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The $\alpha 3\beta 4^*$ nicotinic acetylcholine receptor subtype mediates nicotine reward and physical nicotine withdrawal signs independently of the $\alpha 5$ subunit in the mouse

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Abstract

The 15q25 gene cluster contains genes that code for the $\alpha 5$, $\alpha 3$, and $\beta 4$ nicotinic acetylcholine receptor (nAChRs) subunits, and in human genetic studies, has shown the most robust association with smoking behavior and nicotine dependence to date. The limited available animal studies implicate a role for the $\alpha 5$ and $\beta 4$ nAChR subunits in nicotine dependence and withdrawal; however studies focusing on the behavioral role of the $\alpha 3\beta 4^*$ nAChR receptor subtype in nicotine dependence are lacking. Because of the apparent role of the $\alpha 3\beta 4^*$ nAChR subtype in nicotine dependence, the goal of the current study was to better evaluate the involvement of this subtype in nicotine mediated behavioral responses. Using the selective $\alpha 3\beta 4^*$ nAChR antagonist, α -conotoxin AuIB, we assessed the role of $\alpha 3\beta 4^*$ nAChRs in acute nicotine, nicotine reward, and physical and affective nicotine withdrawal. Because $\alpha 5$ has also been implicated in nicotine dependence behaviors in mice and can form functional receptors with $\alpha 3\beta 4^*$, we also evaluated the role of the $\alpha 3\beta 4\alpha 5^*$ nAChR subtype in nicotine reward and somatic nicotine withdrawal signs by blocking the $\alpha 3\beta 4^*$ nAChR subtype in $\alpha 5$ nAChR knockout mice with AuIB. AuIB had no significant effect on acute nicotine behaviors, but dose-dependently attenuated nicotine reward and physical withdrawal signs, with no significant effect in affective withdrawal measures. Interestingly, AuIB also attenuated nicotine reward and somatic signs in $\alpha 5$ nAChR knockout mice. This study shows that $\alpha 3\beta 4^*$ nAChRs mediate nicotine reward and physical nicotine withdrawal, but not acute nicotine behaviors or affective nicotine withdrawal signs in mice. The $\alpha 5$ subunit is not required in the receptor assembly to mediate these effects. Our findings suggest an important role for the $\alpha 3\beta 4^*$ nAChR subtype in nicotine reward and physical aspects of the nicotine withdrawal syndrome.

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Keywords

nicotine; nicotinic acetylcholine receptors; nicotine withdrawal; somatic signs; nicotine reward; alpha3 beta4 nicotinic receptors

1. Introduction

A major goal in nicotine research is to gain a better understanding of the molecular and receptor mediated mechanisms of nicotine dependence and withdrawal in order to generate more effective smoking cessation therapies. The initial targets for nicotine are nicotinic acetylcholine receptors (nAChRs), ligand-gated ion channels which have been identified throughout the central and peripheral nervous systems, as well as at skeletal neuromuscular junctions. While animal studies implicate a significant role for $\beta 2$ -containing nAChRs in nicotine reward, dependence, and withdrawal (Picciotto et al., 1998; Walters et al., 2006; Jackson et al., 2008; Jackson et al., 2009b), to date, the 15q25 gene cluster, which contains the CHRNA5/CHRNA3/CHRNA4 genes, coding for the $\alpha 5$, $\alpha 3$, and $\beta 4$ nAChR subunits respectively, has shown the most robust findings in human genetic studies as a candidate region contributing to risk of heavy smoking, nicotine dependence, and smoking related diseases in humans (Berrettini et al., 2008; Bierut, 2010; Liu et al., 2010; Lips et al., 2010; Ware et al., 2011).

The $\alpha 5$ nAChR subunit coassembles with $\alpha 3\beta 4^*$ nAChR subtypes (where * denotes the possible inclusion of additional subunits) to form functional receptors in the peripheral ganglia, as well as centrally, in the medial habenula (MHb) and interpeduncular nucleus (IPN) (Wada et al., 1990; Zoli et al., 1995; Quick et al., 1999; Whiteaker et al., 2002). Despite findings of robust associations with the 15q25 gene cluster, rodent studies assessing the role of $\alpha 3$, $\beta 4$, and $\alpha 5$ nAChRs are limited, though available studies do implicate a role for these nAChRs in nicotine mediated behaviors.

In the central nervous system (CNS), the mesolimbic dopamine pathway contains some $\alpha 3\beta 4^*$ (where * denotes the possible inclusion of additional subunits) subtypes; however, the majority of this receptor subtype resides in the medial habenula (MHb) and interpeduncular nucleus (IPN) (Wada et al., 1990; Zoli et al., 1995; Quick et al., 1999; Whiteaker et al., 2002). In these brain regions, the $\alpha 5$ nAChR subunit co-assembles with $\alpha 3\beta 4^*$ nAChR subtypes to various degrees (15 to 35% of the $\alpha 3\beta 4^*$ subtypes at the MHb and IPN, respectively) (Grady et al., 2009). Importantly, $\alpha 5$ -containing nAChRs in the MHb and IPN have recently been implicated in nicotine self-administration and reward. $\alpha 5$ nAChR knockout ($-/-$) mice are less sensitive to the acute behavioral effects of nicotine, and maintain expression of a significant nicotine conditioned place preference (CPP) at nicotine doses that are aversive in wild-type ($+/+$) littermates (Jackson et al., 2010). An increased nicotine intake was also observed in $\alpha 5$ nAChR $-/-$ mice. This effect was 'rescued' in $-/-$ mice by re-expressing $\alpha 5$ subunits in the MHb, and recapitulated in rats through $\alpha 5$ subunit knockdown in the MHb (Fowler et al., 2011). In addition, mice null for the $\alpha 5$ nAChR subunit exhibit a reduction in nicotine withdrawal somatic signs (Jackson et al., 2008; Salas et al., 2009).

