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The Necessity of α4* Nicotinic Receptors in Nicotine-Driven Behaviors: Dissociation Between Reinforcing and Motor Effects of Nicotine

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Here we utilize a mouse line with a targeted deletion of the α4 subunit (α4−/− mice), to investigate the role of α4* nAChRs in reinforcing and locomotor effects of nicotine. Within a conditioned place preference paradigm, both α4−/− mice and wild-type (WT) littermates showed a similar place preference to nicotine (0.5 mg/kg i.p.) conditioning. When assessed for operant intravenous self-administration of nicotine (0.05 mg/kg/infusion), α4−/− mice did not differ from their WT littermates in self-administration behavior. To further examine a modulatory role for α4* nAChRs in the reinforcing effects of nicotine, a transgenic mouse with a point mutation of the α4 subunit (α4-S248F) that renders increased sensitivity to low dose nicotine, was assessed for nicotine self-administration over a range of doses. At higher doses examined (0.05 and 0.07 mg/kg/infusion) there was no difference in intravenous nicotine self-administration; however, when mice were offered a lower dose of nicotine (0.03 mg/kg/infusion), α4-S248F mice showed greater nicotine intake than controls. Acute administration of 0.5 mg/kg nicotine caused significant locomotor depression in WT mice but α4−/− mice instead showed significant hyperactivity. Following chronic, intermittent administration of this dose of nicotine only WT mice displayed significant tolerance. Analogous experiments utilizing administration of the nicotinic antagonist mecamylamine in WT mice confirmed a dissociation between the putative nicotinic receptor subtypes required for mediating psychomotor and reinforcing effects of nicotine. These data demonstrate a necessary role for α4* nAChRs in the locomotor depressant effect of nicotine but not the reinforcing effects that support ongoing self-administration of nicotine.

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INTRODUCTION

Cigarette smoking is the single largest preventable cause of death and disease in the developed world (WHO, 2008), yet many individuals continue to smoke tobacco products due to addiction to nicotine. Less than 5% of individuals successfully quit smoking without the use of nicotine replacement therapies, and no more than one-third are successful with them. Buproprion and varenicline may aid in smoking cessation; however, they are not always effective and adverse side effects may occur (Doggrell, 2007; Rollema *et al*, 2007).

Nicotine acts centrally on neuronal nicotinic acetylcholine receptors (nAChRs), pentameric ion-channels for which 12 subunits (α2-α10, β2-β4) have so far been identified (Dani and Bertrand, 2007; Gotti *et al*, 2009; McGehee and Role, 1995). The α7–α9 subunits form homomeric receptors, although the majority of subunits form functional heteromeric pentamers with diverse properties. Of the heteromeric receptors, α4β2* (* denoting the potential presence of other subunits), is the most abundantly expressed in the brain (Drago *et al*, 2003; Gotti *et al*, 2006; Picciotto *et al*, 1995).

Dopaminergic projections from the VTA to the NAc are critical in mediating nicotine addiction (Corrigall *et al*, 1992, 1994). Numerous nAChR subunits are expressed in these DA neurons, including the β2, β3, α3, α4, α5, and α6 subunits (Champtiaux *et al*, 2003; Changeux, 2010; Gotti *et al*, 2005; Grady *et al*, 2007, 2009; Klink *et al*, 2001); α4 and β2 subunits are also expressed on GABAergic midbrain neurons with α7 homomeric receptors on the terminals of glutamatergic inputs. The β2* nAChRs have a critical role in mediating the rewarding effects of nicotine (Maskos *et al*, 2005; Picciotto *et al*, 1998; Pons *et al*, 2008), and studies have suggested roles for α6, β4, and α4 subunits (Brunzell *et al*, 2006, 2010; Changeux, 2010; Jackson *et al*, 2009; Pons

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et al, 2008; Tapper et al, 2004). The α5* nAChRs have recently been shown to mediate an inhibitory motivational signal that limits nicotine intake (Fowler et al, 2011). Receptors containing β2, α6, and α4 subunits have been implicated in mediating locomotor effects of acute and chronic nicotine (Brunzell et al, 2006, 2010; Changeux, 2010; Marubio et al, 2003; McCallum et al, 2006; Ross et al, 2000; Tapper et al, 2004, 2007; Tritto et al, 2004).

Here we utilize a mouse line with a targeted deletion of the α4 subunit (α4−/− mice (Ross et al, 2000)). The targeting construct was designed to create a non-functioning allele by removing a 750 bp fragment from exon five, encoding the first hydrophobic transmembrane domain through to the second intracytoplasmic loop. The lack of mRNA corresponding to this deleted sequence was validated and [^{3}H]epibatidine and [^{3}H]nicotine labeling was restricted to several discrete nuclei, whereas [^{125}I] α-bungarotoxin showed preservation of binding in mutant mouse brains (Ross et al, 2000).

We have employed these well-characterized α4−/− mice to further investigate the necessity of α4* nAChRs in the reinforcing and locomotor effects of nicotine. The locomotor effects of acute and chronic nicotine were investigated over a highly resolved timeframe, consonant with the rapid uptake into brain and short half-life of nicotine in mice (Petersen et al, 1984). Nicotine reward and reinforcement were investigated in both conditioned place preference and operant intravenous self-administration of nicotine. The chronic self-administration of nicotine employed here allowed for analysis of the contribution of α4* nAChRs in a paradigm thought to closely model nicotine abuse in humans (Corrigall, 1999) and that allows investigation of the motivation for nicotine and relapse-like behavior following a period of abstinence. In addition to a genetic approach, the contributions of different subunit configurations of nAChRs to nicotine-mediated behaviors were also further elucidated utilizing the nicotinic receptor antagonist mecamylamine.

Further investigations into the potential role of α4* nAChRs in mediating nicotine reward and reinforcement were made with the α4-S248F mouse (Teper et al, 2007). These mice possess a single point mutation, resulting in the substitution of a phenylalanine for a serine residue within the M2 channel lining region of the mutant α4 subunit. In humans, the hereditary disorder autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) occurs in those heterozygous for the mutation (Scheffer et al, 1995). Heterozygous α4-S248F mice do not display spontaneous seizures, though moderate to high doses of nicotine can induce an ADNFLE-like syndrome. Most importantly for the present study, the α4-S248F mutation confers increased sensitivity of the α4* receptor to low doses of nicotine (Teper et al, 2007).

MATERIALS AND METHODS

Animals

All experiments were performed in adherence to the Prevention of Cruelty to Animals Act, 1986, under the guidelines of the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. The α4−/− mice (Ross et al, 2000) were backcrossed 10 generations to a C57Bl6 background. α4 +/− mice were bred to provide null and wild-type littermates for experimentation. The α4-S248F mice (Teper et al, 2007) were also backcrossed 10 generations to C57Bl6; heterozygous α4-S248F mice were compared with WT littermate controls. C57Bl6 mice used in the mecamylamine experiments were from Animal Resource Centre (Perth, WA, Australia). All experiments were performed with adult male mice, 9–13 weeks of age at commencement. Mice used in locomotor and conditioned place preference experiments were group housed in a standard 12 h light-dark cycle (light 0700–1900 h) with nesting material and free access to food (standard mouse chow) and water. Mice used for intravenous self-administration studies were singly housed in a 12 h reverse light-dark cycle (light 1900–0700 h).

Drugs

Nicotine hydrogen tartrate, mecamylamine hydrochloride (Sigma-Aldrich, St Louis, MO) and Ketamine (Parnell Laboratories, Alexandria, NSW, Australia), were dissolved in sterile 0.9% saline. All nicotine doses quoted refer to nicotine free base. Neomycin sulfate (Delta Veterinary Laboratories, Hornsby, NSW, Australia), was diluted in heparinized (90 U) 0.9% saline. Meloxicam was obtained from Boeringher Ingleheim (Ingleheim, Germany).

