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Differential Role of Nicotinic Acetylcholine Receptor Subunits in Physical and Affective Nicotine Withdrawal Signs

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Abstract

It has been suggested that the negative effects associated with nicotine withdrawal promote continued tobacco use and contribute to the high relapse rate of smoking behaviors. Thus, it is important to understand the receptor-mediated mechanisms underlying nicotine withdrawal to aid in the development of more successful smoking cessation therapies. The effects of nicotine withdrawal are mediated through nicotinic acetylcholine receptors (nAChRs); however, the role of nAChRs in nicotine withdrawal remains unclear. Therefore, we used mecamylamine-precipitated, spontaneous, and conditioned place aversion (CPA) withdrawal models to measure physical and affective signs of nicotine withdrawal in various nAChR knockout (KO) mice. 2, 7, and 5 nAChR KO mice were chronically exposed to nicotine through surgically implanted osmotic minipumps. Our results show a loss of anxiety-related behavior and a loss of aversion in the CPA model in 2 KO mice, whereas 7 and 5 KO mice displayed a loss of nicotine withdrawal-induced hyperalgesia and a reduction in somatic signs, respectively. These results suggest that 2-containing nAChRs are involved in the affective signs of nicotine withdrawal, whereas non-2-containing nAChRs are more closely associated with physical signs of nicotine withdrawal; thus, the nAChR subtype composition may play an important role in the involvement of specific subtypes in nicotine withdrawal.

Tobacco dependence is the leading cause of preventable mortality in the United States. Maintenance of this dependence is largely due to nicotine (nic), the main addictive component of tobacco (Stolerman and Jarvis, 1995; Bardo et al., 1999). Although there are smoking cessation therapies available, which include nicotine replacement therapies, the antidepressant bupropion (Zyban), and the partial nicotinic agonist varenicline (Chantix) (Cummings and Mahoney, 2006; Jorenby et al., 2006), the success rate of these therapies is modest.

Smoking cessation after chronic tobacco use produces a negative withdrawal syndrome. In humans, the nicotine withdrawal syndrome is characterized by somatic signs, which include bradycardia, gastrointestinal discomfort, and increased appetite, and affective signs, including irritability, anxiety, depressed mood, difficulty concentrating, disrupted cognition, and nicotine cravings (Stolerman and Shoaib, 1991; American Psychiatric Association, 1994; Mendrek et al., 2006). Studies have suggested that affective signs of nicotine withdrawal contribute more to relapse than somatic signs (Koob et al., 1993; Markou et al., 1998). Several groups, including our laboratory, have reported use of rodent models of the

nicotine withdrawal syndrome. From these models, studies have shown that somatic signs of nicotine withdrawal are mediated by central and peripheral nAChRs, whereas affective signs are mediated solely through central nAChR populations (Watkins et al., 2000).

Physical signs in rodents are measured as somatic signs (Malin et al., 1992; Hildebrand et al., 1997; Damaj et al., 2003), hyperalgesia (Salas et al., 2004; Grabus et al., 2005), and changes in locomotor activity (Hildebrand et al., 1999; Nomikos et al., 1999), whereas affective signs are typically measured as anxiety-related behaviors (Damaj et al., 2003), elevated reward thresholds (Kenny and Markou, 2001; Bruijnzeel and Markou, 2004), contextual fear conditioning (Davis et al., 2005), and conditioned place aversion (CPA) (Suzuki et al., 1999; Malin et al., 2006).

Some available studies have used these models to assess nAChR involvement in nicotine withdrawal behaviors, both pharmacologically, and using transgenic animals. It was shown that $\alpha 4$ KO mice did not display somatic signs after nicotine withdrawal, whereas nicotine-withdrawn $\alpha 7$ KO mice did not display withdrawal-induced hyperalgesia or decreases in locomotor activity (Salas et al., 2004; Grabus et al., 2005). Both studies implicate a role for these subunits in physical withdrawal signs. Pharmacologically, methyllycaconitine, an $\alpha 7$ antagonist, was also shown to precipitate mild somatic signs and changes in locomotor activity (Nomikos et al., 1999; Damaj et al., 2003). However, it was found that nicotine-dependent $\alpha 2$ KO mice displayed typical somatic signs after nicotine withdrawal, suggesting that this subunit is not involved in somatic nicotine withdrawal signs (Salas et al., 2004; Besson et al., 2006). Alternatively, the $\alpha 2$ -selective antagonist dihydro- β -erythroidine was shown to precipitate anxiety-related behavior and elevations in reward threshold in nicotine-dependent rodents, implicating a role for $\alpha 2$ -containing nAChRs in affective withdrawal behaviors (Damaj et al., 2003; Bruijnzeel and Markou, 2004). Despite the importance of affective nicotine withdrawal signs, few withdrawal studies assess the receptor-mediated mechanisms associated with this aspect of withdrawal. In addition, many studies use nAChR antagonists, which do not have high selectivity for specific subtypes. A complementary approach would be the use of transgenic mice for specific nicotinic receptors subunits. Indeed, the use of nAChR KO mice provides greater specificity than would be achieved using current pharmacological agents.

