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ORIGINAL INVESTIGATION

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The β2 but not α7 subunit of the nicotinic acetylcholine receptor is required for nicotine-conditioned place preference in mice

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Abstract *Rationale:* Tobacco use is implicated in approximately 440,000 deaths per year, making it the leading cause of preventable death in the United States. Although it is generally recognized that tobacco use is correlated with a variety of health-related complications, many smokers are unsuccessful in their efforts to stop smoking using current cessation therapies. *Objectives:* Given that nicotine is the addictive component of tobacco, successful smoking cessation therapies must address the various processes, including reward, which contribute to nicotine addiction. As such, determining the nicotinic receptor subtypes involved in nicotine reward is of utmost importance to understanding how nicotine addiction progresses. *Methods:* Conditioned place preference (CPP) in three-chamber conditioning boxes was performed. For antagonist studies, drug was given on all conditioning sessions 10 min before nicotine or saline injection and placement in the boxes. *Results:* We have demonstrated that a pretreatment with the α4β2 subunit of the nicotinic acetylcholine receptor (nAChR) antagonist dihydro-β-erythroidine (2.0 mg/kg, s.c.) blocked nicotine (0.5 mg/kg, s.c.) CPP in wild-type mice (C57BL/6 mice). In contrast, pretreatment with an antagonist of the α7 subunit of the nAChR, methyllycaconitine (MLA, 5.0 or 10.0 mg/kg, s.c.), had no effect on this behavior. Finally, we showed that mice lacking the β2 subunit of the nAChR did not exhibit nicotine CPP while α7 knock-out mice did. *Conclusion:* Taken together, these data suggest that the β2 subunit of the nAChR is critically involved in nicotine reward as measured by CPP.

Keywords Nicotine · Reward · Conditioned place preference · Nicotinic acetylcholine receptor · α7 subunit · β2 subunit · Mice

Abbreviations nAChR: Nicotinic acetylcholine receptor · CPP: Conditioned place preference · DHβE: Dihydro-β-erythroidine · MLA: Methyllycaconitine

Introduction

Nicotine is the key factor that underlies tobacco consumption and consequent smoking behavior; however, the mechanism underlying the rewarding effects of nicotine is not well defined. As such, smoking cessation techniques range from ineffective to mildly effective (Vaszar et al. 2002). The influence of nicotine depends on its ability to modulate neuronal nicotinic acetylcholine receptors (nAChRs). nAChRs are pentameric structures comprised of various neuronal subunits that combine to produce many different nAChR types. Therefore, exploring specific nAChR subunits involved in nicotine reward may be the basis for successful smoking cessation programs.

To gain a better understanding of the rewarding potential of nicotine, animal models such as self-administration and conditioned place preference (CPP) have been developed in rats and mice. In a typical CPP experiment, a stimulus of interest is conditioned to distinct environmental cues. If the stimulus is rewarding, the animal will demonstrate a preference for the conditioned environment over the environment conditioned to a nonrewarding (placebo) stimulus. One advantage of CPP is that it is readily adaptable to mice and does not require surgery or extensive training necessary for self-administration paradigms. However, interpretation of CPP can be problematic in terms of

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state-dependent learning and novelty-seeking behavior (for reviews, see Bardo and Bevins 2000; Carr et al. 1989).

While models of nicotine reward have been established in the rodent, receptor-mediated events in this process have not been well elucidated. Attempts to explore receptor subunit involvement have typically utilized nicotinic antagonists with known cross reactivity and lack of specificity (Harvey et al. 1996; Khiroug et al. 2004; Watkins et al. 1999; Williams and Robinson 1984). However, these studies, along with some recent genetic studies, implicate the β2 and α7 nAChR subunits in nicotine reward. Nicotine reward measured by self-administration in rats is blocked by dihydro-β-erythroidine (DHβE, nicotinic antagonist with preferential affinity to the β2 subtype). In addition, nicotine reward measured by self-administration is absent in mice lacking the β2 nAChR subunit (Grottick et al. 2000; Picciotto et al. 1998). While reward has not been studied in mice lacking the α7 subunit of the nAChR, studies with the α7 antagonist methyllycaconitine (MLA) (Klink et al. 2001) have yielded conflicting results with nicotine reward as measured by self-administration in the rat (Grottick et al. 2000; Markou and Paterson 2001). Although previous studies indicate that β2 and possibly α7 containing nAChRs mediate nicotine reward, the lack of highly selective antagonists prevents identification of the precise make up of the receptors involved in this process. Even though nicotine self-administration can be accomplished in mice, a mouse model using drug-naïve mice not restricted in their movement and with a limited access schedule (similar to that frequently used in opiates and psychostimulants studies) is still not available for nicotine. Reward as measured by self-administration in the β2 knock-out mice involved pretreatment with cocaine (Picciotto et al. 1998) and the effects of pre-treating mice in this manner are unknown. Molecular adaptations that occur during the acquisition of cocaine self-administration may be different from those that occur during acquisition of nicotine self-administration. Therefore, coupling the power of pharmacological techniques with genetic advances will form a more complete picture of the receptor subtypes involved in nicotine reward as measured by CPP.

