated hyperalgesia (but not plus-maze behavior) and a mild but significant somatic signs. Recent report by Nomikos et al. (1999) showed that MLA injected in the VTA 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 hyperlagesia 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 hyperlagesia reflects the involvement of peripheral but not central nicotinic receptors. Our data complement the results of recent studies which addressed the issue of the involvement of different subtypes nAChRs. Taken together, our data suggest that the activity of many subtypes of nAChRs are 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 the levels of nicotine and its main metabolite cotinine in serum or saliva correlated positively with withdrawal severity (McNeill et al., 1986) or relapse (Piasecki 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 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 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 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 targeted mutant mice. The 129 family of substrains is genetically complex, but we have used a single substrain in our experiments and relatively very few behavioral data were available (including the degree of tolerance) on the effects of nicotine in this strain. The genetic differences in nicotine withdrawal sensitivity reported here can also be due to difference in nicotine pharmacokinetics between the two strains. However, a direct assessment of this possibility is beyond the scope of the present investigations,

In summary, the results of our study demonstrate that our mouse nicotine withdrawal model using chronic s.c. infusion will be useful for studying the pharmacological, biochemical and genetic mechanisms involved in nicotine dependence. It provides a model, which mimics some of the smokers' withdrawal signs and uses a regimen that avoids stressful chronic intermittent s.c. injections. Our present results suggest that withdrawal from nicotine can be modulated by daily nicotine intake, duration of nicotine exposure and withdrawal history.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1 Spontaneous withdrawal following termination of chronic nicotine infusion using minipumps. Mini-pumps were removed from animals at day 15, and withdrawal data were collected at 24h after. Somatic withdrawal signs (Fig. 1A), % time spent in open arms (Fig. 1B), hot-plate time (Fig. 1C) and tail-flick time (Fig. 1D) 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 (sec) ± SE of 8 to 12 mice. *P < 0.05 compared to saline correspondent zero time point.

Fig. 2 Reversal of nicotine somatic (Fig. 2A) and decrease in time spent in open arms (% open) (Fig. 2B) withdrawal signs with an acute injection of nicotine (0.1 and 0.5 mg/kg) and metanicotine (2 mg/kg) in mice infused chronically with nicotine (24 mg/kg/day for 14 days). Mini-pumps were removed from animals at day 15, and withdrawal data were collected 5 min after injection of nicotinic agonists. Sal = Saline; Nic = Nicotine; Meta = Metanicotine. Each point represents the mean ± SE of 8 to 12 mice. *P < 0.05 compared to saline/saline.

Fig. 3 Time-course of spontaneous withdrawal following termination of chronic nicotine infusion. Mice were infused chronically with nicotine (24 mg/kg/day) (—●—) or saline (—■—) for 14 days. Mini-pumps were then removed from animals at day 15, and somatic (Fig. 3A) and time spent in open arms (% open) (Fig. 3B) withdrawal data were collected at 24-h intervals for seven days following termination of nicotine. Each point represents the mean ± SE of 8 to 12 mice. *P < 0.05 compared to saline.

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 immediately after withdrawal signs were measured for 20 min. 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 ± SE of 8 to 12 mice. *P < 0.05 compared to saline.

Fig. 5 Non-somatic withdrawal signs (Elevated plus maze performance and hyperalgesia) 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 thereafter withdrawal signs were measured immediately. Elevated plus maze performance was measured as % time spent in open arms (% open) and hyperalgesia as time (sec) spent in the tail-flick and the hot-plate tests. 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 mk/kg; H1 = Hexamethonium 1 mg/kg. Each point represents the mean ± SE of 8 to 12 mice. *P < 0.05 compared to saline.

Fig. 6 Nicotine withdrawal signs precipitated by an acute dose of mecamylamine (2 mg/kg, s.c.) in C57/BL (Fig. 6A) and 129/SvEv (Fig. 6B) 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 immediately after withdrawal signs were measured. Elevated plus maze performance was measured as % time spent in open arms (% open) and hyperalgesia as time (sec) spent in the tail-flick and the hot-plate tests Each point represents the mean ± SE of 8 to 12 mice. *P < 0.05 compared to saline.