In the current study, we assessed two physical and two affective signs of nicotine withdrawal in various nAChR KO mice. The $\alpha 2$ nAChR subunit is a central, highly expressed subunit that coassembles with many $\alpha 4$ and $\alpha 6$ subunits in the mesocorticolimbic drug pathway to form functional receptors (Wada et al., 1989, 1990; Klink et al., 2001). The homomeric $\alpha 7$ nAChR is also a major subtype found on neurons in the mesocorticolimbic drug pathway and has been implicated in physical withdrawal behaviors. In addition, we evaluated the role of the $\alpha 5$ subunit in nicotine withdrawal behaviors. The $\alpha 5$ subunit cannot form functional receptors alone but can alter the calcium permeability, desensitization rate, and other biophysical and pharmacological properties of nAChRs depending on the subunit composition (Ramirez-Latorre et al., 1996; Gerzanich et al., 1998). The $\alpha 5$ subunit is also present centrally in the mesocorticolimbic pathway (Wada et al., 1990), as well as peripherally in sympathetic and parasympathetic ganglia (De Biasi, 2002). Using precipitated, spontaneous, and CPA models of nicotine withdrawal, the overall goal of our study was to determine the role of the $\alpha 2$, $\alpha 7$, and $\alpha 5$ nAChR subunits in both physical and affective signs of nicotine withdrawal using nAChR KO mice.

Materials and Methods

Animals

Mice 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 AM). Mice were approximately 8 to 10 weeks of age and weighed approximately 20 to 25 g at the start of the experiment. All experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Male mice were used for all experiments unless otherwise noted. In the pharmacological studies, C57BL/6 mice from Jackson Laboratory (Bar Harbor, ME) were used. Mice lacking the $\alpha 7$ subunit of the nicotinic receptor (C57BL/6 background) and wild-type (WT) litter mates were purchased from Jackson Laboratories (B6.129S7-*charna7tm1*bay, no. 003232; for information regarding initial breeders, see Orr-Urtreger et al., 1997). Breeding pairs of mice lacking the $\alpha 2$ subunit of the nicotinic receptor (C57BL/6 background) and wild-type litter mates were shipped from Institut Pasteur, Paris, France (for information regarding initial breeders, see Picciotto et al., 1995). Mice null for the $\alpha 5$ nicotinic receptor subunit (C57BL/6 background) and wild-type litter mates were shipped from Baylor College of Medicine, Houston, TX (for information regarding initial breeders, see Salas et al., 2003). For all experiments, $\alpha 7$ and $\alpha 5$ KO mice were backcrossed to at least eight to 10 generations, and $\alpha 2$ KO mice were backcrossed at least 10 to 12 generations. Mutant and wild-type controls were obtained from crossing heterozygote mice. This breeding scheme controlled for any irregularities that might occur with crossing solely mutant animals.

Drugs

(–)-Nicotine ditartrate salt was purchased from Sigma Chemical (Milwaukee, WI). Mecamylamine (mec) hydrochloride (2-methylamino-2,3,3-trimethylnorbornane) was a gift from Merck, Sharp, Dohme (West Point, PA). Drugs were dissolved in physiological saline (sal) (0.9% sodium chloride) and injected s.c. at a volume of 10 ml/kg body weight. Mecamylamine (2 mg/kg for precipitated studies or 3.5 mg/kg for CPA studies) and nicotine (36 mg/kg/day for minipumps) doses were based on published and unpublished studies from our laboratory. Mecamylamine doses were within a range of doses effective at blocking behavioral effects of nicotine and inducing nicotine withdrawal (Damaj et al., 2003).

Induction of Nicotine Dependence

Mice were implanted with Alzet osmotic minipumps (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 minipump flow rate, resulting in 36 mg/kg/day for 14 or 28 days. The minipumps were surgically implanted s.c. under sterile conditions with sodium pentobarbital anesthesia (35 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.