The role of $\alpha 3$ and $\beta 4$ nAChR subunits in nicotine's behavioral effects is starting to emerge. Studies using nicotinic knock-out mice, $\alpha 3$ and $\beta 4$ nAChRs were found to be necessary for nicotine-induced seizures in mice (Kedmi et al., 2004; Salas et al., 2004), while $\beta 4$ nAChRs partially mediate acute nicotine-induced hypolocomotion (Salas et al., 2004), hypothermic response (Sack et al., 2005), and antinociception (Semenova et al., 2012). Furthermore, withdrawal somatic signs and hyperalgesia are reduced in $\beta 4$ nAChR $-/-$ mice after nicotine

withdrawal (Salas et al., 2004; Stoker et al., 2012). $\beta 4$ nAChR subunits were also found to be involved in affective nicotine withdrawal measures, as $\beta 4$ nAChR $-/-$ mice do not display elevated intracranial self-stimulation thresholds following nicotine withdrawal (Stoker et al., 2012). Currently, evidence of a clear behavioral role for $\alpha 3$ nAChR subunits in nicotine withdrawal is lacking. Selective $\alpha 3\beta 4^*$ antagonists are becoming available to delineate the role of $\alpha 3\beta 4^*$ nAChR subtypes in nicotine dependence. For example, the peptide α -conotoxin AuIB (AuIB), which was originally isolated from the venom of the predatory cone snail, *Conus aulicus*, was shown to most potently block $\alpha 3\beta 4$ nAChRs as expressed in *Xenopus* oocytes (Luo et al., 1998). The peptide was subsequently synthesized, and synthetic material was used in subsequent studies to block native CNS $\alpha 3\beta 4$ nAChRs in hippocampus (Luo et al., 1998; Kulak et al., 2001), locus coeruleus (Fu et al., 1999), interpeduncular nucleus (Grady et al., 2001), and medial habenula (McCallum et al., 2012). More recently, AT-1001, a relatively selective $\alpha 3\beta 4^*$ antagonist, was reported to dose-dependently block nicotine intravenous self-administration in rats after systemic injection (Toll et al., 2012).

Because of the apparent role of the $\alpha 3\beta 4^*$ nAChR subtypes in nicotine dependence, the goal of the current study was to better evaluate the involvement of this subtype in nicotine mediated behavioral responses. Thus, we used the $\alpha 3\beta 4^*$ selective antagonist AuIB to question $\alpha 3\beta 4^*$ nAChR contributions in acute nicotine behaviors, nicotine reward using the CPP model, and physical (somatic signs, hyperalgesia) and affective (anxiety-related behavior) nicotine withdrawal using a spontaneous nicotine withdrawal model. As an additional measure of affective nicotine withdrawal, we assessed the effects of AuIB in the conditioned place aversion (CPA) model. Further, we determined the contributions of $\alpha 5$ subunits to the effects of the selective $\alpha 3\beta 4^*$ antagonist AuIB to nicotine reward and somatic nicotine withdrawal by measuring expression of nicotine CPP and nicotine withdrawal somatic signs in $\alpha 5$ nAChR $-/-$ mice after pre-treatment with AuIB.

2. Methods

2. 1. Animals

Male adult C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice null for the $\alpha 5$ nicotinic receptor subunit (C57BL/6 background) and $+/+$ littermates were shipped from Baylor College of Medicine, Houston, Texas (see Salas et al., 2003 for information regarding initial breeders) and were subsequently bred in an animal care facility at Virginia Commonwealth University. Mutant and $+/+$ mice were obtained by breeding heterozygote pairs. Male adult $\alpha 5$ nAChR $+/+$ and $-/-$ mice were used for all $\alpha 5$ studies. Animals were 8-10 weeks of age, were group-housed in a 21°C humidity-controlled AAALAC-approved animal care facility with *ad libitum* access to food and water. Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2. 2. Drugs

(-)-Nicotine hydrogen tartrate salt and mecamylamine hydrochloride were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). The $\alpha 3\beta 4^*$ -selective antagonist AuIB was synthesized as described by Luo et al., 1998. AuIB, purified from the venom of the “court cone”, *Conus aulicus*, blocks the $\alpha 3\beta 4^*$ receptor subtype with > 100 fold higher potency than other receptor combinations, such as $\alpha 3\beta 2^*$ and $\alpha 4\beta 4^*$ (Luo et al., 1998). The compounds were dissolved in physiological saline (0.9% sodium chloride). Nicotine was administered by subcutaneous (s.c.) injection or through minipump infusion. AuIB was administered via intracerebroventricular (i.c.v.) injection. AuIB doses were calculated based on the functional IC_{50} at $\alpha 3\beta 4$ nAChRs (Luo et al., 1998).