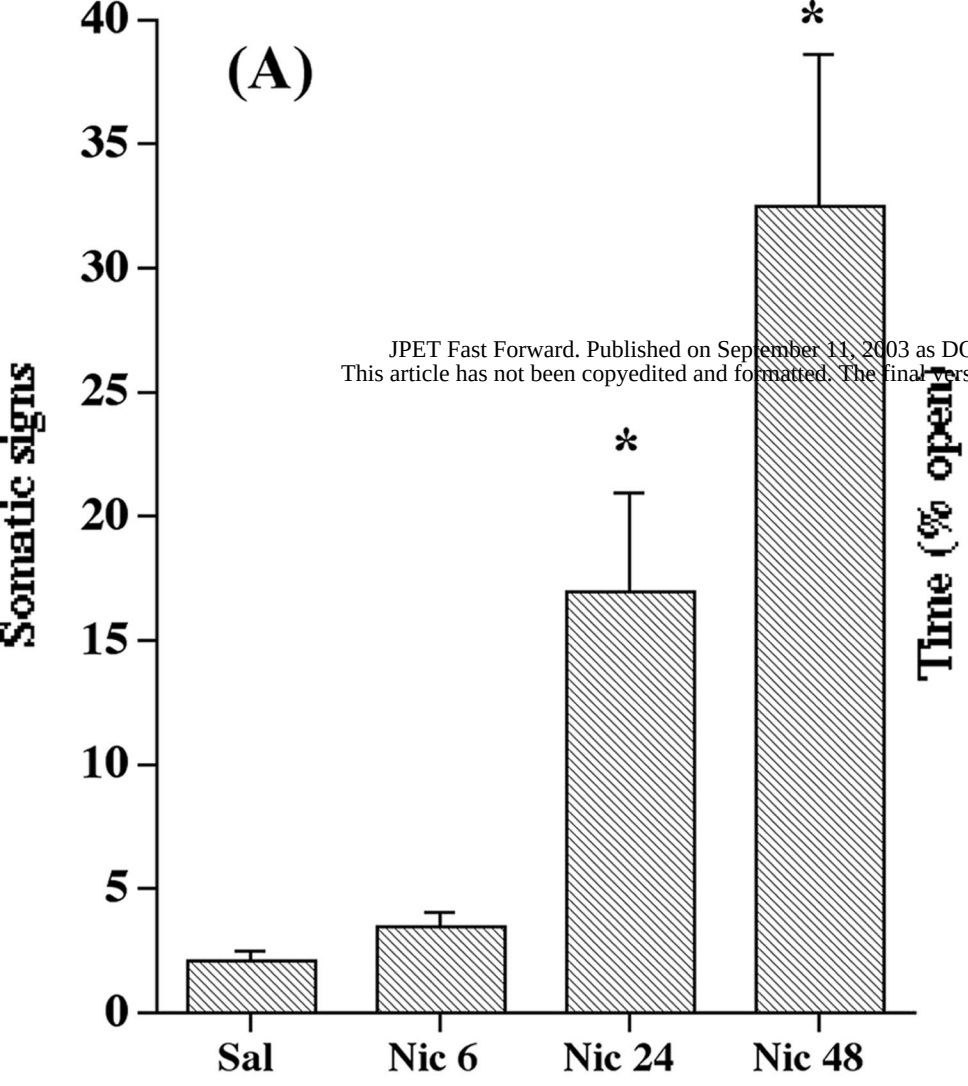
Table 1: Influence of the duration of infusion on the severity of nicotine withdrawal. Mice were implanted with osmotic mini-pumps for 7, 14, 30 and 60 days filled with either (-)-nicotine (24 mg/kg/day) or sterile physiological saline solutions. At days 7, 14, 30 and 60, different groups of mice were injected with mecamylamine (2 mg/kg, s.c.) and assessed for withdrawal as described in the Methods section. Results are expressed the mean ± S.E.M. of 6 - 8 mice. *P < 0.05 compared to saline treatment.