Nicotine Withdrawal Assessment

For mecamylamine-precipitated withdrawal studies, on the morning of day 15, mice previously infused with saline or nicotine were given s.c. injections with mecamylamine (2 mg/kg) or saline, and withdrawal signs were measured 10 min after injection. The mice were first evaluated for 5 min in the plus-maze test for anxiety-related behavior. The mice were then observed for somatic signs of withdrawal for 20 min. Hyperalgesia was evaluated

immediately after the somatic sign observation period. The specific testing sequence was chosen because prior studies in our laboratory showed that this particular scheme reduced within-group variability and produced the most consistent results. For spontaneous withdrawal studies, minipumps were removed under ether anesthesia on the evening of day 14. Testing was initiated on day 15, approximately 18 h after minipump removal. Spontaneous withdrawal experiments were conducted using the same testing scheme as mentioned with precipitated studies. All studies were conducted under blind conditions.

Elevated Plus Maze

An elevated plus maze, prepared with gray Plexiglas, consisted of two open arms (23×6.0 cm) and two enclosed arms ($23 \times 6 \times 15$ 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 allowed to roam freely between the open and closed arms. The time spent in the open and closed arms was automatically recorded by a photocell beam system. The test lasted 5 min, and the apparatus was thoroughly cleaned after removal of each animal. A decrease in the amount of time spent on the open arms was indicative of increased anxiety-related behavior. Results were expressed as the mean $\pm$ S.E.M. number of seconds spent in the open arms. As a control, we also measured the number of times each animal crossed from one side of the plus maze to the other, noted as the total average number of arm crosses. This was to ensure that the reduction in time spent on the open or closed arms was not a reflection of a lack of overall activity.

Somatic Signs

Mice were observed for 20 min in empty transparent activity cages (32×18 cm) for typical somatic withdrawal behaviors. Typical nicotine withdrawal signs that were tallied included head shakes, paw tremors, body tremors, and backing. Ptosis, curls, and jumps were also tallied collectively as “other” somatic signs. Results were expressed as the mean $\pm$ S.E.M. number of signs displayed by mice during the 20-min observation period.

Hyperalgesia

The nicotine withdrawal-induced hyperalgesia response was evaluated using the hot-plate test (Thermojust Apparatus). The hot-plate is a rectangular heated surface surrounded by Plexiglas and maintained at 52°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). The timer has an automatic cut-off of 40 s to avoid tissue damage. A decreased latency on the hot-plate was counted as increased pain sensitivity (hyperalgesia). Results were expressed as the mean $\pm$ S.E.M. latency (reaction time for jumping or paw licking) displayed by the mice.

CPA

The CPA paradigm is a valid measure of the negative affective state associated with nicotine withdrawal. It is a form of classic Pavlovian conditioning where the animal learns to avoid a compartment, which was previously paired with an aversive stimulus. Previous work using this model in the rat has shown that nicotine withdrawal is associated with a negative affective state, and place aversion to previously neutral environmental stimuli represents a motivational component in the maintenance of drug use (Suzuki et al., 1999). To date, this model has not been assessed in mice using nicotine. Nicotine CPA testing for 2, 5, and 7 KO mice was conducted using male and female KO and wild-type litter mates. Data from our laboratory indicated no sex differences in KO or wild-type mice in the CPA paradigm

[2 KO mice, $F(1,13) = 0.484$, $p = 0.5028$, all male wild-type mice were used; 5 wild-type mice, $F(1,12) = 1.088$, $p = 0.2661$, all male KO mice were used, all male 7 wild-type and KO mice were used]; therefore, data from male and female 2 and 5 mice were pooled. All mice were surgically implanted with 28-day minipumps containing either saline or nicotine. Mice were chronically exposed to nicotine or saline for 14 days before initiation and throughout the duration of CPA testing. The CPA protocol was conducted over the course of 4 days in a biased fashion. The CPA apparatus consists of a three-chambered box with a white compartment, a black compartment, and a center gray compartment. The black and white compartments also have different floor textures to help the mice further differentiate between the two environments. On day 1 of CPA testing, the mice were placed in the gray center compartment for a 5-min habituation period, followed by a 15-min test period. During habituation, mice did not have access to the other compartments. During the test period, mice were allowed to roam freely between compartments. The CPA boxes are connected to a computer, which records the amount of time the mouse spends on each side of the compartment. A prepreference score was determined for each mouse and was used to pair the mouse with mecamlamine (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 nonpreferred compartment for 30 min. No less than 4 h later, mice received an injection of mecamlamine and were immediately confined to their preferred compartment for 30 min. Day 4 was the drug-free test day. The procedure was the same as day 1 of the protocol, and a postpreference score was recorded. Aversion was counted as mice spending less time in their initially preferred compartment on test day when compared with time spent in the same compartment before drug conditioning.