2.3. I.c.v. surgery

C57Bl/6J and $\alpha 5$ nAChR $+/+$ and $-/-$ mice were anesthetized with sodium pentobarbital (45 mg/kg i.p.) on the evening prior to testing, and a scalp incision was made to expose the bregma. Unilateral injection sites were prepared using a 26-gauge needle with a sleeve of polyurethane tubing to control depth of the needle at a site 2 mm rostral and 2 mm lateral to the bregma at a depth of 2 mm. Animals were sutured in such a way to enable an injection volume of 5 μ l using a 26-gauge needle with a sleeve of polyurethane tubing into the lateral ventricle on the morning of testing. The needle was held in place for 20 s to ensure drug delivery.

2.4. Acute nicotine assessment

Naïve C57Bl/6J mice were injected i.c.v. with vehicle or AuIB (14 or 70 pmol) 5 min prior to a single s.c. injection with nicotine or saline. Antinociception using the tail-flick and hot-plate tests, changes in body temperature, and changes in locomotor activity were measured 5 or 30 min following nicotine or saline injection.

2.4.1. Antinociception—Antinociception was assessed by the tail-flick method of D'Amour and Smith (1941) and the hot plate test ($n=6$ per group). In the tail-flick test, mice were lightly restrained while a radiant heat source was directed onto the upper portion of the tail. A control response (2-4 s) was determined for each mouse before treatment, and test latency was determined 5 min after nicotine administration (2.5 mg/kg, s.c.). The apparatus has an automatic cut-off of 10 s to minimize tissue damage. The response for the tail-flick was calculated as percentage of maximum possible effect (% MPE), where % MPE = [(test - control)/(10 - control)] $\times$ 100. In the hot plate test, mice were placed into a 10-cm wide glass cylinder on a hot-plate (Thermojust Apparatus, Columbus, OH). The hot plate is a rectangular heated surface surrounded by plexiglass and maintained at 55°C. The device is connected to a manually operated timer that records the amount of time the mouse spends on the heated surface before showing signs of nociception (e.g. jumping, paw licks). A control response (8-12 s) was determined for each mouse before treatment, and test latency was determined 5 min after nicotine administration (2.5 mg/kg, s.c.). The timer has an automatic cut-off of 40 seconds to avoid tissue damage. The hot plate response was calculated as percentage of maximum possible effect (% MPE), where % MPE = [(test - control)/(40 - control)] $\times$ 100. While the dose of nicotine used in these studies (2.5 mg/kg) does significantly impact locomotor activity of the animal in motor activity assessments, by our observation it does not impair the mouse to the point that it is unable to flick its tail or exhibit nociceptive behaviors (e.g., paw licking, jumping) in the antinociceptive tests.

2.4.2. Body temperature—Rectal temperature ($n=6$ per group) was measured by a thermistor probe (inserted 24 mm) and digital thermometer (YSI Inc., Yellow Springs, OH). Readings were taken just before and at 30 min after nicotine injection (1.5 mg/kg, s.c.). The difference in rectal temperature before and after treatment was calculated for each mouse. The ambient temperature of the laboratory varied from 21-24°C from day to day.

2.4.3. Locomotor activity—Mice ($n=6$ per group) were placed into individual photocell activity cages (28 $\times$ 16.5 cm; Omnitech, Columbus, OH) 5 min after saline or nicotine (1.5 mg/kg, s.c.) administration. Interruptions of the photocell beams (two banks of eight cells each) were then recorded for the next 10 min. Data are expressed as number of photocell interruptions.

2.5. Nicotine CPP protocol

Nicotine CPP was conducted using an unbiased design as previously described by Kota et al., (2007). In brief, separate groups of C57Bl/6J and $\alpha 5$ nAChR $+/+$ and $-/-$ mice ($n=6-9$ per group) were handled for three days prior to initiation of CPP testing. The CPP apparatus consisted of a three-chambered box with a white compartment, a black compartment, and a center grey compartment. The black and white compartments also had different floor textures to help the mice further differentiate between the two environments. On day 1, mice were placed in the grey center compartment for a 5 min habituation period, followed by a 15 min test period to determine baseline responses. A pre-preference score was recorded and used to counterbalance and randomly pair each mouse with either the black or white compartment. Drug-paired sides were randomized so that an even number of mice received drug on the black and white side. Over the next 3 days, mice were conditioned for 20 min twice a day with conditioning sessions no less than 5 hr apart. The saline group receiving saline on both sides of the boxes and drug groups receiving nicotine (0.5 mg/kg, s.c.) on one side of the box and saline on the opposite side. Animals in the drug group received drug each day. On test day (day 5), mice were treated with AuIB (3.5, 7, or 14 pmol, i.c.v.) or vehicle 5 min prior to exposure to the chambers, and day 1 procedure was repeated. Data were expressed as time spent on the drug-paired side post-conditioning minus time spent on the drug-paired side pre-conditioning. A positive number indicated a preference for the drug-paired side, whereas a negative number indicated an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

2.6. Chronic nicotine administration

C57Bl/6J ($n=6-8$) and $\alpha 5$ nAChR $+/+$ and $-/-$ mice ($n=4-8$) were anesthetized with sodium pentobarbital (45 mg/kg, intraperitoneal; i.p.) and implanted with Alzet osmotic minipumps [model 2002 (14 days) or model 2004 (28 days) Durect Corporation, Cupertino, CA] filled with (-)-nicotine or saline solution as described in Jackson et al., 2008. 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. This dose of nicotine was chosen based on results from previous studies in our lab (Jackson et al., 2009a), where we found that for spontaneous withdrawal assessments, using a higher nicotine dose for 14 day chronic infusion produced a more robust nicotine withdrawal syndrome, particularly in the plus maze measure, as withdrawal signs tend to be less robust and observable in spontaneous nicotine withdrawal assessments compared to precipitated nicotine withdrawal (Stoker et al., 2008).