The goal of the present work was twofold. We first wanted to establish a reliable model of nicotine reward in an inbred strain of mouse (C57BL/6). We next sought to determine the role of the β2 and α7 nAChR subunits in nicotine reward as measured by conditioned place preference using pharmacology and genetically altered mice.

Materials and methods

Subjects

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 A.M.). Mice were about 8 weeks of age and weighed approximately 25–30 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.

In the pharmacological studies, C57BL/6 mice from Jackson Laboratories were used. Mice lacking the α7 subunit of the nicotinic receptor (C57BL/6 background) and wild-type littermates were purchased from Jackson Laboratories (B6.129S7-charna7tm1bay, number 003232). Breeding pairs of mice lacking the β2 subunit of the nicotinic receptor (C57BL/6 background) and wild-type littermates were shipped from Institut Pasteur (Paris, France). Mice about 8 weeks of age (together with age- and sex-matched wild-type controls) were used for the CPP experiments. β2 and α7 knock-out mice are maintained on a C57BL/6 background and both have been backcrossed to at least N10. For all experiments, mutant and wild-type controls are obtained from crossing heterozygote mice. This breeding scheme allows us to rigorously control for any anomalies that may occur with crossing solely mutant animals.

Drug administration

(-)-Nicotine was purchased from Sigma Chemical (Milwaukee, WI, USA). Mecamylamine hydrochloride [1.0 mg/kg, subcutaneously (s.c.)] was a gift from Merck, Sharp, Dohme (West Point, PA, USA). Dihydro-β-erythroidine (DHβE) and methyllycaconitine citrate were purchased from Sigma-RBI (Natick, MA, USA). Drugs were dissolved in saline and injected subcutaneously at a volume of 10 ml/kg body weight. The pH of the nicotine solution is checked and neutralized if necessary. The mecamylamine (1.0 mg/kg, s.c.) and DHβE doses (2.0 mg/kg, s.c.) were based on published and unpublished studies from our lab and was within a range of doses effective at blocking behavioral effects of nicotine (Damaj et al. 1995, 2003). The MLA doses (5.0 or 10.0 mg/kg, s.c.) were within the range reported to block α7 nicotinic receptors (Turek et al. 1995) and the effects of nicotine on multiple behavioral assays (Damaj et al. 1999). All doses are expressed as the free base of the drug.

Conditioned place preference

Place conditioning chambers (MED Associates, St. Albans, VT, USA) consist of three distinct compartments separated by manually operated doors. The center compartment measures 8.25×12.5×13 cm (*w*×*d*×*h*) and is grey with a smooth polyvinyl chloride floor. This compartment serves as a thoroughfare between the two pairing sides. The pairing compartments are 16.5×12.5×13 cm (*w*×*d*×*h*). One compartment has black walls with a stainless steel grid rod floor consisting of 0.31-cm rods placed on 0.78-cm centers. The other compartment has white walls with a 0.625×0.625-cm stainless steel mesh floor. The design used is an unbiased procedure as preliminary data from our lab indicate that mice do not show any initial bias to one side or the other.