Statistical Analysis

For all data, statistical analyses were performed using StatView (SAS, Cary, NC). Studies using transgenic mice were analyzed with two-way analyses of variance [with genotype (KO versus wild-type) and treatment (saline versus mecamlamine or saline versus nicotine) as between-subject factors] using the Newman-Keuls post-hoc test. Studies using only C57BL/6J mice were analyzed with one-way analyses of variance [with treatment (saline versus mecamlamine) as the between-subject factor] using the Newman-Keuls post-hoc test. p Values of less than 0.05 were considered significant.

Results

Evaluation of Affective and Physical Signs of Nicotine Withdrawal Using the Precipitated Model

To assess the involvement of specific nAChR subtypes in nicotine withdrawal, we adapted a nicotine withdrawal model that would allow us to measure both physical and affective aspects of nicotine withdrawal in one setting. Mecamlamine (2 mg/kg) precipitated significant nicotine withdrawal signs in C57BL/6J mice chronically exposed to nicotine (36 mg/kg/day) for 14 days. Our results show a significant reduction in the amount of time spent on the open arms of the elevated plus maze in nicotine-dependent mice treated with mecamlamine when compared with control groups, indicating an anxiety-related response in these mice [Fig. 1A; $F(3,28) = 4.158$, $p < 0.05$]. No significant changes in the number of crosses between arms were noted [$F(3,28) = 0.241$, $p = 0.7895$; Table 1]. The decrease in open arms time is not attributed to a lack of activity on the plus maze, as opposed to a preference for the closed arms or aversion for the open arms, because no differences in the average number of times the animals crossed from one side of the plus maze to the other for the different treatments was seen (Table 1). This indicates that the observed effects in the plus maze were not attributed to differences in activity between groups in our mecamlamine-precipitated model. Nicotine-dependent mice also showed enhanced nicotine

withdrawal somatic signs after mecamylamine injection [Fig. 1B; $F(3,28) = 74.130$, $p < 0.0001$], as well as significant nicotine withdrawal-induced hyperalgesia as measured by a decreased latency on the hot-plate [Fig. 1C; $F(3,28) = 7.774$, $p < 0.05$]. Saline minipump mice that received an injection of mecamylamine and nicotine minipump mice that received an injection of saline on test day did not differ from saline-saline control animals, indicating that the mecamylamine dose used did not produce effects on its own and that the nicotine minipump mice were not experiencing nicotine withdrawal on test day.

Role of the $\beta 2$ nAChR Subunit in Precipitated Nicotine Withdrawal

We used our 14-day nicotine withdrawal model to measure the affective and physical signs of nicotine withdrawal in nicotine-dependent $\beta 2$ KO mice. Our results in Fig. 2A show that nicotine-dependent $\beta 2$ KO mice displayed a loss of withdrawal induced anxiety-related behavior when compared with wild-type counterparts, indicated by no difference in the amount of time spent on the open arms of the place maze compared with control animals [$F(1,16) = 4.931$, $p < 0.05$ for main effects of treatment; $F(1,16) = 5.983$, $p < 0.05$ for main effects of genotype; no significant interaction]. No significant changes in the number of crosses between arms were noted [$F(1,16) = 0.435$, $p = 0.5159$; Table 1]. However, nicotine-dependent $\beta 2$ KO and wild-type mice both displayed significantly more somatic signs than saline-treated wild-type and KO mice [Fig. 2B; $F(1,16) = 164.450$, $p < 0.0001$ for main effects of treatment] and displayed a similar decrease in hot-plate latency, indicating a significant hyperalgesia response [Fig. 2C; $F(1,16) = 22.154$, $p < 0.05$ for main effects of treatment]. Saline control $\beta 2$ KO mice did not differ from wild-type counterparts in any withdrawal test.