2.7. Nicotine withdrawal assessment

Nicotine withdrawal studies were conducted using the spontaneous nicotine withdrawal model. C57Bl/6J ($n=6-8$) and $\alpha 5$ nAChR $+/+$ and $-/-$ mice ($n=4-8$) were anesthetized under isoflurane anesthesia and minipumps were removed on the morning of day 14. On day 15, C57Bl/6J mice were injected i.c.v. with vehicle or AuIB (3, 3.5 or 7 pmol), 18-24 hr after minipump removal, and withdrawal signs (anxiety-like behavior, somatic signs, and hyperalgesia response) were measured 5 min after injection. Testing was as described in Jackson et al., (2008). The specific testing sequence was chosen based on our prior studies showing that this order of testing reduced within-group variability and produced the most consistent result. For studies involving $\alpha 5$ nAChR $+/+$ and $-/-$ mice, only nicotine withdrawal somatic signs were assessed.

2.7.1. Elevated Plus Maze—The mice were first evaluated for 5 min in the elevated plus maze test for anxiety-related behavior. Briefly, the 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 the time spent in the open arms was automatically recorded by a photocell beams system. The apparatus was thoroughly cleaned after removal of each animal using a solution made up of 50% water and 50% Windex®. Results were expressed as the time spent in open arms. The number of arm crosses in the plus maze test was also counted as a measure of locomotor activity.

2.7.2. Somatic signs—The elevated plus maze test was immediately followed by a 20 min observation of somatic signs. Animals were placed in individual, Plexiglas containers [28.5 cm (*L*)x18 cm (*W*)x13 cm (*H*)] and observed occurrences of paw and body tremors, head shakes, backing, jumps, curls, and ptosis. For each animal, the total score for this assay was the sum of these individual behaviors. For the $\alpha 5$ nAChR somatic sign assessment, $\alpha 5$ nAChR $-/-$ mice and $+/+$ littermates were injected with vehicle or AuIB (3.5 pmol, i.c.v.) and somatic signs were measured 5 min after injection for 20 min.

2.7.3. Hyperalgesia—Hyperalgesia was evaluated using the hot plate immediately following the somatic sign observation period as described in section 2.4.1.

2.8. Nicotine CPA

The CPA protocol was conducted over the course of four days in a biased fashion as described in Jackson et al., 2009b. Briefly, C57Bl/6J ($n= 8-10$) mice were implanted with 28 day mini pumps 14 days prior to initiation of CPA testing to induce tolerance. Infusion continued throughout the duration of testing. The conditioning chambers were the same as those used for CPP studies. On day 1 of the CPA procedure, after a 5 minute habituation period in the center compartment, mice were allowed to roam freely between compartments for 15 minutes to determine baseline responses. The pre-preference score was used to pair each mouse with mecamylamine (3.5 mg/kg) to its initially preferred compartment. On days 2 and 3 of CPA training, all nicotine infused mice received injections of saline in the morning and mecamylamine in the afternoon. Saline infused mice received saline in the morning and afternoon. After the mecamylamine conditioning session on day 3, i.c.v. injection sites were prepared. On day 4, mice received i.c.v. injections of vehicle, or AuIB (3.5 pmol), 5 min before being placed in the test chambers. Mice moved freely between compartments as on day 1 and time spent in each compartment was recorded for each mouse. A reduction in time spent in the initially preferred compartment was interpreted as CPA.

2.9. Statistical analysis

For all data, statistical analyses were performed using StatView® (SAS, Cary, NC, USA). Data were analyzed with one-way analysis of variance with treatment as the between subject factor or three-way analysis of variance with i.c.v. pre-treatment, treatment, and genotype as between subject factors for the $\alpha 5$ nAChR $-/-$ nicotine CPP and somatic sign study. *P* values of less than 0.05 were considered significant. Significant results were further analyzed using the Neuman-Keuls post-hoc test.

3. Results

3.1. AuIB has no significant effect on acute nicotine-mediated behaviors

C57Bl/6J mice were treated with AuIB (14 or 70 pmol, i.c.v.) 5 min prior to a single injection of nicotine and tested in a battery of behavioral tests. Results are shown in Table 1. Nicotine produced significant antinociception indicated by a decrease in latency in the tail-flick ($F(5,30)= 36.45$, $p < 0.0001$) and hot plate ($F(5,30)= 95.00$, $p < 0.0001$) tests, a

significant decrease in body temperature ($F(5,30) = 291.51$, $p < 0.0001$), and a decrease in locomotor activity ($F(5,30) = 17.45$, $p < 0.0001$). AuIB at 14 pmol did not significantly alter these behaviors in mice, as AuIB treated nicotine mice did not differ from vehicle treated nicotine mice in any measure. AuIB also failed to significantly affect acute nicotine behaviors at a dose 5x higher (70 pmol, i.c.v.). The highest dose of AuIB used in this (70 pmol) study had no significant effect in any behavioral test.