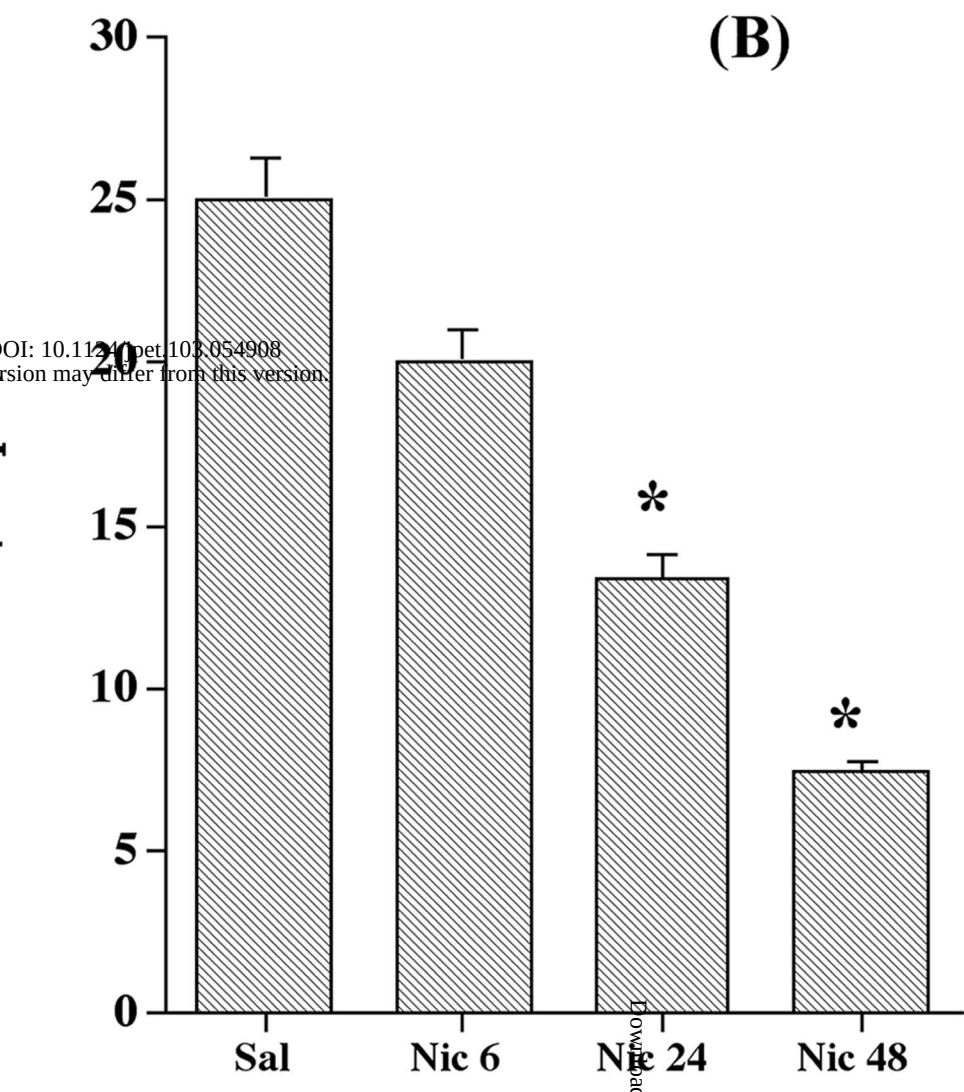
Measure (Mean ± SEM)		Week 1	Week 2	Week 4	Week 8
Somatic Signs (Mean/animal)	Saline	3 ± 1	2.5 ± 0.5	2 ± 0.3	4 ± 1
	Mecamylamine	16 ± 3*	17 ± 2*	11 ± 3*	23 ± 2*
Plus-Maze (% Open)	Saline	31 ± 4	29 ± 3	32 ± 6	25 ± 3
	Mecamylamine	29 ± 4	21 ± 3	20 ± 3	12 ± 3*
Hot-Plate (Sec)	Saline	10 ± 0.4	11 ± 0.3	9.6 ± 0.4	10.5 ± 0.5
	Mecamylamine	8.7 ± 0.4	7 ± 0.3*	8.5 ± 0.3	7.8 ± 0.2
Tail-Flick (Sec)	Saline	6 ± 0.4	7.5 ± 0.3	7.7 ± 0.5	7.8 ± 0.5
	Mecamylamine	5.4 ± 0.4	5.5 ± 0.5*	5 ± 0.4*	5.1 ± 0.5*