Conditioned place preference occurs in three phases: preconditioning, conditioning, and test (or expression). Day 1 is a preconditioning day when mice are allowed to roam freely among the three compartments for 900 s (15 min) and time spent in each compartment is recorded. These data are used to segregate the animals into groups with equal bias so that all groups have a preference score at or near 0 indicating no preference for either side. On the morning of day 2, animals were injected with saline or 0.5 mg/kg nicotine (s.c.) and immediately confined to one of the pairing compartments for 20 min (n=6–8 animals per group) Five hours later, animals were injected with the alternate drug condition and immediately confined to the opposite chamber for 20 min. Saline control groups receive saline on both sides of the cage. This procedure was repeated on days 3–4 so that each animal receives a total of three drug and three saline conditioning trials (unless they belong to the saline control group). The groups are counterbalanced to assure that some animals get nicotine in the white side while others get nicotine in the black side and some get nicotine in the morning while others get nicotine in the afternoon. On day 5, animals once again are allowed to roam freely among the three compartments for 900 s (15 min) and time spent on each side is recorded. Animals are drug-free on preconditioning and test days. Preference score is expressed in seconds and is calculated by subtracting preconditioning day data from test day data.

For conditioning studies with antagonists, the antagonist is administered 10 min before all conditioning sessions but not on test day.

Statistics

For all data, statistical analyses were performed using StatView (SAS, Cary, NC, USA). Conditioned place preference data were analyzed with ANOVAs using Bonferroni–Dunn post-hoc test. p values of less than 0.05 were considered significant.

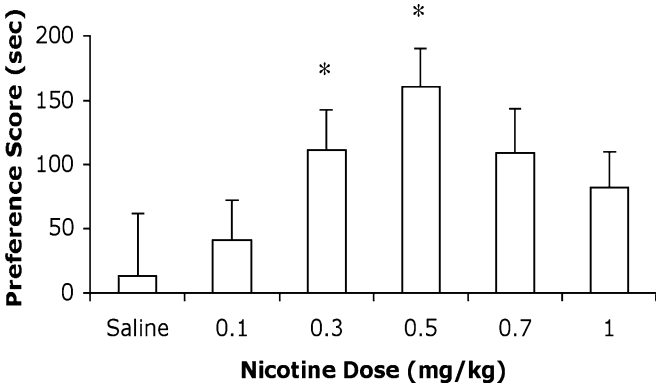

Fig. 1 Dose response curve for nicotine conditioned place preference in C57BL/6 mice. All doses of nicotine were administered via s.c. injections. The *x-axis* represents nicotine dose in milligram per kilogram and the *y-axis* represents the preference score (test day minus preconditioning day) in seconds. *Indicates p<0.05 from saline group (n=8–9 animals per group)

Results

A range of doses was utilized to find the best dose to produce significant nicotine place preference. Conditioned place preference on C57BL/6 inbred mice was performed with five doses of nicotine: 0.1, 0.3, 0.5, 0.7, and 1.0 mg/kg. Reward as measured by CPP is dose dependent. Specifi-