Role of the $\alpha 7$ nAChR Subunit in Precipitated Nicotine Withdrawal

The role of $\alpha 7$ nAChR receptors in nicotine withdrawal is shown in Fig. 3. Nicotine-dependent wild-type and $\alpha 7$ KO mice spent significantly less time on the open arms of the plus maze than saline-treated mice, indicating the presence of an anxiety-related response [Fig. 3A; $F(1,4) = 48.876$, $p < 0.0001$ for main effects of treatment; no significant main effects of genotype or interaction; no significant changes in the number of crosses between arms were noted, $F(1,14) = 0.143$, $p < 0.7110$; Table 1]. Significant withdrawal somatic signs were also observed in nicotine-dependent wild-type and $\alpha 7$ KO mice compared with saline treated wild-type and KO mice [Fig. 3B; $F(1,14) = 108.401$, $p < 0.0001$]. Significant hyperalgesia was observed in nicotine-dependent wild-type mice after mecamylamine treatment; however, nicotine-dependent $\alpha 7$ KO mice showed a loss of nicotine withdrawal-induced hyperalgesia, indicated by no difference in hot-plate latency between saline mice and nicotine-dependent $\alpha 7$ KO animals [Fig. 3C; $F(1,14) = 5.217$, $p < 0.05$ for main effects of treatment; $F(1,14) = 7.772$, $p < 0.05$ for treatment $\times$ genotype interaction; no significant main effects of genotype]. Saline-treated KO mice did not differ from wild-type mice in any withdrawal test.

Assessment of $\beta 2$ and $\alpha 7$ nAChR KO Mice in a Spontaneous Withdrawal Model

It was important to demonstrate that the precipitated nicotine withdrawal behaviors observed in transgenic mice were not an assessment of the mecamylamine-dependent behavioral effects in nAChR subunits. Therefore, we used the spontaneous withdrawal model to assess nicotine withdrawal signs in $\beta 2$ and $\alpha 7$ KO mice 18 to 20 h after withdrawal from nicotine. Results of the spontaneous withdrawal assessment in $\beta 2$ KO mice are shown in Fig. 4. As observed in the mecamylamine-precipitated model, nicotine-withdrawn $\beta 2$ KO mice displayed significant somatic signs and hyperalgesia but a loss in anxiety-related behavior after cessation of nicotine treatment. Likewise, spontaneous withdrawal studies using $\alpha 7$ KO mice were comparable with mecamylamine-precipitated studies as shown in Fig. 5.

Nicotine-withdrawn 7 KO mice showed significant somatic signs and anxiety-related behavior but not withdrawal-induced hyperalgesia.

Role of the $\alpha 5$ nAChR Subunit in Precipitated Nicotine Withdrawal

The evaluation of nicotine withdrawal in 5 KO mice is shown in Fig. 6. Nicotine-dependent wild-type and 5 KO mice spent significantly less time on the open arms of the elevated plus maze than saline-treated wild-type and KO mice, indicating that an anxiety-related response was still present [Fig. 6A; $F(1,12) = 37.343$, $p < 0.0001$ for main effects of treatment]; no significant changes in the number of crosses between arms were noted [$F(1,12) = 0.165$, $p = 0.6921$; see Table 1]. Somatic sign observation of nicotine-dependent

5 KO mice revealed a significant reduction in paw tremors, backing, and total somatic signs compared with nicotine-dependent wild-type litter mates; however, total somatic signs in nicotine-dependent 5 KO mice were significantly higher than saline control mice [Fig. 6B; $F(1,12) = 196.130$, $p < 0.005$ for main effects of treatment; $F(1,12) = 21.100$, $p < 0.05$ for main effects of genotype; $F(1,12) = 25.418$, $p < 0.05$ for treatment $\times$ genotype interaction]. Both wild-type and 5 KO nicotine-dependent mice displayed a decreased latency on the hot-plate, indicating a significant hyperalgesia response [Fig. 6C; $F(1,12) = 23.986$, $p < 0.05$ for main effects of treatment]. There were no differences between saline-treated KO and wild-type mice for any withdrawal test.

Role of the $\beta 2$, $\alpha 7$, and $\alpha 5$ nAChR Subunits in Affective Signs Using the CPA Model

We adapted a nicotine CPA model to further assess affective withdrawal signs in transgenic mice. Figure 7 shows that a dose of 3.5 mg/kg of mecamylamine was sufficient to consistently precipitate aversion in C57BL/6J mice chronically exposed to nicotine for 14 days before conditioning. The dose of mecamylamine used did not precipitate aversion in saline-treated mice. Our CPA assessment using 2, 7, and 5 nAChR KO mice is shown in Fig. 8. There was a loss of mecamylamine-precipitated aversion in nicotine-dependent 2 KO mice [Fig. 8A; $F(1,29) = 4.568$, $p < 0.05$ for main effects of treatment; $F(1,29) = 2.982$, $p < 0.05$ for main effects of genotype]. However, mecamylamine-precipitated aversion was present in nicotine-dependent 7 and 5 KO mice [Fig. 8B; $F(1,29) = 5.948$, $p < 0.05$ for main effects of treatment; no main effects of genotype or interaction; Fig. 8C; $F(1,26) = 14.375$, $p < 0.005$ for main effects of treatment; no main effects of genotype or interaction].