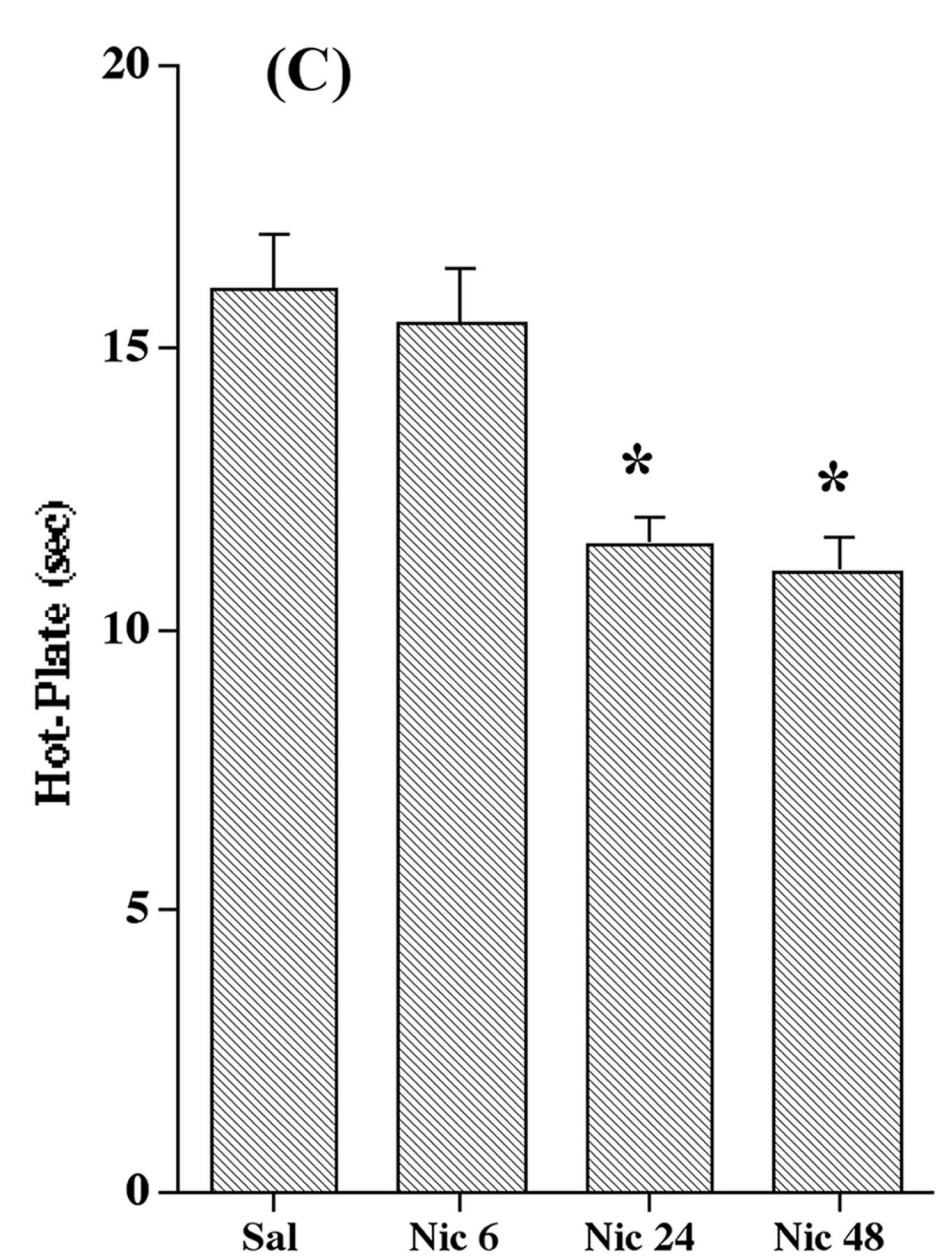
Table 2: Influence of multiple exposure to mecamylamine on the severity of nicotine withdrawal. Mice were implanted with osmotic mini-pumps for 30 days filled with either (-)-nicotine (24 mg/kg/day) or sterile physiological saline solutions. At day 7, 14, 21 and 28, the same mice were injected with mecamylamine (2 mg/kg, s.c.) and assessed for withdrawal as described in the Methods section. Results are expressed the mean ± S.E.M. of 6 - 8 mice. *P < 0.05 compared to saline treatment.