3.2. AuIB dose-dependently attenuates expression of nicotine CPP in mice

The role of $\alpha 3\beta 4^*$ nAChRs was assessed using the CPP model nicotine reward. Results are shown in Figure 1. A robust CPP was observed in C57Bl/6J mice conditioned with nicotine (0.5 mg/kg, s.c.) for three days ($F(5,36) = 3.03$, $p < 0.05$). Treatment with AuIB dose-dependently attenuated expression of nicotine CPP. The highest dose of AuIB used in this assessment (14 pmol, i.c.v.) did not produce significant behavioral effects alone.

3.3. AuIB attenuates nicotine CPP in $\alpha 5$ nAChR $-/-$ mice

To examine the involvement of $\alpha 3\beta 4\alpha 5^*$ nAChRs in nicotine CPP, $\alpha 5$ nAChR $+/+$ and $-/-$ mice were treated with AuIB (14 pmol, i.c.v.). Results are shown in Figure 2. There were significant main effects of pre-treatment ($F(1,43) = 15.48$, $p < 0.001$) and treatment ($F(1,43) = 11.31$, $p < 0.01$) and a significant pre-treatment $\times$ treatment interaction ($F(1,43) = 16.22$, $p < 0.01$). A significant CPP was observed in both $+/+$ and $\alpha 5$ nAChR $-/-$ nicotine-conditioned mice pre-treated with vehicle. AuIB pre-treatment significantly blocked CPP in nicotine-conditioned $\alpha 5$ nAChR $-/-$ mice and $+/+$ counterparts. The highest dose of AuIB used in this assessment (14 pmol, i.c.v.) did not produce significant behavioral effects in $\alpha 5$ nAChR $+/+$ or $-/-$ mice.

3.4. AuIB dose-dependently attenuates physical signs of nicotine withdrawal

Male C57Bl/6J mice chronically infused with saline or nicotine were treated i.c.v. with vehicle or AuIB at different doses. Compared to saline treated counterparts, vehicle pre-treated nicotine withdrawn mice displayed a significant anxiety-related response ($F(5,38) = 10.29$, $p < 0.001$), a significant increase in the number of somatic signs ($F(4,31) = 56.47$, $p < 0.0001$), and a decreased latency in the hot plate test indicative of hyperalgesia ($F(5,38) = 7.63$, $p < 0.001$) (Figure 3). Pre-treatment with AuIB had no significant effect in the plus maze assessment, but dose-dependently decreased total somatic signs in nicotine withdrawn mice. Blockade of somatic signs was observed at 3.5 pmol, thus we did not evaluate a higher AuIB dose in this test. Similarly, AuIB at the highest dose tested (7 pmol) reversed the hyperalgesia response in nicotine withdrawn mice. The 7 pmol dose on its own had no significant effect on behaviors in saline treated mice in any paradigm. The number of arm crosses did not significantly differ between treatment groups, indicating no significant effects on locomotor activity in the plus maze test.

3.5. AuIB attenuates nicotine withdrawal somatic signs in $\alpha 5$ nAChR $-/-$ mice

Evaluation of nicotine withdrawal somatic signs in $\alpha 5$ nAChR $+/+$ and $-/-$ mice revealed significant main effects of pre-treatment ($F(1,22) = 11.27$, $p < 0.01$), treatment ($F(1,22) = 19.48$, $p < 0.0001$), and a significant pre-treatment $\times$ treatment ($F(1,22) = 14.24$, $p < 0.001$) interaction. As expected, the increase in total somatic signs observed in vehicle treated nicotine-infused $\alpha 5$ nAChR $+/+$ mice was significantly reduced in $\alpha 5$ nAChR $-/-$ mice (Figure 4). Pre-treatment with AuIB eliminated the expression of somatic signs in $\alpha 5$ nAChR $-/-$ mice. There were no significant main effects of genotype in this assessment.

3.6. AuIB has no effect on expression of nicotine CPA

To further evaluate the role of the $\alpha 3\beta 4$ nAChR subtype in another affective nicotine withdrawal measure, C57Bl/6J mice were treated with AuIB and evaluated in the CPA model (Figure 5). Mecamylamine precipitated a significant aversion in vehicle treated nicotine infused mice ($F(4,42) = 4.41$, $p < 0.005$). Nicotine infused mice treated with 3.5 pmol AuIB expressed a significant aversion similar to that observed in nicotine infused vehicle treated mice. The doses of AuIB and mecamylamine used in this assessment did not precipitate aversion in saline infused mice.

4. Discussion

The goal of the present study was to elucidate the role of $\alpha 3\beta 4^*$ nAChRs in nicotine mediated behaviors. These behavioral studies using pharmacological agents and genetically modified mice suggest that $\alpha 3\beta 4^*$ nAChRs are not involved in acute nicotine behaviors, but mediate nicotine reward and physical, but not affective nicotine withdrawal signs. Interestingly, the $\alpha 5$ nAChR subunit, which can coassemble with the $\alpha 3\beta 4^*$ subtype to form functional receptors, is not required in this receptor combination for mediating nicotine reward or nicotine withdrawal somatic signs.