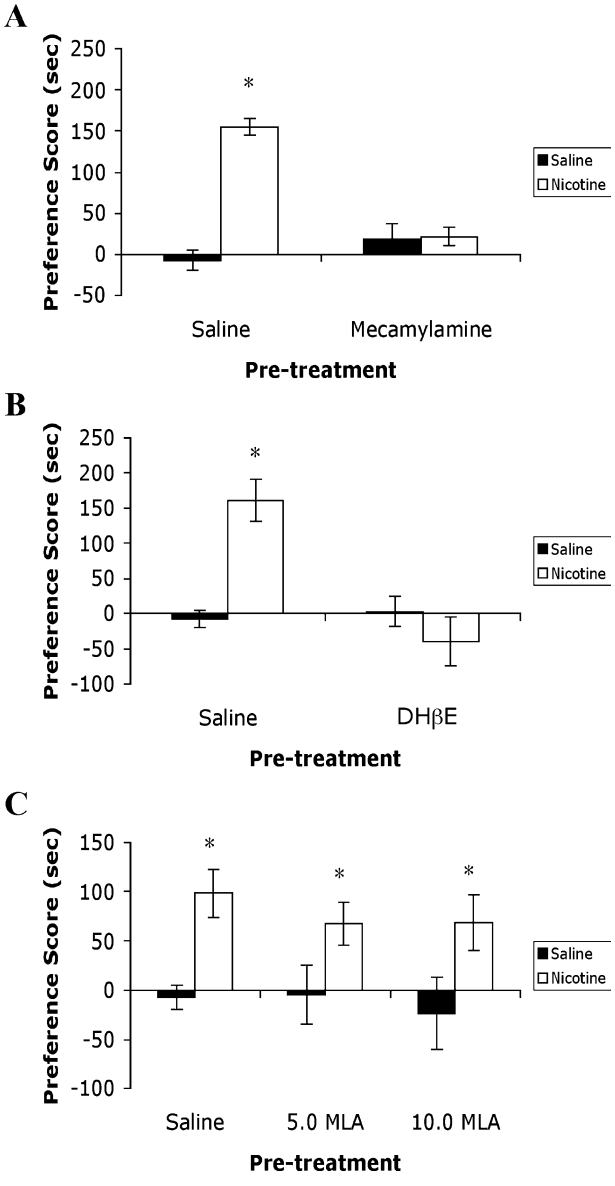

Fig. 2 Antagonists that work at β2 but not α7 receptors block nicotine-induced place preference. All drugs were administered via s.c. injections and the nicotine dose was 0.5 mg/kg. The *x-axes* represent antagonist pretreatment and the *y-axes* represent the preference score (test day minus preconditioning day) in seconds. **a** Pretreatment with mecamylamine (1 mg/kg), a nonselective nicotinic receptor antagonist, blocks nicotine-conditioned place preference. *Indicates p<0.05 from corresponding saline group (n=6–7 animals per group). **b** Pretreatment with DHβE (2 mg/kg), a selective β2 antagonist, blocks nicotine place preference. *Indicates p<0.05 from corresponding saline group (n=8 animals per group). **c** Pretreatment with MLA (5 or 10 mg/kg), a selective α7 antagonist, does not block nicotine place preference. *Indicates p<0.05 from corresponding saline groups (n=6–7 animals per group)

cally, the doses that produced significant place preference were 0.3 and 0.5 mg/kg (Fig. 1; $F_{(5,51)}$=2.603, p<0.05). For 0.5 mg/kg nicotine, an example of raw data is test day (paired 417.16 s, unpaired 283.23 s) vs pre-conditioning day (paired 256.81 s, unpaired 293.42 s). At the higher range of doses, there is no effect, which results in an inverted U-shaped dose response curve. Even though a dose of 0.3 mg/kg produced significant place preference, 0.5 mg/kg was used, which produced larger preference (though not significantly higher than preference seen at 0.3 mg/kg).

To determine if nicotine-conditioned place preference could be blocked, the non-selective nicotinic receptor antagonist mecamylamine (1 mg/kg, s.c.) was administered 10 min before each conditioning session to C57BL/6 inbred mice. Pretreatment with mecamylamine blocked place preference to nicotine while mice pretreated with saline show significant preference to nicotine (Fig. 2a; $F_{(3,25)}$=31.175, p<0.05).

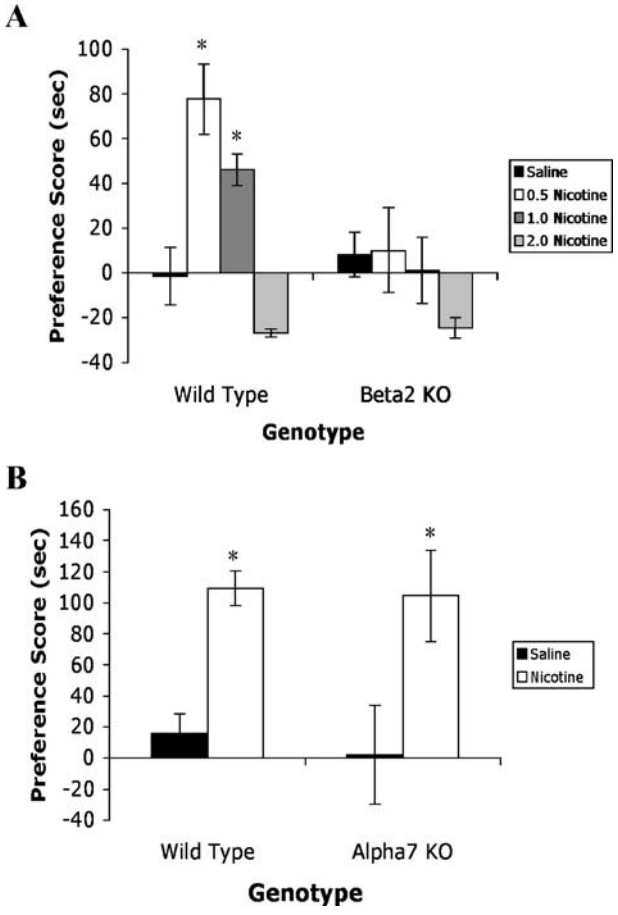

Fig. 3 Mice lacking the β2 nicotinic receptor subunit do not exhibit nicotine-induced place preference while α7 knock-out animals do. Nicotine was administered via s.c. injections. The *x-axes* represent genotype and the *y-axes* represent the preference score (test day minus preconditioning day) in seconds. **a** Mice lacking the β2 nicotinic receptor subunit do not show significant nicotine place preference. *Indicates p<0.05 from corresponding saline group (n=6–7 animals per group). **b** Mice lacking the α7 nicotinic receptor subunit show significant nicotine place preference. *Indicates p<0.05 from corresponding saline groups (n=6 animals per group)