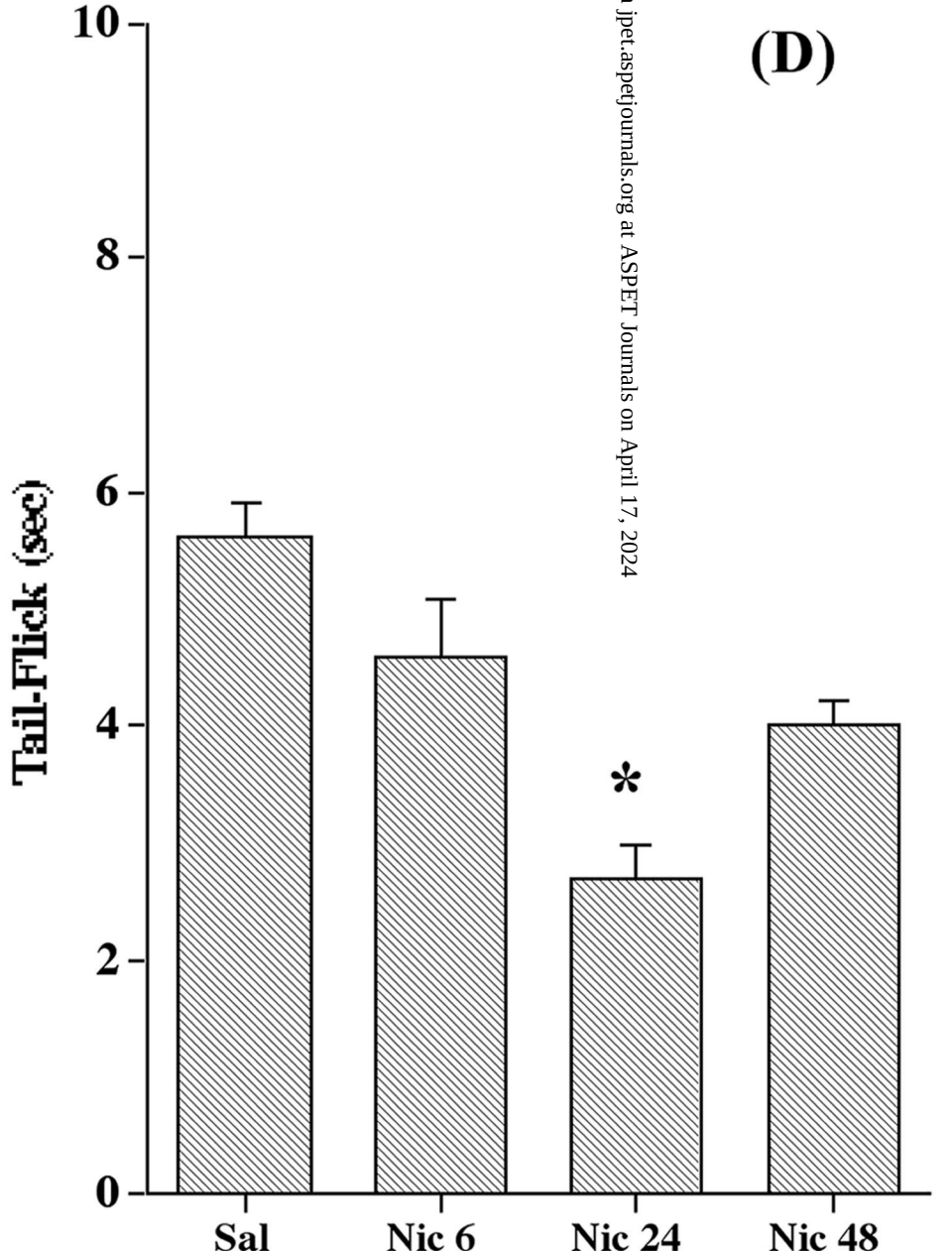
Measure (Mean ± SEM)		Injection 1	Injection 2	Injection 3	Injection 4
Somatic Signs (Mean/animal)	Saline	1 ± 0.5	1.5 ± 0.5	2 ± 0.7	1.5 ± 0.7
	Mecamylamine	14 ± 2*	11.6 ± 2*	7.5 ± 0.5*	9.5 ± 1*
Plus-Maze (% Open)	Saline	23 ± 5	24 ± 3	21 ± 4	22 ± 4
	Mecamylamine	20 ± 4	15 ± 2	8.5 ± 2*	7 ± 2*
Hot-Plate (Sec)	Saline	10.5 ± 0.5	11 ± 0.4	10.5 ± 0.3	10 ± 0.7
	Mecamylamine	7.5 ± 0.2*	7.1 ± 0.3*	8.1 ± 0.2*	8.3 ± 0.3