In the current study, we injected doses of 3-14 pmol of AuIB peptide i.c.v., and a high dose of 70 pmol to further assess $\alpha 3\beta 4^*$ in acute nicotine behaviors. The 14 pmol dose, based on diffusion studies from Matta et al. (1995) translates to an estimated tissue concentration of $\approx 2.8 \mu\text{M}$, a value similar to the IC_{50} value ($0.75 \mu\text{M}$) obtained with rat $\alpha 3\beta 4$ nAChRs expressed in *Xenopus oocytes* (Luo et al., 1998) and the IC_{50} value ($2.2 \mu\text{M}$) that inhibits nicotine-induced hippocampal norepinephrine secretion (Fu et al., 1999). AuIB is 100-fold more potent at $\alpha 3\beta 4$ -containing nAChRs compared to other heteromeric nicotinic receptor combinations, and 10-fold more potent at $\alpha 3\beta 4^*$ than at the $\alpha 7$ homomeric subtype (Luo et al., 1998). Doses as high as 25 pmol AuIB, however, were not sufficient to block $\alpha 7$ nAChRs (Fu et al., 1999). Based on this information, we are confident that the doses used in our study produced relatively selective blockade of $\alpha 3\beta 4^*$ nAChRs.

Our acute assessment revealed that $\alpha 3\beta 4^*$ nAChRs are not significantly involved in mediating several nicotine-induced behaviors (antinociception, body temperature, locomotor activity) after acute nicotine administration in the mouse, even at very high AuIB doses. These findings contrast with previous studies suggesting involvement of $\beta 4$ nAChRs in acute nicotine-induced hypolocomotion (Salas et al., 2004), hypothermia (Sack et al., 2005), and antinociception (Semenova et al., 2012). The major difference between our study and the aforementioned studies is the use of a selective antagonist vs. $\beta 4$ nAChR knockout ($-/-$) mice. $\beta 4$ nAChRs can form functional receptors with $\alpha 6$ and $\alpha 4$ nAChR subunits, including in the MHb and the IPN (Barabino et al., 2001; Moretti et al., 2004; Gotti et al., 2007; Grady et al., 2009). Thus, these differences may reflect involvement of different populations of $\beta 4$ -containing nicotinic receptors, such as $\alpha 6\beta 4^*$ or $\alpha 4\beta 4^*$, which would be targeted in studies using $\beta 4$ nAChR $-/-$ mice, but not in studies using the AuIB antagonist. We also cannot rule out the possible contribution of compensatory mechanisms to these effects, as $\beta 4$ nAChR $-/-$ mice were found to have altered $\alpha 3$ nAChR expression in the brain (Salas et al., 2004). Further, $\beta 4$ nAChR $-/-$ mice were less sensitive to nicotine-induced hypomotility at low nicotine doses (0.5 mg/kg) (Salas et al., 2004), and it is unclear if this effect is present at higher nicotine doses such as those used in the current study (1.5 mg/kg). It is also possible that peripheral $\alpha 3\beta 4^*$ nAChRs contribute to the acute behaviors measured, thus, effects were not observed in the current study since AuIB was administered i.c.v.

In the nicotine reward assessment, AuIB dose-dependently blocked expression of nicotine CPP, suggesting a role for $\alpha 3\beta 4^*$ nAChRs in nicotine conditioned reward. Our finding

complements the recent finding that a relatively selectively $\alpha 3\beta 4^*$ antagonist blocks nicotine reinforcement in the rat intravenous self-administration model (Toll et al., 2012), as well as a study by Glick et al. (2011) implicating the involvement of MHb $\alpha 3\beta 4^*$ nAChRs in AuIB and 18-methoxycoronaridine induced decreases in nicotine self-administration. While the brain regions that mediate the blocking effect of AuIB on nicotine preference were not investigated in our experiments, the limited brain distribution of $\alpha 3\beta 4^*$ nAChRs suggest the MHb-IPN pathway as a possible site. The $\alpha 3\beta 4^*$ subtype was in fact shown to dominate function in the MHb (Quick et al., 1999). In line with this suggestion, a recent study showed that nicotine-induced increases in extracellular dopamine levels in the nucleus accumbens are blocked by intra-habenular administration of AuIB (McCallum et al., 2012). In addition, our results suggest that incorporation of the $\alpha 5$ nAChR subunit into the $\alpha 3\beta 4^*$ subtype is not required for its mediation of nicotine CPP, as AuIB blocked expression of nicotine CPP in $\alpha 5$ nAChR $-/-$ mice. Indeed, results using $\alpha 5$ nAChR $-/-$ mice in CPP are the opposite of that observed in the current study, as the $\alpha 3\beta 4^*$ antagonist blocked expression of nicotine CPP, while the effect appears to be enhanced at higher doses in $\alpha 5$ nAChR $-/-$ mice (Jackson et al., 2010). Overall, our results along with previous reports (Fowler et al., 2011; Toll et al., 2012), suggest that $\alpha 3\beta 4^*$ and $\alpha 5$ nAChRs differentially mediate nicotine reward and reinforcement. The lack of $\alpha 5$ nAChR subunit involvement in the effects of a selective $\alpha 3\beta 4^*$ antagonist is not very surprising since most $\alpha 3\beta 4$ subunits in the MHb-IPN do not contain $\alpha 5$, and the $\alpha 3\beta 4^*$ nAChR subtype controls acetylcholine release in the IPN independently of $\alpha 5$ subunits (Grady et al, 2009).