Conditioned Place Preference

The conditioned place preference apparatus has been previously described (Brown et al, 2009; McPherson et al, 2010). WT ($n=18$) and α4−/− mice ($n=16$) were habituated to the experimental room for at least 30 min before each session. On day 1 (pre-conditioning), mice were placed in the neutral zone and allowed free access to the apparatus for 10 min, with time spent in each compartment recorded (Motor Monitor; Kinder Scientific, CA, USA). Drug pairing was biased to the least preferred chamber. This strategy resulted in a balanced allocation between chambers and genotypes, indicating no true drug-naïve side preference. On days 2–5, mice underwent twice daily conditioning sessions separated by 5–6 h where they received alternating injections of nicotine (0.5 mg/kg, i.p.) or saline (0.1 ml/10 g body weight, i.p.) and were immediately confined into a conditioning chamber for 10 min. On day 6 (test day) mice were allowed free access to all three chambers for 10 min. Place preference was determined as a significant increase in time spent in the drug-paired chamber, as a percentage of time spent in the conditioning chambers (preference score), post-conditioning compared with pre-conditioning.

Following habituation, a separate cohort of C57Bl6 mice were administered mecamylamine (0.5 mg/kg i.p., $n=12$) or saline ($n=12$), 10 min before nicotine conditioning sessions. All mice were administered saline 10 min before saline conditioning sessions. Locomotor activity was also measured, by distance (in cm) traveled during conditioning sessions. Place preference was measured as above.

Operant Self-Administration

Mice were engaged in natural reward-based instrumental learning task, as previously described (Brown *et al*, 2009; McPherson *et al*, 2010). Delivery of 10% sucrose solution to a liquid receptacle in the chamber (5 ml) was contingent upon an active lever press. A fixed ratio of 1 (FR1) was employed. Daily 2 h sessions were held in the dark phase.

Intravenous self-administration of nicotine (0.05 mg/kg/infusion) was assessed in WT ($n=10$) and α4−/− littermates ($n=11$) according to an established protocol previously used for morphine and cocaine (Brown *et al*, 2009; McPherson *et al*, 2010). Mice were anaesthetized (isoflurane 1.5–1.8%) and indwelling venous cannulae were implanted into the jugular vein. After surgery mice were injected with meloxicam (1 mg/kg i.p.) and allowed to recover. For up to 5 days following surgery, the catheters were flushed twice daily, once with 10 U heparinized saline and once with 90 U heparinized saline containing 6 mg/ml neomycin sulfate, and thereafter with heparinized saline twice daily. The patency of the catheters was evaluated regularly, and before mice being placed into withdrawal, using 0.02–0.03 ml infusion of ketamine (15 mg/ml). If prominent signs of hypnosis were not apparent within seconds of infusion the mouse was excluded.

For IV self-administration of nicotine (0.05 mg/kg/infusion) mice were connected via the jugular catheter to an intravenous line (Tygon, inner diameter 0.02 in, outer diameter 0.06 in) connected to a 22-gauge swivel (Instech Solomon, Plymouth Meeting, PA, USA). The swivel was connected to a syringe held in the infusion pump (model PHM-100SVA; Med Associates) with Bcoex-T22 tubing (inner diameter 0.24 in, outer diameter 0.64 in; Instech Soloman). S+ and CS+ were present and an FR1 schedule was employed during 2 h self-administration sessions held daily during the dark phase. The infusion volume was 20 μl, duration 1.7 s. A maximum of 80 drug infusions (at which the session terminated) was set, as well as a 10 s timeout period after each drug infusion. During the timeout period active lever presses were recorded but no drug infusion occurred. Mice who failed to meet criteria of at least 10 active lever presses, with at least 70% lever discrimination over 3 days were excluded from the study.

'Breakpoint' was assessed using a progressive ratio schedule, where an increasing number of active lever presses were required to receive each infusion (1, 3, 9, 13, 16, 18, 20, 22, 24, 25, 27, 28, 29, 31, 32, 34, 35, 37, 39, 41, 44, 47, 52, 64, 76, 88, 100, 112, 124, 136 lever presses for each subsequent infusion) (Brown *et al*, 2009). The breakpoint was defined as the last completed ratio, after which a period of 60 min ensued where no reinforcer was earned or the final ratio completed within the 2 h session. After 3 weeks abstinence in the home cage, drug-seeking was precipitated by placing mice into the operant chambers with S+ present. Drug-seeking was assessed under extinction conditions (FR1 response resulted in CS+ but no infusion of nicotine) for 1 h (Brown *et al*, 2009).

The preceding protocol was also used to compare IV self-administration of nicotine in heterozygous α4-S248F knock-in mice and their WT littermates for 0.03 mg/kg/infusion ($n=7$ WT, 10 α4-S248F), 0.05 mg/kg/infusion ($n=13$ WT, 9 α4-S248F), and 0.07 mg/kg/infusion ($n=14$ WT, 6 α4-S248F). For 0.07 mg/kg/infusion maximum infusions were reduced to 60 per session, whereas for 0.03 mg/kg/infusion a maximum of 120 was allowed.

A modified version of the protocol was employed to investigate the effect of mecamylamine on nicotine IV self-administration of (0.07 mg/kg/infusion) in a separate cohort of WT C57Bl6 mice ($n=11$). The half-life of mecamylamine in rodents is ~1.2 h (Debruyne *et al*, 2003) so self-administration sessions were reduced to 1 h. Following acquisition of nicotine self-administration mice were administered saline (0.1 ml/10 g body weight, i.p.) before the following three daily self-administration sessions, then before the fourth daily session administered mecamylamine at 0.5, 1 or 2 mg/kg, i.p., randomly assigned. Mice were then administered saline before a further three daily sessions, before again being administered an alternate dose of mecamylamine.

Locomotor Response to Nicotine

Locomotor responses to chronic, intermittent nicotine were investigated in WT ($n=15$) and α4−/− mice ($n=18$) using photo-optic locomotor cells (Truscan Photobeam; Coulbourn Instruments, Allentown, PA, USA) in a low luminosity (20 lux), controlled environment. Movement was measured over x and y axes by optic sensor beams. Before each session, mice were habituated to the experimental room for at least 30 min. Mice were habituated to the locomotor cells for 30 min per day over 3 consecutive days (habituation days 1–3). On the following 5 days (treatment days 1–5), mice were placed in the locomotor cells immediately after nicotine (0.5 mg/kg, i.p.) or saline injection, and their locomotor activity was recorded for 30 min. After completing chronic nicotine or vehicle administration, mice remained without treatment in their home cage for 7 days. The following day (challenge day), all mice received a challenge dose of nicotine (0.5 mg/kg, i.p.) and their locomotor activity was again measured for 30 min.

Data Analysis

Sigma Stat version 3.5 (Jandel, San Jose, CA, USA) was used to analyze all the data, and significance was set at $p<0.05$. Two-way repeated measures ANOVA was used to compare the preference score before and after conditioning with conditioning and genotype (for α4−/− experiments) or treatment (for mecamylamine experiments) as the factors. Acquisition and progressive ratio data for IV self-administration experiments was compared between the genotypes over time by two-way repeated measures ANOVA, average response rates compared between the genotypes by Student's t-test when comparing α4−/− mice with their WT littermates and by two-way ANOVA for analyzing the effect of genotype and nicotine dose in α4-S248F mice and their WT littermates. Locomotor responses to nicotine were compared within each genotype by two-way repeated measures ANOVA with treatment and time as the factors and within each treatment group by genotype and time. For all ANOVAs, Tukey's tests were employed for *post-hoc* pairwise comparisons, with the exception of mecamylamine IVSA experiments where a Holm–Sidak test was used to compare the effect of each dose with vehicle treatment.

RESULTS

The α4 nAchR Subunit is not Critical for Conditioned Place Preference to Nicotine in Mice

A role for α4* nAChRs in mediating a conditioned place preference to nicotine has been suggested (Pons *et al*, 2008; Tapper *et al*, 2007). To establish if the α4 subunit is critical in mediating this nicotine-induced behavior, α4−/− mice and their WT littermates were investigated.