Discussion

The major goal of this study was to determine the role of several nAChR subtypes in the physical and affective signs of nicotine withdrawal. Therefore, we used precipitated, spontaneous, and CPA nicotine withdrawal models to measure physical and affective signs of nicotine withdrawal in 2, 7, and 5 nAChR KO mice. Our spontaneous withdrawal tests using transgenic mice confirmed that the observed precipitated withdrawal behavioral effects were not an assessment of mecamylamine-dependent effects on nAChR subunits. The data showed that 2 nAChRs are not involved in the physical signs of nicotine withdrawal, as indicated by the lack of a reduction in somatic signs and the presence of hyperalgesia in nicotine-withdrawn 2 KO mice. These data are consistent with previous studies assessing the role of 2 nAChRs in somatic signs of nicotine withdrawal using 2 KO mice (Salas et al., 2004; Besson et al., 2006). Both studies found that mecamylamine-precipitated nicotine withdrawal somatic signs in nicotine-dependent 2 KO in a similar fashion to what was observed in nicotine-dependent wild-type litter mates. In addition, we assessed two affective signs of nicotine withdrawal in 2 KO mice and found that 2 nAChRs are involved in both affective measures of nicotine withdrawal. Nicotine-dependent 2 KO mice displayed a lack of anxiety-related behavior in the plus maze, as well as a lack of mecamylamine-precipitated aversion in the CPA model. Our studies using transgenic

mice are complemented by previous pharmacological nicotine withdrawal studies that used the $\alpha 2$ -selective antagonist, dihydro- β -erythroidine, to precipitate anxiety-related behavior and elevations in reward threshold, also measures of affective signs of nicotine withdrawal (Damaj et al., 2003; Bruijnzeel and Markou, 2004). Taken together, these studies suggest an important role for $\alpha 2$ -containing nicotinic receptors in affective nicotine withdrawal behaviors.

Assessment of the $\alpha 7$ nAChR subunit using transgenic mice showed that nicotine-dependent $\alpha 7$ KO mice displayed anxiety-related behavior and somatic signs but a loss of hyperalgesia. A previous $\alpha 7$ nAChR KO study from our laboratory using the oral route of chronic nicotine administration and the spontaneous nicotine withdrawal model produced similar findings (Grabus et al., 2005). However, the oral route of administration yields variable amounts of nicotine intake, making it difficult to control the actual dose of nicotine reaching the receptor; therefore, we wanted to assess the role of the $\alpha 7$ subunit using a more consistent exposure method. It was noted that $\alpha 7$ KO mice displayed anxiety-related behavior in the plus maze, indicating that this subtype is not involved in affective withdrawal signs. Upon further evaluation of affective signs in the CPA model, we found that mecamylamine precipitated aversion in $\alpha 7$ KO mice. Taken together, these results suggest that $\alpha 7$ nAChRs are involved in physical, not affective, nicotine withdrawal signs. The data also suggest that our two physical measures of nicotine withdrawal (somatic signs and hyperalgesia) are mediated by different nAChR subtype populations. Although $\alpha 7$ KO mice showed a loss of the hyperalgesia response, these mice exhibited somatic signs of nicotine withdrawal. The $\alpha 7$ nAChR subunit is expressed in the peripheral ganglia, as well as centrally. Although hyperalgesia is measured as a physical sign in the mouse, studies have suggested that spinal and supraspinal nAChR populations mediate the hyperalgesia response (Schmidt et al., 2001; Damaj and Flores, 2002). Our hyperalgesia measure was conducted using the hot-plate, which measures supraspinal mechanisms; thus, we cannot rule out the possibility that the nicotine withdrawal-induced hyperalgesia response is mediated by neuronal $\alpha 7$ nAChRs. It is also noted that MLA, the $\alpha 7$ antagonist, precipitated mild somatic signs of withdrawal in wild-type mice (Damaj et al., 2003), whereas our assessment revealed the presence of typical nicotine withdrawal somatic signs in $\alpha 7$ KO mice. However, it was also shown that MLA can antagonize $\alpha 6$, $\alpha 3$, and $\alpha 3$ nAChR subunits at doses typically used to block $\alpha 7$ nAChRs (Mogg et al., 2002); thus, it is possible that the observed behavioral responses were attributed to effects on other nAChR subtypes.

