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Deletion of α5 nicotine receptor subunits abolishes nicotinic aversive motivational effects in a manner that phenocopies dopamine receptor antagonism

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Abstract

Nicotine addiction is a worldwide epidemic that claims millions of lives each year. Genetic deletion of α5 nicotinic acetylcholine receptor (nAChR) subunits has been associated with increased nicotine intake, however, it remains unclear whether acute nicotine is less aversive or more rewarding, and whether mice lacking the α5 nAChR subunit can experience withdrawal from chronic nicotine. We used place conditioning and conditioned taste avoidance paradigms to examine the effect of α5 subunit-containing nAChR deletion (α5 −/−) on conditioned approach and avoidance behaviour in nondependent and nicotine-dependent and -withdrawn mice, and compared these motivational effects with those elicited after dopamine receptor antagonism. We show that nondependent α5 −/− mice find low, non-motivational doses of nicotine rewarding, and do not show an aversive conditioned response or taste avoidance to higher aversive doses of nicotine. Furthermore, nicotine-dependent α5 −/− mice do not show a conditioned aversive motivational response to withdrawal from chronic nicotine, although they continue to exhibit a somatic withdrawal syndrome. These effects phenocopy those observed after dopamine receptor antagonism, but are not additive, suggesting that α5 nAChR subunits act in the same pathway as dopamine and are critical for the experience of nicotine's aversive, but not rewarding motivational effects in both a nondependent and nicotine-dependent and -withdrawn motivational state. Genetic deletion of α5 nAChR subunits leads to a behavioural phenotype that exactly matches that observed after antagonizing dopamine receptors, thus we suggest that modulation of nicotinic receptors containing α5 subunits may modify dopaminergic signalling, suggesting novel therapeutic treatments for smoking cessation.

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Author contributions

TEG, OG, and DVDK designed the experiments. TEG, MAB, MY, HVP, MC, and GMB performed the research. TEG and DVDK wrote the paper.

Data accessibility

On acceptance for publication the raw data will be deposited on the website figshare.com.

Keywords

conditioned place preference; genetic knockout mice; motivation; nicotine withdrawal; ventral tegmental area

Introduction

Nicotine is the main psychoactive component in tobacco smoke that causes dependence and withdrawal after repeated self-administration through smoking (Koob & Le Moal, 2006), behaviours that can lead to disease and premature death. Although the multitude of risks associated with smoking are well known (CDC, 2015), many people begin or continue to smoke every day, and therefore the study of nicotine's rewarding and aversive motivational components is necessary for understanding the initiation of smoking and for the treatment of nicotine dependence.

Nicotine stimulates the brain by activating nAChRs, which are ligand-gated ion channels with five membrane-spanning subunits (Le Novere *et al.*, 2002). There are 12 neuronal nAChR subunits, designated α2-α10 and β2–β4, that combine to form a large variety of homomeric and heteromeric receptors (Le Novere *et al.*, 2002), of which those containing the α4 and β2 subunits have been implicated most often in nicotine motivation (Picciotto *et al.*, 1998; Tapper *et al.*, 2004; Maskos *et al.*, 2005). More recently, studies have focused on other nAChR subtypes, such as those containing the α5 subunit, revealing that mice with a null mutation of the gene encoding the α5 nAChR subunit (α5 −/−) will dramatically increase their nicotine consumption in a self-administration paradigm (Fowler *et al.*, 2011), although they have normal brain anatomy and are generally healthy (Salas *et al.*, 2003). α5 −/− mice also have been shown to be less sensitive to nicotine-induced seizures and locomotor effects in the open field than wildtype mice (Salas *et al.*, 2009) and to increase intracranial electrical self-stimulation (ICSS) thresholds after being given acute nicotine (Fowler *et al.*, 2013). Furthermore, polymorphisms in the α5 subunit gene lead to increased vulnerability for smoking dependence (Bierut *et al.*, 2008; Kuryatov *et al.*, 2011). These effects have been attributed to a decrease in nicotine's aversive motivational properties (Fowler *et al.*, 2011, 2013). However, like most other drugs of abuse, nicotine has both rewarding and aversive properties (Jorenby *et al.*, 1990), which are mediated by separate neurobiological substrates: acute nicotine reward in nondependent animals involves activation of the tegmental pedunculopontine nucleus (TPP) of the brainstem (Iwamoto, 1990; Laviolette *et al.*, 2002), and nicotine's aversive motivational effects are mediated by the dopaminergic system (Laviolette & van der Kooy, 2003; Grieder *et al.*, 2010, 2012). Interestingly, the nAChR subtypes that regulate dopaminergic transmission in the brain motivational system depend critically upon α5 nAChR subunits (Exley *et al.*, 2012; Chatterjee *et al.*, 2013; Morel *et al.*, 2014). We thus questioned whether the α5 subunit-containing nAChRs are affecting nicotine's acute motivational properties by modulating dopaminergic signaling to decrease the aversiveness or increase the rewarding properties of nicotine. Although previous studies using self-administration and ICSS have suggested that nicotine is less aversive in α5 −/− mice (Fowler *et al.*, 2011, 2013), observed increases in self-administration or decreases in brain reward thresholds could be due to either an increase