A conditioned place preference to nicotine (0.5 mg/kg, i.p) was established in WT and α4−/− mice (Figure 1). Two-way repeated measures ANOVA revealed no effect of genotype ($F_{(1,67)} = 0.918$, $p > 0.05$), but a significant effect of conditioning ($F_{(1,67)} = 34.255$, $p < 0.001$). *Post-hoc* analysis (Tukey's test) revealed a significant increase in time spent in the drug-paired chamber for both WT and α4−/− mice (Figure 1a, WT: $q = 7.490$, $p < 0.001$, B, α4−/−: $q = 4.314$, $p = 0.005$).

The α4 nAchRs Subunit is not Critical for Chronic IV Self-Administration of Nicotine in Mice

WT and α4−/− mice both acquired reliable IV self-administration of nicotine, with two-way repeated measures ANOVA revealing no significant differences between the genotypes in the number of infusions of nicotine administered (Figure 2a, $F_{(1,169)} = 0.118$, $p > 0.05$) or percentage discrimination between lever presses on the active and inactive levers (Figure 2b, $F_{(1,169)} = 0.197$, $p > 0.05$). 76.9% of WT and 84.6% of α4−/− mice achieved self-administration criteria. Analysis of the micro-architecture of self-administration sessions through event records also revealed no substantive difference in the pattern of responding between genotypes (Figure 2d). Furthermore, student's *t*-test analysis of aggregate data from 5 days of stable responding did not reveal a significant difference between the genotypes for these measures (Figure 2c, $t = 0.931$, $p > 0.05$).

Motivation to self-administer nicotine was assessed using a progressive ratio schedule of responding (Brown *et al*, 2009). WT and α4−/− mice showed a similar breakpoint (Figure 2e; student's *t*-test, $t = -0.185$, $p > 0.05$). Two-way repeated measures ANOVA revealed no effect of genotype in cumulative active lever presses over the 2 h PR session (Figure 2f, $F_{(1,187)} = 0.970$, $p > 0.05$).

WT and α4−/− mice were then tested for cue-induced drug-seeking following a period of withdrawal. Following 21 days of withdrawal, mice were returned to the experimental chambers for a 1 h session, with S + and CS + present but no drug available (extinction). During these sessions WT and α4−/− mice displayed similar cue-driven initial drug-seeking behavior, with no significant difference between the genotypes for the number of active lever presses performed or discrimination between the active and inactive levers (Figure 2g, H; Student's *t*-test, active lever presses: $t = 0.456$, $p > 0.05$, lever discrimination: $t = -0.0349$, $p > 0.05$).

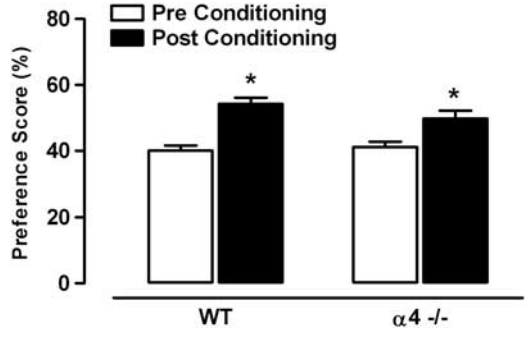

Figure 1 Conditioned place preference to nicotine (0.5 mg/kg, i.p.) in wild-type (WT, $n = 18$) and α4−/− ($n = 16$) mice. The preference score is the time spent in the nicotine-paired compartment divided by the total time spent in the both the nicotine- and saline-paired compartments, multiplied by 100. Time spent in the neutral zone is disregarded. *$p < 0.05$ compared with pre-conditioning session (*post-hoc* Tukey's test following two-way repeated measures ANOVA). The data are expressed as mean (± SEM) of the preference score.

The α4 nAchR Subunit Modulates the Reinforcing Effects of Nicotine

Numerous studies have suggested a role for α4* nAChRs in nicotine reinforcement (George *et al*, 2010; Lam and Patel, 2007; Li *et al*, 2005; Metaxas *et al*, 2010; Pons *et al*, 2008; Rollema *et al*, 2007; Tapper *et al*, 2007). Although our results suggest the α4 subunit is not critical in mediating the reinforcing effects of nicotine, this does not preclude a modulatory role for the α4 subunit in this context.

To investigate this possibility we compared IV self-administration of nicotine in WT and α4-S248F mice over a range of doses, 0.03 mg/kg/infusion, 0.05 mg/kg/infusion, and 0.07 mg/kg/infusion (Figure 3). WT mice self-administered nicotine in a dose-related fashion, showing decreased responding at 0.03 mg/kg/infusion, whereas responding by α4-S248F mice was more stable across the doses. Two-way ANOVA revealed a significant effect of genotype for the number of infusions received (Figure 3a, $F_{(1,\ 58)} = 4.219$, $p = 0.045$) and number of active lever presses made (Figure 3b, $F_{(1,58)} = 5.079$, $p = 0.028$), as well as genotype × dose interactions for infusions received (Figure 3a, $F_{(1,58)} = 3.188$, $p = 0.049$) and active lever presses (Figure 3b, $F_{(1,58)} = 3.193$, $p = 0.049$). *Post-hoc* analysis revealed that WT mice received significantly less infusions and made significantly less active lever presses at 0.03 mg/kg/infusion, compared with WT mice administering 0.07 mg/kg/infusion of nicotine (Figure 3a, $q = 4.514$, $p = 0.007$, 3B, $q = 3.869$, $p = 0.023$), and with α4-S248F mice at 0.03 mg/kg/infusion (Figure 3a, $q = 4.458$, $p = 0.003$, 3B $q = 4.613$, $p = 0.002$). Both genotypes displayed a high level of lever discrimination across all doses, with no effect of genotype (Figure 3c, $F_{(1,58)} = 1.234$, $p > 0.05$), dose (Figure 3c, $F_{(1,58)} = 0.217$, $p > 0.05$) or interaction between the two (Figure 3c, $F_{(1,58)} = 1.670$, $p > 0.05$). Although 71.4% of α4-S248F mice achieved criteria at 0.03 mg/kg/infusion of nicotine, only 53.8% of WT mice did. A more similar proportion of mice achieved criteria at 0.05 mg/kg/infusion (WT = 76.5%, α4-S248F = 60.0%), whereas at 0.07 mg/kg/infusion only 50.0% of α4-S248F mice achieved criteria compared with 87.5% of WT mice (Figure 3d).

The α4 nAchRs Subunit is Required for Mediating the Acute Locomotor Depressant Effects of Nicotine in Mice

Tolerance and/or sensitization to the psychomotor effects of drugs of abuse are thought to be indicative of long-term neuroplastic changes occurring within the basal ganglia