To determine if nicotine-conditioned place preference could be blocked with a more selective antagonist, the α4β2 antagonist DHβE (2 mg/kg, s.c.), another test was conducted. Similar to mecamylamine pretreatment, pretreatment with DHβE blocks place preference to nicotine while mice pretreated with saline exhibited significant preference to the nicotine paired side (Fig. 2b; $F_{(3,32)}$=18.549, p<0.05).

In contrast to mecamylamine and DHβE pretreatments, the α7 antagonist MLA (5 or 10 mg/kg) failed to affect place preference induced by nicotine. Nicotine remains effective in producing conditioned place preference regardless of pretreatment with saline or MLA (Fig. 2c; $F_{(1,40)}$=21.328, p<0.05).

To further delineate the role of β2 and α7 nAChR subunits in nicotine reward, genetically altered mice were utilized. β2 knock-out mice did not exhibit nicotine place preference to nicotine at any of the doses that their wild-type littermates exhibited preference (Fig. 3a; $F_{(3,45)}$=9.526, p<0.05). In contrast, a comparison of α7 knock-out mice to their wild-type littermates shows that mice lacking the α7 subunit of the nAChR exhibit nicotine-conditioned place preference similar to their wild-type littermates. Nicotine remains effective in producing conditioned place preference regardless of genotype (Fig. 3b; $F_{(1,24)}$=16.758, p=0.0004).

Discussion

We have found that mice dose dependently exhibited significant conditioned place preference to nicotine. Nicotine place preference displayed a narrow, inverted U-shaped dose response curve. We chose a dose of 0.5 mg/kg for the rest of the experiments which is consistent with previous work in mice showing a significant increase in dopamine release in the striatum after an acute injection of 0.5 mg/kg of nicotine (Picciotto et al. 1998).

In addition to establishing nicotine CPP in mice, we have shown that mecamylamine pretreatment on conditioning days alone blocks nicotine CPP in C57BL/6 inbred mice. Previous studies in rats have shown that nicotine place preference is attenuated by mecamylamine (non-selective nicotinic antagonist) but not hexamethonium (a peripheral non-selective nicotinic antagonist) (Fudala et al. 1985). These results indicate that the reinforcing effects of nicotine as measured by CPP are mediated by activation of central nicotinic acetylcholine receptors.

We next examined the involvement of the β2 nAChR subunit in nicotine reward. In this study, we showed that administration of the competitive α4β2 antagonist dihydro-β-erythroidine (DHβE) before conditioning sessions blocked nicotine CPP and that mice lacking the β2 nAChR subunit did not exhibit nicotine CPP. These data are consistent with previous studies that showed both systemic and local intra-VTA injections of DHβE blocked nicotine self-administration (Corrigall et al. 1994; Watkins et al. 1999). In addition, wild-type and β2 knock-out mice both exhibited cocaine self-administration; however, when nicotine was

substituted for cocaine, wild-type mice continued to self-administer nicotine while β2 knock-out mice did not (Picciotto et al. 1998). Finally, nicotine stimulated release of dopamine in the striatum of wild-type mice (Grady et al. 1992), but these increases were absent in β2 knock-out mice (Picciotto et al. 1998). Taken together, these data suggest that the β2 nAChR subunit is critically involved in nicotine reward as measured by both self-administration and CPP.

We have shown that administration of the α7 antagonist methyllycaconitine does not block nicotine CPP. In addition, we found that the α7 knock-out mice still find nicotine rewarding in the CPP paradigm. Previous work with MLA has yielded conflicting results with one study showing MLA reduces intravenously administered nicotine self-administration (Markou and Paterson 2001) and another showing MLA has no effect on nicotine self-administration in rats (Grottick et al. 2000). These inconsistent results may be due to differences in methodology between the two studies. Our results provide further evidence that the α7 nAChR subunit is not involved in nicotine reward as measured by CPP.