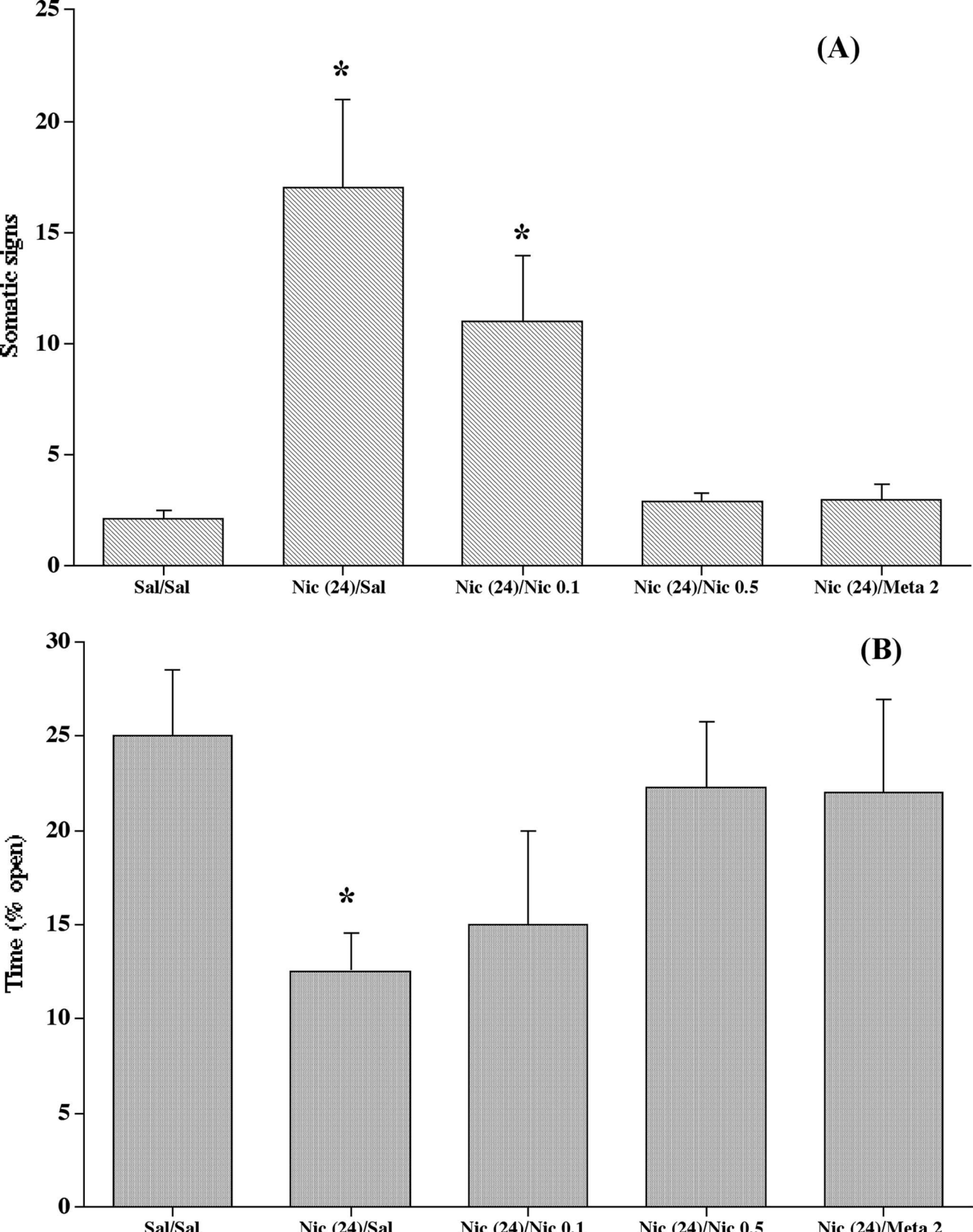
Index words: Nicotine Withdrawal Precipitated Genotype Mice