in the rewarding properties of nicotine or a decrease in nicotine's aversive properties, effects that cannot be differentiated by the self-administration or ICSS paradigms (Mucha *et al.*, 1982). Further, if a neuroleptic is given during responding in these paradigms, changes in responding could be due to motor deficits rather than changes in rewarding or aversive drug properties. By using α5 −/− mice in a place conditioning paradigm, which can distinguish between the rewarding and aversive conditioned motivational effects of abused drugs like opiates and nicotine (Mucha *et al.*, 1982; Laviolette & van der Kooy, 2003), we determined that α5 −/− mice behaviourally phenocopy mice treated with dopamine receptor antagonists: they find acute nicotine less aversive, but not more rewarding, and after chronic nicotine they fail to show a conditioned aversive response to nicotine withdrawal. Although these conditioned effects were the same, they were not additive. These data support the hypothesis that α5 subunit-containing receptors modify dopaminergic signalling (Exley *et al.*, 2012; Chatterjee *et al.*, 2013; Morel *et al.*, 2014) in brain reward pathways.

Materials and methods

All animal use procedures were approved by the University of Toronto Animal Care Committee, in accordance with the guidelines of the Canadian Council on Animal Care.

Adult male C57BL/6 mice ($n = 48$) were purchased from Charles River (Montreal, Canada). Male and female heterozygous α5 nAChR breeding pairs, that were backcrossed a minimum of 10 times on to a C57BL/6 background, were a gift from M. Marks and P. Kenny (The Scripps Research Institute, Jupiter, Florida). Breeding in our facility was undertaken to produce homozygous male α5 −/− ($n = 81$) and +/+ ($n = 103$) littermate control mice. Mice were at least 10 weeks old at the beginning of experiments. All mice ($n = 232$) were housed in a temperature-controlled room with lights on from 7 AM to 7 PM.

Drugs

Nicotine hydrogen tartrate salt (Sigma-Aldrich, Ontario) was dissolved in saline at pH 7.0 ± 0.4 and administered via s.c. injection (0.35 or 1.75 mg/kg) or osmotic minipumps (chronic nicotine, 7 mg/kg/day for 12 days, minipump model 1002, Alzet, Cupertino, California). We previously have shown that this dose of chronic nicotine in mice leads to arterial blood concentrations similar to human chronic smokers (Grieder *et al.*, 2010). Nicotine-dependent and -withdrawn mice had their minipumps removed 8 h prior to experimentation at a time that corresponded to peak motivational withdrawal (Grieder *et al.*, 2010). The dopamine receptor antagonist α-flupenthixol (0.08 mg/kg) was purchased from Sigma-Aldrich, Ontario, dissolved in saline and administered i.p. 60 min prior to conditioning. The opioid antagonist naloxone (0.6 mg/kg) was purchased from Sigma-Aldrich, Ontario, dissolved in saline and administered i.p. immediately prior to conditioning. Morphine sulfate (Almat Pharmachem) was dissolved in physiological saline at pH 7.0 ± 0.4 and administered via i.p. injections (0.5 mg/kg and 5 mg/kg). All doses of drugs are expressed as mg of free base/kg of body weight. Doses and time of injections were selected based on previous studies (Laviolette *et al.*, 2004; Grieder *et al.*, 2010).