The role of the $\alpha 5$ nAChR subunit in nicotine withdrawal has not previously been addressed. Assessment of the $\alpha 5$ subunit role showed a reduction in somatic signs in nicotine-dependent $\alpha 5$ KO mice after mecamylamine treatment, whereas anxiety-related behavior and hyperalgesia were still present. Additional evaluation of affective signs revealed the expression of mecamylamine-precipitated aversion in $\alpha 5$ KO mice. These findings suggest that the $\alpha 5$ subunit is involved to an extent in some physical aspects of nicotine withdrawal but not affective nicotine withdrawal signs. It has been reported that the $\alpha 4$ subunit is involved in the physical signs of nicotine withdrawal (Salas et al., 2004). The $\alpha 4$ subunit is coexpressed with the $\alpha 3$ and $\alpha 5$ subunits in the peripheral ganglia (Salas et al., 2004). Somatic signs of nicotine withdrawal were shown to be partially mediated by peripheral nAChR populations; therefore, we propose that ganglionic $\alpha 5 \alpha 4^*$ -containing nAChR subtypes mediate somatic signs of nicotine withdrawal. Because the $\alpha 5$ subunit can coassemble with both $\alpha 2$ and $\alpha 4$ nAChRs, it is possible that the role of $\alpha 5$ in nicotine withdrawal differs depending on nAChR subunit composition. One of the problems in interpretation of results obtained with KO mice is whether compensatory changes in expression of other genes occur as a result of deletion of a particular gene. Although this issue has not yet to be directly explored with the nicotinic KO mice, it should be noted that behavioral differences observed with these KO mice were reproduced using various

nicotinic antagonists (at least for the $\alpha 2$ and $\alpha 7$ receptor subtypes) (Damaj et al., 2003; Grabus et al., 2005). In addition, no compensatory changes of other nicotinic subunits were reported in these particular KO strains (Picciotto et al., 1995; Orr-Urtreger et al., 1997; Salas et al., 2004). Although we cannot completely rule out effects on other systems, these findings argue against a role for compensatory changes and suggest a direct role for these subunits in nicotine withdrawal.

The present study suggests that $\alpha 2$ -containing nAChRs are involved in the affective signs of nicotine withdrawal, whereas non- $\alpha 2$ -containing nAChRs are more closely associated with physical nicotine withdrawal signs. These behavioral findings, in addition to recent studies, suggest an important role for $\alpha 2$ -containing nAChRs in nicotine dependence and provide better insight into potential targets for more effective smoking cessation therapies.

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ABBREVIATIONS

nAChR	nicotinic acetylcholine receptor
CPA	conditioned place aversion
KO	knockout
WT	wild type
sal	saline
mec	mecamylamine
nic	nicotine

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Fig. 1.

Assessment of physical and affective nicotine withdrawal signs in C57BL/6J mice using the precipitated model. After treatment with mecamylamine (2 mg/kg) on test day, nicotine-dependent mice show: A, anxiety-related behavior noted by a reduction in the time spent on the open arms; B, significant somatic signs; and C, withdrawal-induced hyperalgesia, noted by a decreased latency on the hot-plate. Each point represents the mean $\pm$ S.E.M. of six to eight mice per group. *, $p < 0.05$ versus control groups. **, $p < 0.005$ versus control groups. ***, $p < 0.0005$ versus control groups. MP, minipump.

Fig. 2.

The $\alpha 2$ nAChR subunit is involved in the affective signs but not the physical signs of nicotine withdrawal. Compared with wild-type nicotine-dependent mice, nicotine-dependent $\alpha 2$ KO mice show: A, no reduction in the time spent on the open arms of the plus maze, indicating a loss of anxiety-related behavior; but B, significant nicotine withdrawal somatic signs; and C, a decreased hot-plate latency, indicating the presence of the hyperalgesia response. Each point represents the mean $\pm$ S.E.M. of six to eight mice per group. *, $p < 0.05$ versus saline groups and versus nicotine KO group for the plus-maze test. ***, $p < 0.05$ versus saline groups.

Fig. 3.

The $\alpha 7$ nAChR subtype is involved in some physical aspects of nicotine withdrawal but not the affective signs. Nicotine-dependent $\alpha 7$ KO mice showed: A, a reduction in the time spent on the open arms of the plus maze, indicating an anxiety-related response; and B, significant nicotine withdrawal somatic signs. However, in C, a loss of nicotine withdrawal-induced hyperalgesia response, as noted by the lack of decreased hot-plate latency, was observed in nicotine-dependent $\alpha 7$ KO mice compared with their wild-type counterparts. Each point represents the mean $\pm$ S.E.M. of six to eight mice per group. *, $p < 0.05$ versus saline groups and versus nicotine KO group for the hyperalgesia test. **, $p < 0.005$ versus saline control groups. ***, $p < 0.0005$ versus saline control groups.