While our data combine pharmacologic and genetic evidence that the β2 but not the α7 subunit is involved in nicotine reward as measured by conditioned place preference, alternative hypotheses must be considered especially when working with knock-out mice. In the β2 knock-out mice, alternate physiological or behavioral deficits caused by the knock-out may play a role in the ability of the knock-out mice to perform in the conditioned place preference paradigm. In mice lacking the β2 subunit of the nAChR, there are normal levels of α2–α7 and β3–β4 nAChR subunit mRNA (Picciotto et al. 1995). With respect to learning and memory, the β2 knock-out mice do not show nicotine-induced enhancement of passive avoidance, a test of associative memory, and they perform better than their wild-type littermates (Picciotto et al. 1995) suggesting they do not have any deficits in learning an associative memory task. Finally, young (2–4 months) β2 knock-out mice do not show impairments in contextual or tone-conditioned fear (Caldarone et al. 2000) nor do they show deficits in spatial learning deficits as measured by the Morris water maze (Zoli et al. 1999). It is interesting to note that β2 knock-out mice show slightly lower reward to cocaine as measured by conditioned place preference (Zachariou et al. 2001). The procedure used to measure preference in this case was biased and only occurred at the lowest dose of cocaine. A higher dose of cocaine produced preference similar to wild-type animals. It is important to note that, in our studies, we utilized the highest dose of nicotine that produced preference in our dose response study.

Even though the α7 knock-out mice exhibit place preference, it is possible that compensatory mechanisms allow for recruitment of alternate nicotinic receptors to restore some of the lacking function of the α7 subunits. α7 nAChR subunits form a homo-oligomer receptor that is inhibited by alpha-bungarotoxin (α-BGT) with high affinity (Couturier et al. 1990). In α7 knock-out mice, there is no significant α-BGT binding but the high-affinity nicotine binding is similar to wild-type animals (Orr-Urtreger et al. 1997). This is in contrast to the β2 knock-out mice in which the high-affinity nicotine binding sites are absent but the α-BGT sites are unchanged (Picciotto et al. 1995). In addition, there is no evidence for significant upregulation of nicotine binding sites in α7 knock-out mice (Orr-Urtreger et al. 1997).

Our data show that α7 knock-out mice still exhibit nicotine CPP while β2 knock-out mice do not. These data suggest that it may be the initial direct stimulation of dopamine neurons possibly in the ventral tegmental area (VTA) via the β2 containing nAChRs leading to an increase of dopamine in the nucleus accumbens (NAc) that is involved in nicotine reward as measured by CPP. A common feature of many drugs that produce reward is an increase in dopamine release usually in the NAc and nicotine is no exception (Di Chiara and Imperato 1979). Previous studies have shown that nicotine directly activated dopamine neurons in the VTA via β2 containing nAChRs and these receptors desensitize within a few minutes (Mansvelder and McGehee 2000). In addition, blocking β2 containing nAChRs through direct application of DHβE in the VTA decreases nicotine self-administration in rats (Corrigall et al. 1994). Finally, in mice lacking the β2 subunit of the nAChR, nicotine fails to produce an increase in dopamine in the striatum seen in their wild-type littermates (Picciotto et al. 1998). However, when the β2 subunit is re-expressed in the VTA only in these mice, the nicotine-induced dopamine release and nicotine self-administration are both recovered (Maskos et al. 2005). Taken together, nicotine reward as measured by CPP may be mediated by direct stimulation of dopamine neurons via the β2 nAChR subunit in the VTA. However, it is important to note that other studies postulate the VTA and nicotine-induced dopamine release modulate the aversive aspects of nicotine rather than the rewarding effects (Laviolette et al. 2002; Laviolette and van der Kooy 2003).

These studies represent an initial attempt to couple pharmacology and genetics to elucidate the subunits involved in nicotine reward as measured by CPP. We first established reliable, dose dependent nicotine CPP. We then showed that both pharmacological blockade and genetic deletion of the β2 nAChR subunit result in an absence of nicotine CPP. In addition, pharmacologic blockade and genetic deletion of the α7 subunit have no effect on nicotine CPP. These data show that the β2 subunit of the nAChR is critically involved in the rewarding properties of nicotine as measured by CPP. As such, pharmaceuticals targeting the β2 subunit of the nAChR may provide effective intervention for nicotine addiction.

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