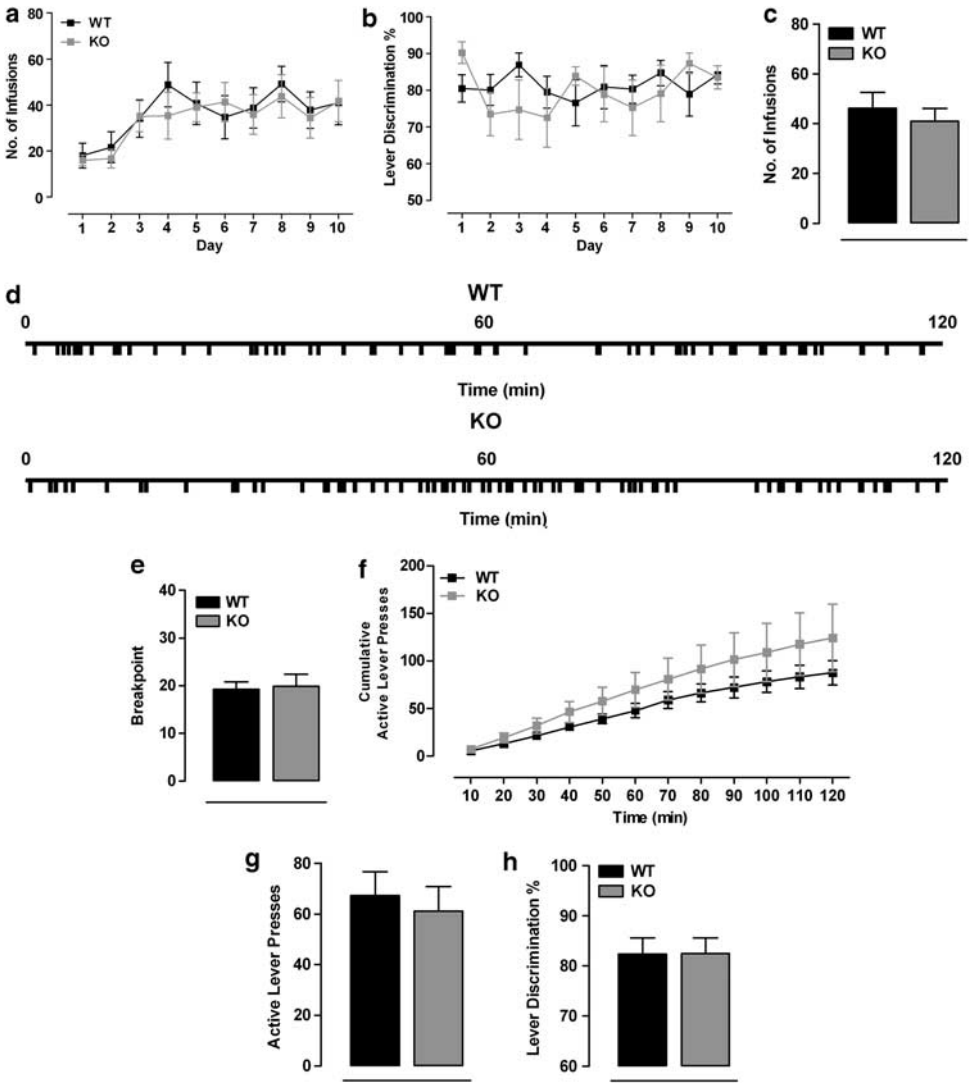

Figure 2 Nicotine (0.05 mg/kg per infusion) self-administration in α4 −/− (KO, $n = 11$) and wild-type (WT, $n = 10$) mice. (a, b) Acquisition of self-administration behavior and (c, d) average responses from 5 days stable responding, both expressed as number of nicotine infusions received (a, c) and percentage discrimination between the active and inactive levers (b) on a fixed ratio (FR) 1 schedule. (d) Typical event record of infusions administered by WT or α4 −/− mice over a 2 h session. (e) Nicotine self-administration on a progressive ratio (PR) schedule is expressed as breakpoint (defined as the last completed ratio, after which a period of 60 min ensued where no reinforcer was earned. If this did not occur the session was terminated after 2 h and the breakpoint was defined as the final ratio completed within the 2 h session) and (f) cumulative active lever presses over 2 h. (g, f) Cue-induced drug-seeking after a period of 21 days withdrawal, expressed as active lever presses (g) and percentage discrimination between the active and inactive levers (h), where no drug infusions are received. All data are expressed as mean (± SEM). No significant differences occur on all measures.

(Robinson and Berridge, 2008). To investigate the potential role of α4* nAChRs in mediating such changes we investigated the response of WT and α4−/− mice to the acute and chronic locomotor effects of nicotine.

Before investigation into the locomotor effects of acute nicotine, mice were habituated to the test chamber over three daily 30 min sessions, and the genotypes did not differ in their locomotor response to a novel or habituated environment (data not shown). Mice of both genotypes were then divided into two groups and administered either saline or nicotine (0.5 mg/kg, i.p.) and returned to the test chamber for 30 min. Two-way repeated measures ANOVA of genotype and time showed there was no effect of genotype or interaction between genotype × time for mice administered saline in any of the measures recorded (Figure 4a and b, distance: genotype $F_{(1,88)} = 0.963$, $p > 0.05$, genotype × time $F_{(5,88)} = 0.636$, $p > 0.05$; C, D, move time: genotype $F_{(1,88)} = 1.452$ $p > 0.05$, genotype × time $F_{(5,88)} = 0.822$, $p > 0.05$; E, F, moves: genotype $F_{(1,88)} = 0.0526$ $p > 0.05$, genotype × time $F_{(5,88)} = 1.124$, $p > 0.05$).

WT mice administered nicotine showed a decrease in locomotor activity compared with WT mice administered saline (Figure 4a, c and e), with significantly less movements initiated over the first 10 min following nicotine administration (Figure 4e, treatment × time: $F_{(1,89)} = 4.722$, $p < 0.001$, *post-hoc* Tukey's test: 5 min, $q = 4.657$, $p = 0.002$; 10 min, $q = 2.910$, $p = 0.046$). Conversely, α4−/− mice administered nicotine showed increased locomotor activity compared with saline-treated α4−/− mice (Figure 4b, d and f). Two-way repeated measures ANOVA of treatment

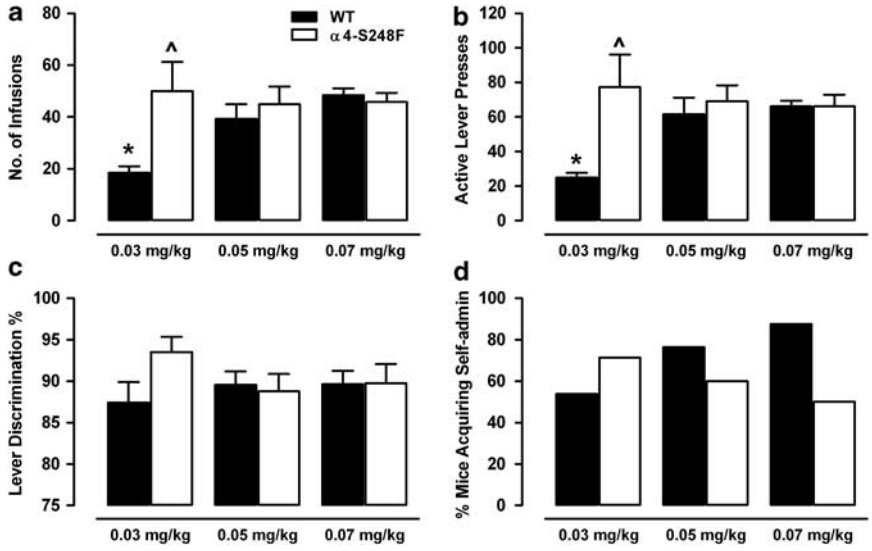

Figure 3 Nicotine self-administration in WT and α4-S248F mice at 0.03 mg/kg/infusion ($n = 7$ WT, 10 α4-S248F), 0.05 mg/kg/infusion ($n = 13$ WT, 9 α4-S248F), and 0.07 mg/kg/infusion ($n = 14$ WT, 6 α4-S248F). (a) Average number of nicotine infusions administered in a 2 h session. (b) Average number of active lever presses. (c) Average percentage of lever presses on the active, drug-paired lever. For (a, b, c) average response was taken as the mean response of each mouse over five stable daily self-administration sessions, and these expressed as mean (± SEM) for each genotype and dose. (d) Percentage of mice achieving self-administration criteria of at least 10 active lever presses, with at least 70% lever discrimination over 3 days for each genotype and dose. *$p < 0.05$, 0.03 *vs* 0.07, within genotype, ^$p < 0.05$, WT *vs* α4-S248F within dose (*post-hoc* Tukey's test following two-way ANOVA).