In the withdrawal assessment, AuIB dose-dependently attenuated somatic signs and hyperalgesia, implicating the $\alpha 3\beta 4^*$ nAChR subtype in physical nicotine withdrawal. Our findings are supported by Salas et al. (2004) and Stoker et al. (2012), who reported that nicotine withdrawal somatic signs and hyperalgesia were significantly reduced in $\beta 4$ nAChR $-/-$ mice. Somatic withdrawal signs were shown to be mediated by central, as well as peripheral nAChR populations by systemic and central administration of chlorisondamine, a noncompetitive nAChR antagonist that does not readily cross the blood brain barrier (Watkins et al., 2000). Although the $\alpha 3\beta 4^*$ subtype is both peripherally and centrally expressed, these studies suggest a role for central $\alpha 3\beta 4^*$ nAChRs in somatic nicotine withdrawal. Previous studies show that microinjection of the non-selective nAChR antagonist, mecamylamine, into the MHb or IPN of mice chronically treated with nicotine precipitated significant somatic signs, indicating that nAChRs in the habenulo-interpeduncular system are necessary for nicotine withdrawal in mice (Salas et al., 2009). Taken together, these studies propose a role for $\alpha 3\beta 4^*$ nAChRs in the MHb and IPN in somatic nicotine withdrawal.

The lack of effect of AuIB in the plus maze and CPA withdrawal assessments suggests that $\alpha 3\beta 4^*$ nAChRs are not involved in mediating affective nicotine withdrawal. However, this effect was surprising since $\beta 4$ nAChR $-/-$ mice do not display anhedonia as measured in the elevated intracranial self-stimulation procedure following nicotine withdrawal (Stoker et al., 2012). As mentioned in the acute assessment, these results may again reflect involvement of $\beta 4$ -containing nAChRs not targeted by AuIB, such as $\alpha 6\beta 4^*$ or $\alpha 4\beta 4^*$. Indeed, $\alpha 6$ -containing nAChRs mediate affective nicotine withdrawal behaviors (Jackson et al., 2009c). While no available studies have specifically addressed the role of the $\alpha 4$ nAChR subunit in nicotine withdrawal, it is likely, due to $\alpha 4\beta 2^*$ being the major heteromeric nAChR subtype in the brain, and based on the role of $\beta 2$ -containing nAChRs in affective nicotine withdrawal (Jackson et al., 2008) that $\alpha 4$ also plays a significant role in these behaviors. Thus, it is possible that the role of $\beta 4$ in nicotine withdrawal varies depending on the subtype composition, with $\alpha 3\beta 4^*$ nAChR subtypes contributing primarily to physical nicotine withdrawal, and $\alpha 6\beta 4^*$ and/or $\alpha 4\beta 4^*$ nAChRs contributing to affective nicotine withdrawal. Additionally, intracranial self stimulation, elevated plus maze, and CPA

measure three different components of the affective nicotine withdrawal syndrome; brain reward/anhedonia (Stoker and Markou, 2011), anxiety (Handley and Mithani, 1984), and the aversive motivational component of withdrawal (Stinus et al., 1990), respectively. Because these components are likely mediated by different brain substrates/pathways, we cannot rule out the possibility that brain pathways, in addition to nAChR assembly, contribute to differences between the findings in these studies.

As previously shown (Jackson et al., 2010), somatic nicotine withdrawal signs were also reduced in $\alpha 5$ nAChR $-/-$ mice in the current study. Interestingly, pre-treatment with AuIB attenuated somatic signs in $\alpha 5$ nAChR $-/-$ mice. These results indicate that while both $\alpha 5$ and $\alpha 3\beta 4^*$ nAChRs mediate somatic nicotine withdrawal signs, $\alpha 5$ subunit coassembly with the $\alpha 3\beta 4^*$ nAChR subtype is not required in the CNS for mediating this aspect of nicotine withdrawal. While $\alpha 3$, $\alpha 5$ and $\beta 4$ subunits are highly expressed in the MHB and IPN (Wada et al., 1990; Broide et al., 2002; Salas et al., 2004), it is possible that $\alpha 5$ nAChRs mediate somatic nicotine withdrawal independently of the $\alpha 3\beta 4^*$ nAChR subtype.

A limitation to these findings using $\alpha 5$ nAChR $-/-$ mice is that our results may be impacted by compensatory mechanisms resulting from up-regulation of nAChR genes in the absence of the $\alpha 5$ subunit, which may take over the function of the missing subunit. In the absence of the $\alpha 5$ nAChR subunit, however, mice have normal brain anatomy, normal mRNA expression of $\alpha 4$, $\alpha 6$, $\alpha 7$, $\beta 2$, and $\beta 4$ nAChR subunits, and normal binding in the brain (Salas et al., 2003). Although there are no differences in expression of other nAChRs, these findings cannot rule out the possibility of functional changes occurring due to alternative mechanisms, such as post-translational modifications. Nonetheless, we feel the Salas et al. (2003) data supports our current findings that the behavioral effects of AuIB in $\alpha 5$ nAChR $-/-$ mice suggest that the behaviors tested are mediated independently of the $\alpha 5$ nAChR subunit.

The major findings of this study are that the $\alpha 3\beta 4^*$ nAChR subtype mediates nicotine reward and physical nicotine withdrawal signs, but not the initial effects of nicotine or nicotine withdrawal affective signs. Mediation of nicotine reward and nicotine withdrawal somatic signs by $\alpha 3\beta 4^*$ is independent of the $\alpha 5$ nAChR subunit. $\alpha 3\beta 4^*$ nAChRs may serve as potential targets for alleviating important aspects of nicotine dependence.