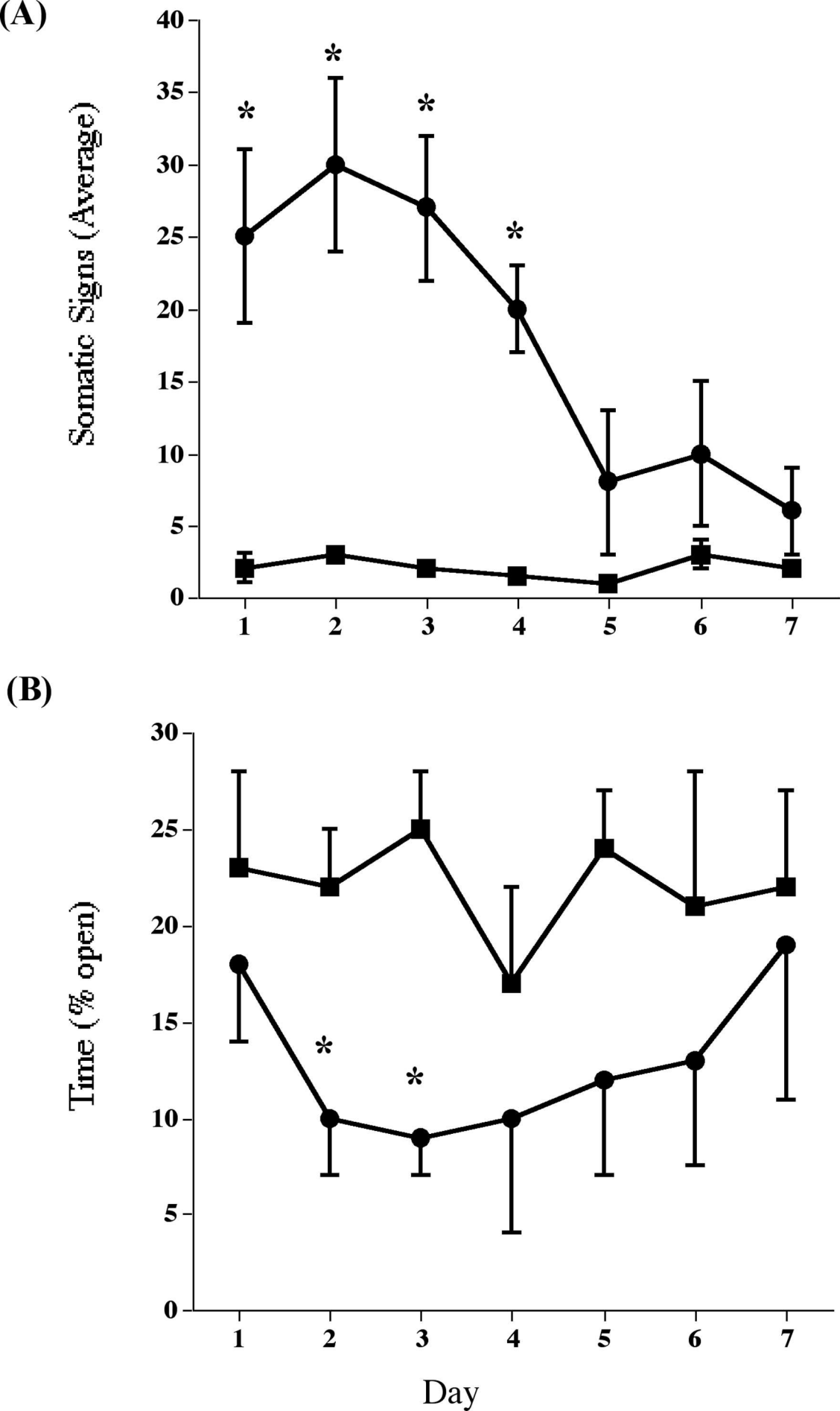


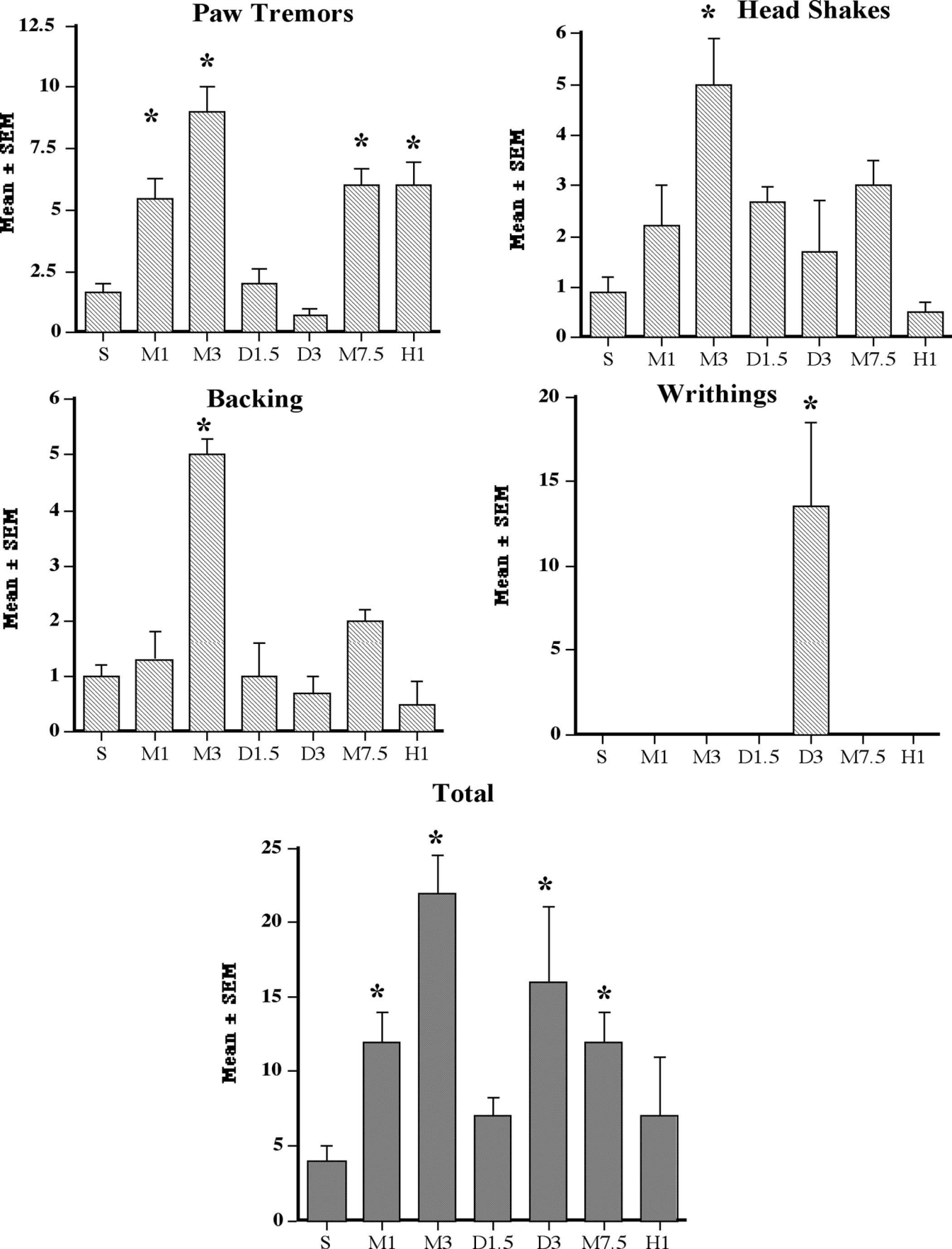


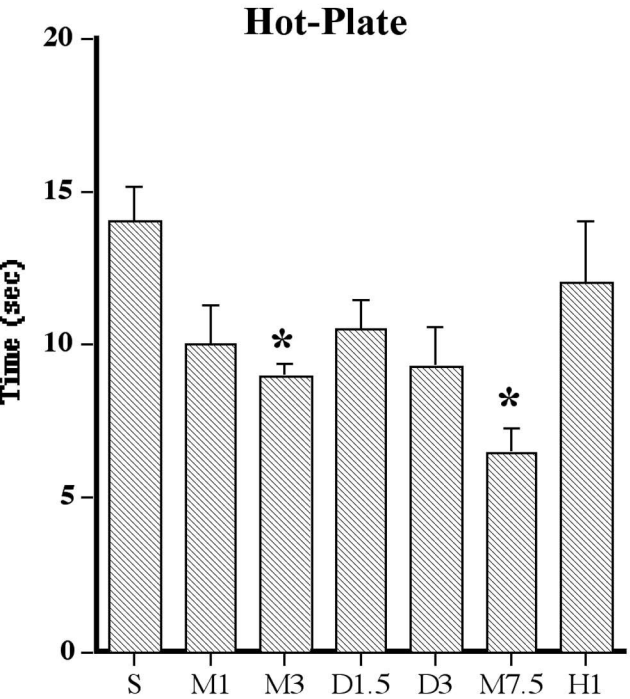


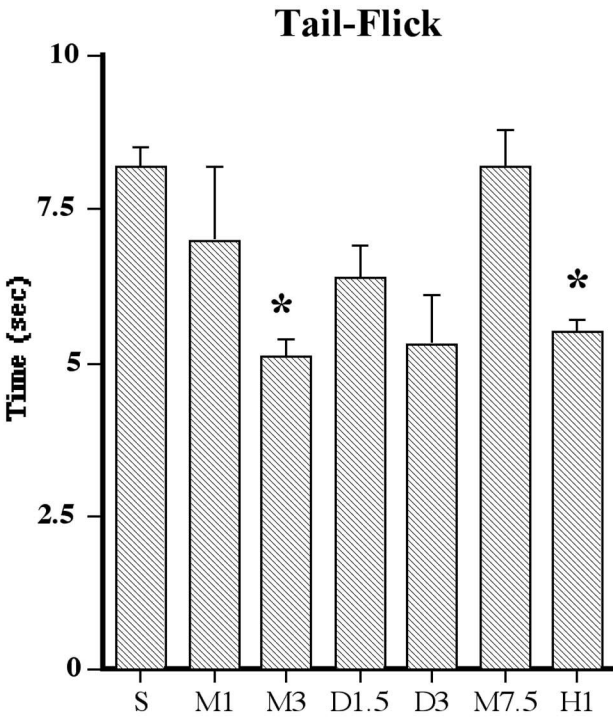


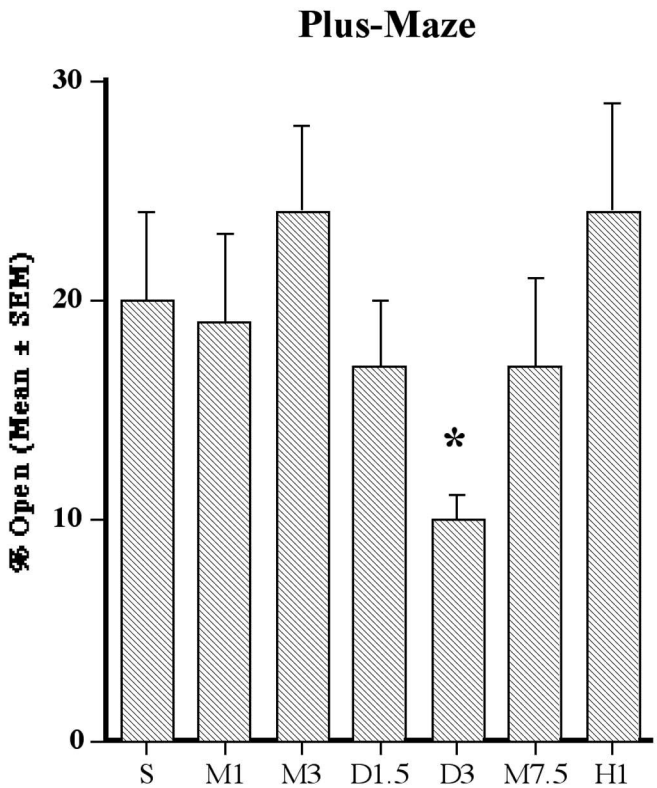

(A)
Somatic signs
25
20
15
10
5
0
*
*
Sal/Sal
Nic (24)/Sal
Nic (24)/Nic 0.1
Nic (24)/Nic 0.5
Nic (24)/Meta 2
(B)
Time (% open)
30
25
20
15
10
5
0
*
Sal/Sal
Nic (24)/Sal
Nic (24)/Nic 0.1
Nic (24)/Nic 0.5
Nic (24)/Meta 2


(A)
Somatic Signs (Average)
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
*
*
*
*
(B)
Time (% open)
30
25
20
15
10
5
0
1
2
3
4
5
6
7
Day
*
*


Paw Tremors
Mean ± SEM
12.5
10
7.5
5
2.5
0
S
M1
M3
D1.5
D3
M7.5
H1
Head Shakes
Mean ± SEM
6
5
4
3
2
1
0
Backing
Mean ± SEM
Writhings
Mean ± SEM
20
15
10
5
0
Total
Mean ± SEM
25
20
15
10
5
0


Hot-Plate
Time (sec)
20
15
10
5
0
*
*
S
M1
M3
D1.5
D3
M7.5
H1


Tail-Flick
Time (sec)
10
7.5
5
2.5
0
*
*
S
M1
M3
D1.5
D3
M7.5
H1


Plus-Maze
% Open (Mean ± SEM)
30
20
10
0
*
S
M1
M3
D1.5
D3
M7.5
H1