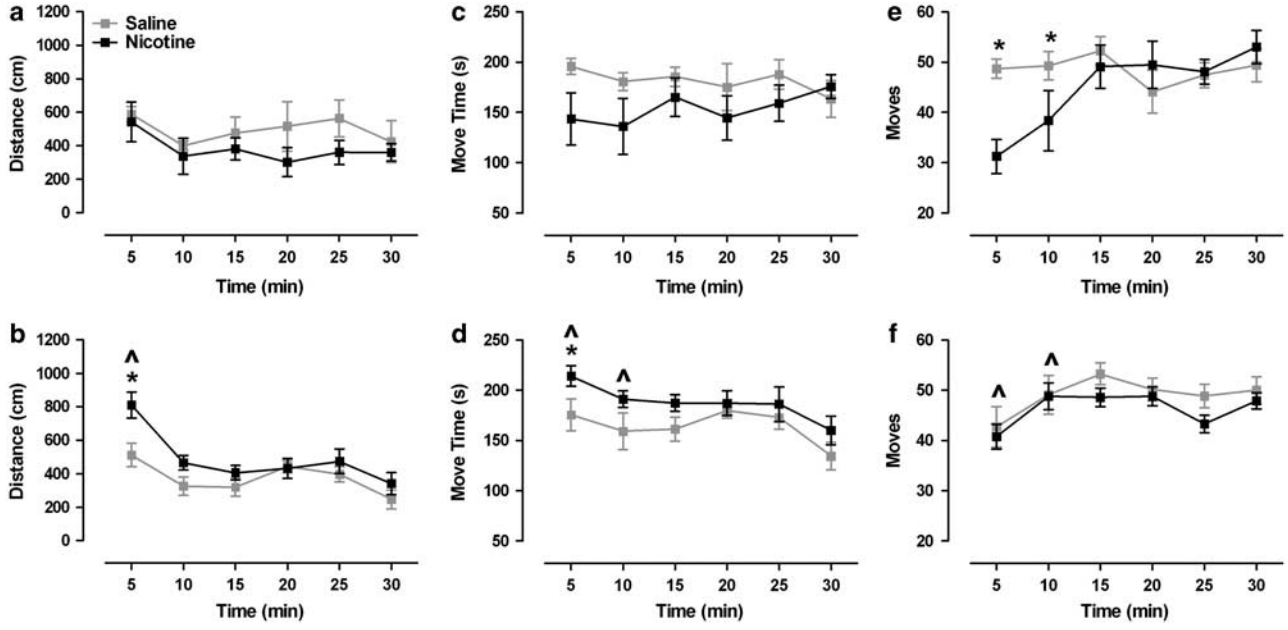

Figure 4 Treatment day 1: timecourse of effect of acute nicotine (0.5 mg/kg, i.p.) or saline administration on locomotor activity in wild-type (a, c, e, $n = 15$) and α4−/− mice (b, d, f, $n = 18$) over 30 min, as measured by distance traveled (a, b), move time (c, d) and number of moves initiated (e, f). All data are expressed as mean (± SEM). *$p < 0.05$ nicotine-treated compared with saline-treated mice of same genotype, ^$p < 0.05$ α4 −/− mice compared with wildtype within the nicotine-treated group (*post-hoc* Tukey's tests following two-way repeated measures ANOVA).

and time followed by *post-hoc* Tukey's test revealed a significant increase in distance covered and time spent moving by α4−/− mice over the first 5 min following nicotine administration, compared with saline administration (Figure 4b, distance: $q = 4.974$, $p < 0.001$, D, move Time: $q = 3.006$, $p = 0.040$). Two-way repeated measures ANOVA confirmed a significant interaction between genotype × time across all measures (Figure 4a and b, distance: $F_{(5,107)} = 2.371$, $p = 0.046$; c, d, move time: $F_{(5,107)} = 5.195$, $p < 0.001$; e, f, moves: $F_{(5,107)} = 3.565$, $p = 0.006$). *Post-hoc* analysis by Tukey's test revealed nicotine-treated α4−/− mice covered significantly greater distance that nicotine-treated WT mice in the initial 5 min following nicotine administration (Figure 4a and b, $q = 3.643$, $p = 0.015$), as well as spending significantly greater time moving and initiating more movements during

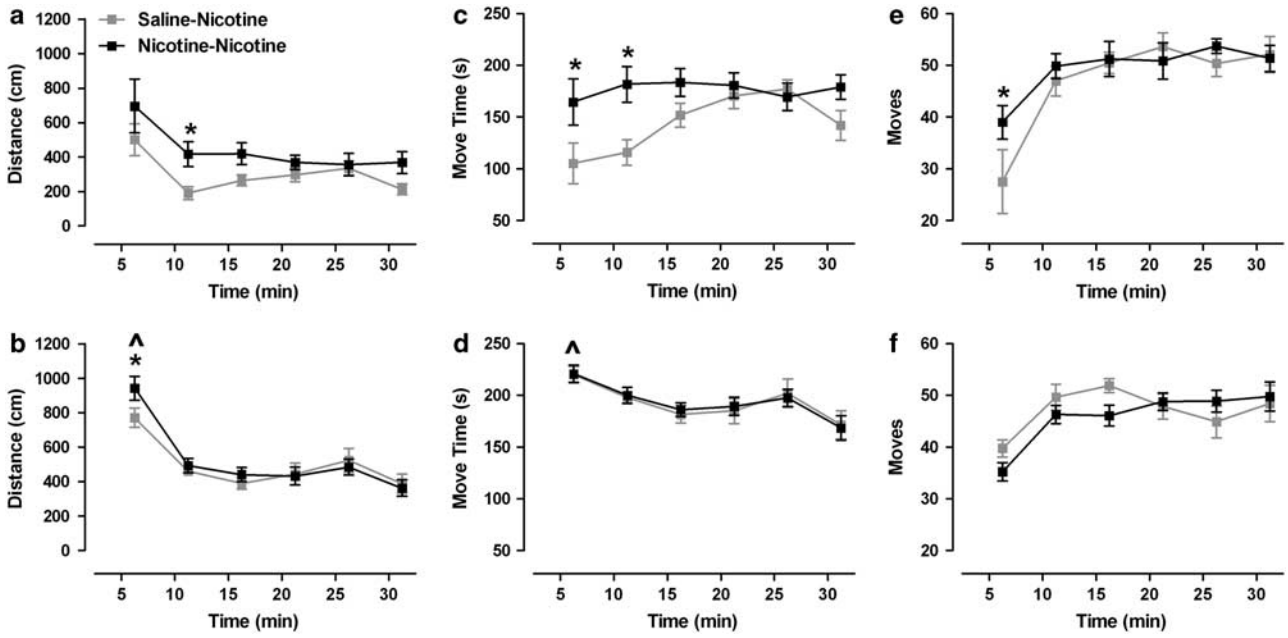

Figure 5 Challenge day: timecourse of effect of nicotine challenge (0.5 mg/kg, i.p.) on locomotor activity in wild-type (a, c, e, $n = 15$) and α4 −/− mice (b, d, f, $n = 18$) chronically treated with saline (saline-nicotine) or nicotine (nicotine-nicotine) over 30 min, as measured by distance traveled (a, b), move time (c, d), and number of moves initiated (e, f). All data are expressed as mean (±SEM). *$p < 0.05$ nicotine-treated compared with saline-treated mice of same genotype, ^$p < 0.05$ α4 −/− mice compared with wildtype within the nicotine-treated group (*post-hoc* Tukey's tests following two-way repeated measures ANOVA).

the 5 (Figure 4c and d, move time: $q = 4.251$, $p = 0.006$; moves: $q = 3.077$, $p = 0.033$) and 10 min time bins (Figure 4c and d, move time: $q = 3.325$, $p = 0.026$; E, F, moves: $q = 3.359$, $p = 0.021$). These data suggest that the α4 nAChR subunit is required for mediating the acute locomotor depressant effects of nicotine at the dose tested.