Fig. 4.

Assessment of nicotine withdrawal signs in 2 nAChR KO mice using the spontaneous withdrawal model. 2 KO mice withdrawn from nicotine 18 to 20 h show: A, a loss of anxiety-related behavior on the plus maze, indicated by the lack of a reduction in the time spent on the open arms; but B, significant somatic signs; and C, withdrawal-induced hyperalgesia, indicated by a decreased hot-plate latency. Each point represents seven to nine mice per group. *, $p < 0.05$ versus saline groups. **, $p < 0.005$ versus saline groups. ***, $p < 0.0005$ versus saline groups.

Fig. 5.

Assessment of nicotine withdrawal signs in 7 nAChR KO mice using the spontaneous withdrawal model. 7 KO mice withdrawn from nicotine 18 to 20 h show: A, anxiety-related behavior on the plus maze, indicated by a reduction in the time spent on the open arms; B, significant somatic signs; but C, a loss of withdrawal-induced hyperalgesia, noted by the lack of a decreased hot-plate latency. Each point represents seven to nine mice per group. *, $p < 0.05$ versus saline groups. ***, $p < 0.0005$ versus saline groups.

Fig. 6.

The $\alpha 5$ nAChR subunit plays a role in some physical aspects of nicotine withdrawal but not the affective signs. Nicotine-dependent $\alpha 5$ KO mice show: A, significant anxiety-related behavior in the plus-maze test, indicated by a reduction in time spent on the open arms; but B, display a significant reduction in total average number of somatic signs when compared with wild-type counterparts. C, nicotine-induced hyperalgesia response is still present in $\alpha 5$ KO mice, indicated by decreased hot-plate latency. Each point represents the mean $\pm$ S.E.M. of six to eight mice per group. *, $p < 0.05$ versus saline groups. **, $p < 0.005$ versus saline groups. ***, $p < 0.005$ versus saline groups. +, $p < 0.05$ versus saline groups and versus WT mec group; ++, $p < 0.05$ versus WT mec group and $p < 0.005$ versus saline groups.

Fig. 7. Mecamylamine (3.5 mg/kg) precipitates aversion in nicotine-dependent C57BL/6J mice in the CPA model. Each point represents the mean $\pm$ S.E.M. of eight to 10 mice per group. *, $p < 0.05$ versus control groups. MP, minipump.

Fig. 8.

Assessment of the role of the $\alpha 2$, $\alpha 7$, and $\alpha 5$ nAChR subunits in affective nicotine withdrawal signs using the CPA model. A, nicotine-dependent $\alpha 2$ KO mice show a loss of mecamylamine-precipitated aversion. B, nicotine-dependent $\alpha 7$ and C. $\alpha 5$ nAChR KO mice express mecamylamine-precipitated aversion in the CPA model. Each point represents the mean $\pm$ S.E.M. of eight to 10 mice per group. *, $p < 0.05$ versus saline groups and versus mec KO group in the $\alpha 2$ assessment. **, $p < 0.005$ versus saline groups.

TABLE 1
Total average number of arm crosses in the plus maze test during withdrawal

Assessment of total number of crosses in the plus-maze test during nicotine withdrawal. Using the precipitated model, nicotine-dependent mice were treated with mecamylamine (2 mg/kg) on test day (day 14), and the total number of crosses between open and closed arms of the plus-maze test was counted. Numbers are presented as the total average number of arm crosses $\pm$ S.E.M. for six to eight mice per group.

C57BL/6J mice	Sal-Sal	Sal-Mec	Nicotine-Sal	Nicotine-Mec
	3.7 $\pm$ 0.64	4.2 $\pm$ 0.67	3.3 $\pm$ 0.42	4.2 $\pm$ 0.51
2 mice	Sal-WT	Sal-KO	Mec-WT	Mec-KO
	3.6 $\pm$ 0.59	3.6 $\pm$ 0.32	4 $\pm$ 0.46	3.3 $\pm$ 0.59
7 mice	Sal-WT	Sal-KO	Mec-WT	Mec-KO
	2.7 $\pm$ 0.42	3.5 $\pm$ 0.48	3 $\pm$ 0.53	2.7 $\pm$ 0.28
5 mice	Sal-WT	Sal-KO	Mec-WT	Mec-KO
	2.8 $\pm$ 0.51	3 $\pm$ 0.49	3.3 $\pm$ 0.42	3.2 $\pm$ 0.40