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Paper Highlights

- AuIB, a selective $\alpha 3\beta 4$ antagonist, was used to assess $\alpha 3\beta 4$ in nicotine behaviors
- $\alpha 3\beta 4$ nicotinic receptors do not mediate acute nicotine behaviors
- $\alpha 3\beta 4$ nicotinic receptors mediate nicotine reward and withdrawal independent of $\alpha 5$
- $\alpha 3\beta 4$ nicotinic receptors are involved in somatic, but not affective withdrawal

Figure 1. AulB dose-dependently attenuates nicotine CPP in C57Bl/6J mice

C57Bl/6J mice were conditioned for 3 days with nicotine (0.5 mg/kg) or saline. A robust CPP was observed in nicotine –conditioned mice pre-treated with vehicle. AulB dose-dependently blocks expression of nicotine CPP. Each point represents the mean $\pm$ S.E.M. of 6-9 mice per group. * denotes $p < 0.05$ vs. saline groups and nicotine-AulB 7 and 14 pmol. Veh, vehicle

Figure 2. Nicotine CPP is attenuated in $\alpha 5$ nAChR $-/-$ mice

$\alpha 5$ nAChR $-/-$ and $+/+$ littermates express a significant CPP after 3 days conditioning with nicotine (0.5 mg/kg, s.c.). AuIB (14 pmol, i.c.v.) blocks expression of nicotine CPP in both $\alpha 5$ nAChR $+/+$ and $-/-$ mice. Each point represents the mean $\pm$ S.E.M. of 6-9 mice per group. * denotes $p < 0.05$ vs. saline controls and AuIB pre-treated mice. Veh, vehicle

Figure 3. AuIB dose-dependently reduces physical nicotine withdrawal signs, but has no effect on anxiety-related behavior in C57Bl/6J mice

Nicotine withdrawn **C57Bl/6J** mice treated with 3, 3.5 and 7 pmol AuIB display **A.** a significant increase in anxiety-related behavior similar to that observed in vehicle treated counterparts, but **B.** attenuation of total somatic signs and **C.** hyperalgesia response. Each point represents the mean $\pm$ S.E.M. of 6-8 mice per group. * denotes $p < 0.05$ vs. all groups; + denotes $p < 0.05$ vs. saline, nic-vehicle, and nic-AuIB 3.5 pmol groups. Sal, saline; nic, nicotine

Figure 4. AuIB attenuates nicotine withdrawal somatic signs in $\alpha 5$ nAChR $-/-$ mice

Nicotine withdrawal somatic signs are reduced in vehicle treated $\alpha 5$ nAChR $-/-$ mice compared to $+/+$ counterparts, and attenuated in AuIB treated $\alpha 5$ nAChR $-/-$ mice. Each point represents the mean $\pm$ S.E.M. of 4-8 mice per group. * denotes $p < 0.05$ vs. all groups. + denotes $p < 0.05$ vs. saline controls, $\alpha 5$ nAChR $+/+$ nic-vehicle, and $\alpha 5$ nAChR $-/-$ nic-AuIB 3.5 pmol groups

Figure 5. AuIB does not reverse expression of nicotine CPA in C57Bl/6J mice

Mecamylamine precipitated a significant CPA in chronic nicotine-infused **C57Bl/6J** mice. Pre-treatment with AuIB failed to reverse expression of mecamylamine-precipitated aversion. The legend is listed as mini pump infusion-conditioning drug/i.c.v. treatment. Each point represents the mean $\pm$ S.E.M. of 8-10 mice per group.* denotes $p < 0.05$ vs. saline groups. Veh, vehicle; mec, mecamylamine

Table 1
AuIB does not mediate acute nicotine responses

Antinociception (tail-flick and hot plate), body temperature, and locomotor activity were measured after nicotine or saline administration (s.c.). AuIB (14 and 70 pmol, i.c.v.) administered 5 min prior to nicotine or saline failed to significantly alter acute nicotine behaviors in any test. Each point represents the mean $\pm$ S.E.M. of 6 mice per group.

Treatment Group	Tail-flick % MPE $\pm$ SEM	Hot Plate % MPE $\pm$ SEM	Body Temp $\Delta^{\circ}\text{C} \pm$ SEM	Locomotor Activity Interrupts Nb. $\pm$ SEM
Saline- vehicle	1 $\pm$ 1	7 $\pm$ 4	0.3 $\pm$ 0.2	221 $\pm$ 58
AuIB (14)- Saline	1 $\pm$ 1	10 $\pm$ 6	0.2 $\pm$ 0.2	331 $\pm$ 78
AuIB (70)- Saline	1 $\pm$ 1	5 $\pm$ 4	0.2 $\pm$ 0.1	279 $\pm$ 89
Vehicle- Nicotine	87 $\pm$ 9 *	89 $\pm$ 7 *	-5.6 $\pm$ 0.2 *	11 $\pm$ 4 *
AuIB (70)- Nicotine	80 $\pm$ 6 *	94 $\pm$ 6 *	-5.7 $\pm$ 0.2 *	17 $\pm$ 11 *
AuIB (70)- Nicotine	81 $\pm$ 12 *	91 $\pm$ 8 *	-5.4 $\pm$ 0.4 *	25 $\pm$ 14 *

* denotes $p < 0.0001$ vs. saline controls.