The α4 nAchRs Subunit is Required for Mediating Tolerance to the Locomotor Effects of Chronic Nicotine in Mice

Following investigation of the locomotor effects of acute nicotine, α4−/− and WT mice were again administered nicotine (0.5 mg/kg, i.p.) or saline for a further 4 days, after which a 7 day period of withdrawal ensued. All mice, nicotine- and saline-treated, then received a challenge dose of nicotine (0.5 mg/kg, i.p.). Figure 4 shows the locomotor responses of these mice; with chronic nicotine-treated mice (nicotine-nicotine) compared with saline-pretreated mice administered their first acute dose of nicotine (saline-nicotine). WT mice chronically treated with nicotine developed tolerance to the locomotor depressant effects of nicotine, such that the acute saline-nicotine WT mice showed significantly less locomotor activity in response to nicotine challenge than their chronically treated nicotine-nicotine counterparts (Figure 5a, c and e). Two-way repeated measures ANOVA within WT mice revealed a significant interaction between treatment × time in WT mice for time spent moving (Figure 5c, $F_{(5,95)} = 3.611$, $p = 0.006$). *Post-hoc* Tukey's tests further revealed significant differences between the treatment groups across all measures for WT mice. Saline-nicotine WT mice covered significantly less distance than nicotine-nicotine mice (Figure 5a, 10 min: $q = 3.213$, $p = 0.028$), moved for significantly less time (Figure 5c, 5 min: $q = 4.066$, $p = 0.007$, 10 min: $q = 4.520$, $p = 0.003$), and made significantly less moves (Figure 5e, 5 min: $q = 3.568$, $p = 0.014$). No tolerance was observed to the chronic locomotor effects of nicotine in α4−/− mice. The only difference apparent between saline-nicotine and nicotine-nicotine α4−/− mice was that chronically nicotine-treated mice covered significantly more distance than the acute nicotine group, during the first 5 min of nicotine re-challenge (Figure 5b, *post-hoc* Tukey's test, $q = 3.332$, $p = 0.021$).

This lack of readily apparent adaptation to the psychomotor effects of nicotine in α4−/− appears to be specific to nicotine, as the ability of a separate cohort of drug-naïve α4−/− mice to sensitize to the locomotor effects of chronic cocaine (20 mg/kg i.p.) was intact (data not shown).

Low Dose Mecamylamine Attenuates the Locomotor, but not Conditioned Reinforcing, Effects of Nicotine

Mecamylamine is commonly described as a non-selective nicotinic receptor antagonist. However, the potency of mecamylamine as a nicotinic antagonist varies according to the subunit configuration of the nAChR in question (Papke *et al*, 2010). We employed mecamylamine in investigations into the rewarding and psychomotor effects of nicotine in a conditioned place preference paradigm.

C57Bl6 WT mice were pretreated with either saline or mecamylamine (0.5 mg/kg, i.p) before nicotine (0.5 mg/kg, i.p.) conditioning sessions. Mecamylamine did not block the development of a conditioned place preference to nicotine. Paired *t*-tests revealed that both saline and mecamylamine pretreated mice showed a significant increase in time spent

in the nicotine-paired chamber of the conditioning apparatus post-conditioning (Figure 6a, saline pretreatment, $t=-4.276$, $p<0.001$; B, mecamylamine pretreatment, $t=-3.422$, $p=0.006$).

However, locomotor data from conditioning sessions revealed that this dose of mecamylamine blocked the locomotor depressant effect of 0.5 mg/kg nicotine (Figure 6c and d). Saline pretreated mice showed significantly decreased locomotor activity during nicotine conditioning sessions compared with saline conditioning sessions, with two-way ANOVA revealing a significant effect of conditioning ($F_{(1,119)}=10.719$, $p=0.007$) and significant interaction between conditioning session × day ($F_{(4,119)}=13.991$, $p<0.001$) on the distance covered by mice during conditioning sessions. *Post-hoc* analysis by Tukey's test confirmed that saline-pretreated mice covered significantly less distance during nicotine conditioning than during saline conditioning over the first 3 days of conditioning (Figure 6c, day 1 $q=9.052$, $p<0.001$, day 2, $q=4.354$, $p=0.006$, day 3, $q=3.422$, $p=0.025$). Mice pretreated with mecamylamine did not differ in their locomotor activity between saline and nicotine conditioning sessions (Figure 6d, no significant differences). Mecamylamine completely inhibited the locomotor depressant effects of nicotine.

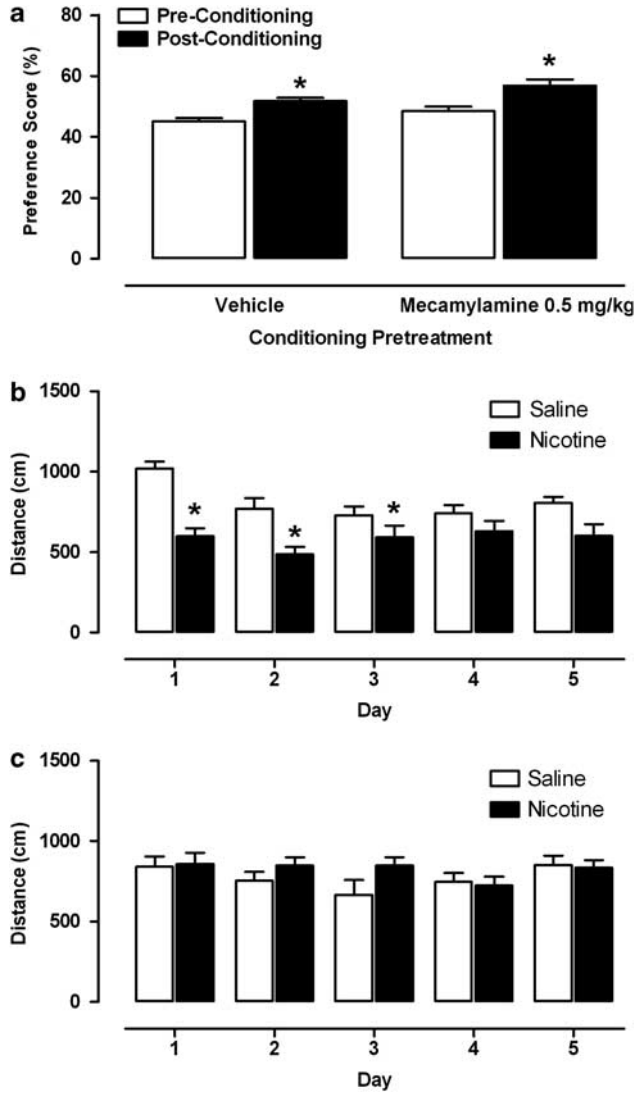

Figure 6 Conditioned place preference to nicotine in saline ($n=12$) or mecamylamine (0.5 mg/kg, i.p., $n=12$) pretreated C57Bl6 mice. The preference score is the time spent in the nicotine-paired compartment divided by the total time spent in both the nicotine- and saline-paired compartments, multiplied by 100. Time spent in the neutral zone is disregarded. Locomotor responses were recorded during conditioning sessions for both saline (b) and mecamylamine (c) pretreated mice. *$p<0.05$ compared with pre-conditioning session (a) or saline conditioning session (b) (*post-hoc* Tukey's test following two-way repeated measures ANOVA). The data are expressed as mean (± SEM).

High Dose Mecamylamine Attenuates the Reinforcing Effects of Nicotine

Operant self-administration of nicotine was employed to further investigate the ability of mecamylamine to inhibit the reinforcing effects of nicotine. As there was no significant difference in response rates between mice administering 0.05 and 0.07 mg/kg/infusion (Figure 3), and a greater proportion of WT mice achieved self-administration criteria at 0.07 mg/kg/infusion of nicotine, the higher nicotine dose was used for this particular study. C57Bl6 mice stably responding for nicotine (0.07 mg/kg/infusion) were habituated to saline injection and then administered 0.5, 1.0 and/or 2.0 mg/kg mecamylamine (i.p.) before nicotine self-administration sessions, in random order, with a further three saline pretreatment sessions between mecamylamine doses. One-way repeated measures ANOVA indicated a significant effect of mecamylamine treatment on nicotine infusions self-administered by the mice (Figure 7a, $F_{(3,32)}=4.523$, $p=0.015$). *Post-hoc* Holm–Sidak analysis revealed that 2.0 mg/kg mecamylamine significantly reduced nicotine infusions compared with saline pretreatment ($t=3.390$, $p=0.003$). Thus, the dose of mecamylamine required to attenuate the reinforcing effects of nicotine was seemingly four times the dose required to prevent the locomotor depressant effects of nicotine.

DISCUSSION

We have utilized mice lacking the α4 nicotinic receptor subunit to investigate the role of α4* nAChR in mediating acute and chronic behavioral effects of nicotine. The data presented suggest that although nAChRs containing the α4 subunit are required for mediating certain psychomotor effects of nicotine, they are not critical for the conditioned reinforcing effects of nicotine or the acquisition and maintenance of nicotine self-administration. Nevertheless, parallel studies employing transgenic α4-S248F confirm a modulatory role for α4* receptors in relation to nicotine self-administration.

The β2 nAChR subunit has been shown to be critically involved in mediating the reinforcing effects of nicotine. β2−/− mice that had been trained to self-administer cocaine failed to continue self-administration when cocaine was substituted with nicotine (Epping-Jordan *et al*, 1999; Picciotto *et al*, 1998). Mice lacking the β2 subunit also do not show intra-VTA self-administration of nicotine (Maskos *et al*, 2005) or exhibit a place preference to nicotine (Walters *et al*, 2006). The α4 subunit is the principal partner of the β2 subunit in forming nAChRs of the mesolimbic

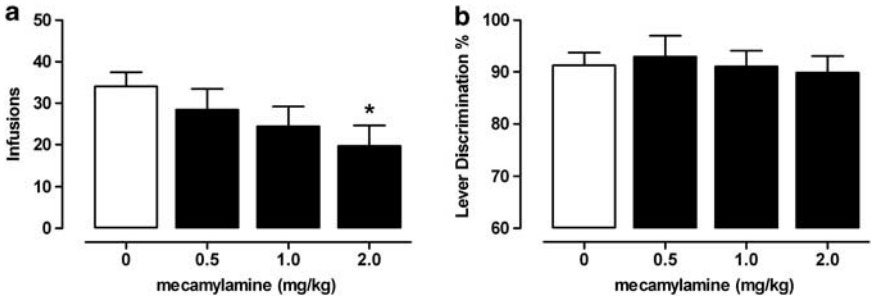

Figure 7 Effect of mecamylamine pretreatment on nicotine (0.07 mg/kg/infusion) self-administration in C57Bl6 mice ($n = 11$). (a) Number of nicotine infusions administered by mice treated with either vehicle (saline) or mecamylamine. (b) Percentage of discrimination between the active, reward-paired lever, and inactive lever of mice treated with either vehicle or mecamylamine. Vehicle data are the average of three separate vehicle treatment sessions, preceding each mecamylamine treatment session. *$p < 0.05$, *post-hoc* Holm–Sidak test following one-way repeated measures ANOVA.

pathway (Champtiaux *et al*, 2003; Changeux, 2010; Grady *et al*, 2007, 2009; Klink *et al*, 2001). The present study suggests, however, that these β2* nAChRs do not require partnership with the α4 subunit to mediate the chronically reinforcing effects of nicotine as measured by either conditioned place preference to nicotine or operant IV self-administration of nicotine. We found that α4−/− mice both show a similar level of nicotine-induced conditioned place preference as do wild-type mice and self-administer a similar amount of nicotine in an operant paradigm. Furthermore, α4−/− mice were similar to their wild-type littermates in their level of motivation to self-administer nicotine under a progressive ratio schedule and also in relapse-like behavior following a period of withdrawal.

In an independently generated line of mice lacking the α4, a different study (Pons *et al*, 2008) indicated a critical role for α4* nAChRs in mediating the rewarding effects of nicotine by utilizing a mouse model of acute self-administration of nicotine in which drug-naïve animals were tested in pairs using a contingent and a yoked control mouse. The α4 knockout mice failed to acutely self-administer nicotine, a finding validated by the ability of lentiviral expression of the α4 subunit in the VTA to restore acute self-administration of nicotine in these α4 knockout mice (Pons *et al*, 2008). However, the acute nature of nicotine administration in this paradigm renders this apparently opposing finding not directly comparable to the lack of a critical role for the α4 subunit in chronic self-administration demonstrated here. The authors noted that their paradigm is thought to assess the initiation rather than the chronic maintenance of drug-taking behavior (Criswell and Ridings, 1983; Pons *et al*, 2008; Rasmussen and Swedberg, 1998). The chronic operant responding for nicotine demonstrated in the current study is thought to more closely model nicotine abuse in humans (Corrigall, 1999).

Few published examples of chronic, operant, IV self-administration of nicotine in mice exist (Bilkei-Gorzo *et al*, 2008; Fowler *et al*, 2011; Martin-Garcia *et al*, 2008; Metaxas *et al*, 2010). A recent study has indicated that mice will respond to visual stimuli that are presented contingently upon lever responding (Olsen and Winder, 2009). Thus, our use of a visual CS contingent with nicotine delivery could, in theory, be a potential confound. However, Olsen and Winder (2009), used randomly varied light responses to maintain novelty, while our CS was consistent throughout the experiment. Furthermore, as can be seen in other published data, mice responding solely for a visual stimulus take up to 5 days to establish significant discrimination between the active, reward-paired lever and the inactive lever, whereas our mice responding for nicotine showed a high level of lever discrimination from the first day ($>75\%$, Figure 2b). This immediate discrimination between the nicotine-paired and inactive levers is consistent with other published accounts of nicotine self-administration (Martin-Garcia *et al*, 2008; Metaxas *et al*, 2010). In addition, the number of nicotine infusions achieved under a progressive ratio is less than under FR1 conditions, using the same dose of nicotine per infusion. Most convincingly, the response to nicotine is dose-related in WT mice, if mice were simply responding for the CS there would be no dose-relationship in the instrumental performance. Likewise, we show that nicotine self-administration can be attenuated by the nAChR antagonist mecamylamine.

An important point is that investigations with the α4−/− mouse allow us to ask the question of whether the α4 subunit is necessary, rather than sufficient, for nicotine reinforcement. Thus, although WT and α4−/− mice both reliably self-administer nicotine, this in itself does not discount a potential role for the α4 subunit in regulating sensitivity to reinforcing *vs* aversive effects of nicotine.

A role for α4* nicotinic receptors in nicotine reinforcement has been established. The *CHRNA4* gene encoding the α4 subunit has been associated with nicotine dependence in humans (Li *et al*, 2005). Varenicline, an $\alpha4\beta2$ receptor partial agonist, shows efficacy as a nicotine cessation aid (Lam and Patel, 2007; Rollema *et al*, 2007) and in reducing chronic nicotine self-administration in rats (George *et al*, 2010). The $\alpha4\beta2$* nAChRs have been shown to be specifically upregulated in response to chronic, low dose nicotine following an operant self-administration paradigm (Metaxas *et al*, 2010). Furthermore, α4* nAChRs have also been shown to be sufficient for mediating nicotine reward (Tapper *et al*, 2004).

While these findings might initially seem at odds with our presentation of intact nicotine reinforcement in α4−/− mice, the two need not be mutually exclusive. The sufficiency of α4* nAChRs, that are presumably of an $\alpha4\beta2$* configuration, in mediating nicotine reward was elegantly displayed by Tapper *et al* (2004) with their use of very low doses of nicotine to induce nicotine place preference only in mice with hypersensitive α4* nAChRs, and not their WT littermates. That the α4 nAChR subunit is

not *critical* for nicotine reinforcement is the important distinction the α4−/− data allow. Other α-subunits expressed in the mesolimbic pathway, including α3, α5 and α6, participate in the formation of functional β2* nAChRs (Azam *et al*, 2002; Champtiaux *et al*, 2002, 2003; Changeux, 2010; Grady *et al*, 2007, 2009; Klink *et al*, 2001; Le Novere *et al*, 1996), and although deletion of the β2 subunit removes high-affinity ligand binding sites of nicotinic agonists throughout the mouse brain (Picciotto *et al*, 1995), some of these nicotinic sites are retained in mice lacking the α4 subunit (Marubio *et al*, 1999, 2003; Ross *et al*, 2000). It is apparent that other (non-α4)β2* nAChR configurations are seemingly capable of mediating nicotine reinforcement in the absence of α4. Here we show evidence from two separate paradigms (operant self-administration and conditioned place preference) that the α4 subunit is not critical for nicotine reinforcement. Nevertheless, necessity and sufficiency are quite different issues; experiments utilizing the α4−/− mice deal solely with the former.

To address a potential modulatory role of the α4 subunit in nicotine self-administration, we have employed the opposite approach to the knock-out paradigm. Transgenic α4-S248F mice show an increased number of infusions received and active lever presses made, compared with WT animals at the low nicotine dose of 0.03 mg/kg/infusion. Furthermore, a greater proportion of α4-S248F mice (71.4%) met criteria for self-administration of nicotine at this dose than WT mice (53.8%). Indeed, the WT data here correspond extremely well with a recent publication (Metaxas *et al*, 2010), which found that almost the same percentage of mice (54.5%) met similar nicotine self-administration criteria at the same dose of nicotine (0.03 mg/kg/infusion). At higher doses of nicotine, the opposite effect occurred, with a greater proportion of WT mice than α4-S248F mice acquiring self-administration of nicotine. This may reflect a leftward shift in the dose-response relationship for nicotine self-administration in these nicotine-sensitive mice. Moreover, the increased responding by α4-S248F mice compared with WT for low dose nicotine is consistent with a modulatory, rather than necessary, role for α4* nAChRs in supporting the ongoing self-administration of nicotine. Nevertheless, the ability of the α4β2 nAChR partial agonist varenicline to decrease nicotine self-administration in rats (George *et al*, 2010) and the contingent relationship between upregulation of α4β2* nicotinic receptors and chronic nicotine reinforcement (Metaxas *et al*, 2010) both demonstrate the biological relevance of this modulatory role for the α4 subunit in aspects of nicotine reinforcement.

Notably, the current results do suggest a *critical* role for α4* nAChRs in mediating the acute locomotor depressant effects induced by low doses of nicotine. Although no differences were detected between the baseline locomotor responses of drug naïve WT and α4−/− mice, administration of nicotine caused opposing effects between the two genotypes. Although WT mice showed the expected decrease in activity, α4−/− mice showed an increase in activity, indicating α4* nicotinic receptors are required for mediating the hypolocomotor effects of nicotine. Indeed, decreased effectiveness of the hypolocomotor effects of nicotine has been reported previously in both α4 and β2 null mice (Marubio *et al*, 2003; McCallum *et al*, 2006; Tritto *et al*, 2004). Furthermore, studies of mice with a point mutation of the α4 subunit gene that renders the α4* receptor hypersensitive to nicotine have shown that nicotine-induced locomotor depression can result from selective activation of α4* nAChRs (Tapper *et al*, 2007).

The shift to a locomotor activating effect of acute nicotine in α4−/− mice, as opposed to simply a lack of locomotor depressant effect, may suggest the activation of non-α4* nAChRs. Interestingly, mice with a point mutation of the α6 subunit gene that renders α6* receptors hypersensitive to nicotine display hyperactivity in response to low dose nicotine (Drenan *et al*, 2010; Drenan *et al*, 2008). Furthermore, the locomotor activating effects of nicotine in rats is dramatically reduced following infusion of α6 antisense oligonucleotides (le Novere *et al*, 1999). That this locomotor activating effect has not been detected in other investigations into the locomotor effects of nicotine in α4 null mice (Drenan *et al*, 2010; Marubio *et al*, 2003; Tapper *et al*, 2007) may be due to the transient nature of the effect.

WT mice showed clear tolerance to the acute locomotor effects of nicotine, with chronically treated WT mice showing significantly less hypolocomotor effects in response to a nicotine challenge than acutely treated mice. This tolerance persisted in mice for at least a week after chronic treatment had ceased, suggesting it is due to a more long-lasting mechanism than receptor desensitization. Tolerance to the acute locomotor effects of nicotine was also absent in chronically treated α4−/− mice, which instead showed a small but significant increase in the opposing hyperlocomotor effect of nicotine. Tolerance to the acute effects of nicotine has been suggested to be due to upregulation of α4β2* nAChRs on midbrain GABAergic neurons (Nashmi *et al*, 2007; Xiao *et al*, 2009). The lack of tolerance observed in α4−/− mice is consistent with such an α4* nAChR dependent mechanism. The nicotine-induced phenotype of α4−/− mice suggests a dissociation between the nAChR configurations required for mediating the locomotor *vs* reinforcing effects of nicotine. Although there is seemingly a critical role for α4* nAChRs in mediating the locomotor depressant effects of nicotine and tolerance, in the absence of the α4 subunit nicotine reinforcement can be sufficiently mediated by other nAChR configurations.

The potential for compensatory neurodevelopmental changes could be an important caveat in interpreting the results from experiments utilizing the α4−/− mice. However, no clear evidence for compensation of nicotinic subunits has been established to date in mice lacking the α4 nAChR subunit. Assessment of mRNA levels in α4−/− mice using quantitative *in situ* hybridization demonstrated no difference to WT in the expression of α3, α6, α7, β2, β3, or β4 nAChR subunits (Marubio *et al*, 1999; Ross *et al*, 2000). Furthermore, we are not aware of the existence of any conditional α4 subunit knockout mice, and therefore the current genetic approach is more definitive than pharmacological manipulation using drugs that may act upon a number of subunits *in vivo*.

Mecamylamine, an open channel blocker, is commonly described as a non-selective nicotinic receptor antagonist. A surprising result has been the dissociation between the doses of mecamylamine required to antagonize different acute behavioral effects of nicotine. Although 0.5 mg/kg mecamylamine readily inhibited the acute hypolocomotor

effect of nicotine in WT mice, this dose was ineffective at blocking the reinforcing effects of nicotine in a conditioned place preference paradigm. In fact, we have found that 2.0 mg/kg mecamylamine, a fourfold higher dose, was required to reduce responding for nicotine in a self-administration paradigm. Although mecamylamine is capable of antagonizing all neuronal nAChRs, inhibition of different nAChRs by mecamylamine does in fact vary depending on the subunit configuration of the receptor (Papke *et al*, 2008, 2001, 2010). Although low dose mecamylamine is sufficient to antagonize the (presumably α4*) nAChRs necessary for mediating locomotor depressant effects of nicotine, much higher doses which are required to inhibit nicotine reinforcement, further supporting the involvement of other, possibly multiple, configurations of nAChRs.

A crucial role for α6* nAChRs in nicotine reinforcement has been suggested (Brunzell *et al*, 2010; Changeux, 2010; Jackson *et al*, 2009; Pons *et al*, 2008). The IC_{50} of mecamylamine inhibition at α4β2 nAChRs is threefold lower than at some α6* nicotinic receptors (Papke *et al*, 2008). Studies have shown that the α6* nAChR-specific antagonist α-conotoxin MII is capable of blocking the reinforcing, but not the locomotor, effects of nicotine (Brunzell *et al*, 2010; Jackson *et al*, 2009). The α6 subunit mRNA expression is intact in α4−/− mice (Ross *et al*, 2000), though a slight decrease in levels of protein have been detected in striatal membrane preparations from other α4-deficient mice (Champtiaux *et al*, 2003). Nevertheless, α6* nAChRs are a likely candidate for mediating nicotine reinforcement in both α4−/− mice and in WT mice that have been treated with low doses of mecamylamine.

The present study demonstrates a critical role for α4* nAChRs in the locomotor depressant but not the reinforcing effects of nicotine, where it appears to have only a modulatory role. The difference in dose of mecamylamine required to block the locomotor versus the reinforcing effects of nicotine further support the notion that nicotinic receptors of different configurations are primarily responsible for mediating these effects. Thus, more broadly, it is apparent that different aspects of nicotine's effects are mediated by nAChRs of different subunit configurations, an important consideration for investigations into the development of targeted, subunit-specific nAChR modulators as drug therapies for nicotine cessation.

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DISCLOSURE

The authors declare no conflict of interest.

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