Somatic withdrawal assessment

Mice were observed for somatic signs of nicotine withdrawal 8 h after minipump removal. Mice were observed over a period of 10 min for head shakes, paw tremors, writhing, scratching, backing and jumping (Isola *et al.*, 1999; Stoker *et al.*, 2008; Grieder *et al.*, 2010). Individual and group average abstinence scores were obtained using total signs observed over a period of 10 min. Experimenters were blind to the drug treatment of each subject.

Place conditioning

The place conditioning apparatus was obtained from Med Associates Inc. (SOF-700RA-25 Two Chamber Place Preference Apparatus, VT, USA). One environment was black with a metal rod floor and the other was white with a wire mesh floor. An intermediate grey area housed a removable partition. Each cage was cleaned between animals and each group was fully counterbalanced. During preference testing, the dividing partition was removed and mice were given free access to both environments. All place conditioning and testing was performed between 10 AM and 6 PM.

To study conditioned approach and avoidance behaviour, a place conditioning procedure was used that has been described previously (Grieder *et al.*, 2010). Briefly, for acute nicotine conditioning sessions in nondependent mice (defined as those with less than 5 life-time administrations of acute nicotine), mice were pre-treated i.p. with saline or the dopamine receptor antagonist α-flupenthixol (0.08 mg/kg) 1 h prior to conditioning and given a subcutaneous injection of nicotine (0.35 or 1.75 mg/kg), morphine (0.5 or 5 mg/kg), naloxone (0.6 mg/kg) or saline immediately prior to conditioning. Each group underwent 8 conditioning trials (4 alternating drug and vehicle pairings) in one of the conditioning environments for 15 min. All conditioning was unbiased and fully counterbalanced for treatment compartment and order of drug presentation. Conditioning of nicotine-dependent and -withdrawn mice (defined as those mice that had been given chronic nicotine for 12 days or more) occurred during peak withdrawal from chronic nicotine so that the motivational effects of withdrawal, but not the direct effects of chronic nicotine, were paired with the place conditioning environment (Grieder *et al.*, 2010). The effects of surgery and having a minipump do not lead to conditioned motivational responses on their own (Grieder *et al.*, 2012). Eight hours after minipump removal, when the mouse was experiencing peak motivational withdrawal from chronic nicotine (Grieder *et al.*, 2010), it was confined to one of the conditioning environments for 50 min. A single 10-min preference testing session was performed 5 days after the last conditioning day, at a time when subjects were drug- and somatic withdrawal symptom-free. The difference score for each animal was calculated by subtracting the time spent in the saline-paired or unpaired environment from the time spent in the nicotine- or withdrawal-paired environment.

Conditioned taste aversion

Mice were trained to consume water on a limited-access regimen of 30 min/d for 5 days before the commencement of conditioning. Training consisted of one exposure per day for 8 days to an unsweetened 0.3% solution of either grape- or cherry-flavoured Kool-Aid (animals display no baseline preference for either of these flavours; Jaeger & van der Kooy, 1996) for 15 min. After flavour exposure, animals received a subcutaneous injection of

nicotine (0.35 or 1.75 mg/kg). On the alternate day, the animal was exposed to the other flavour and then received a subcutaneous injection of saline. The drug-paired flavours and the day of first drug exposure were counterbalanced within groups. After conditioning, animals were left untreated for 2 days, during which time they were given 30 min of normal water access per day. On test day, animals were left drug-free and were presented with both the drug and saline-paired flavour solutions. Amounts consumed of both flavours over a 20-min period were recorded, and the difference score was calculated as the amount of nicotine-paired solution minus the amount of saline-paired solution, in millilitre.

Statistical analysis

Results were analysed using a one- or two-way analysis of variance (ANOVA) or Student's *t*-test with significance level of 0.05 (two tailed). In all cases, a normality test and equal variance test were performed before an ANOVA to ensure its validity. Post hoc Bonferroni tests were used where appropriate. Data are shown as mean ± SEM.

Results

To determine if genetic deletion of the α5 nAChR caused nicotine to be perceived as less aversive or more rewarding, we utilized doses of acute nicotine and treatments that we had demonstrated previously to be aversive or non-motivational in a place conditioning paradigm (Grieder *et al.*, 2010, 2012, 2014). The conditioned aversive response to acute nicotine can be blocked by pre-treatment with the dopamine receptor antagonist α-flupenthixol (Grieder *et al.*, 2010). We pre-treated wild type (+/+) and α5 −/− mice with either saline or α-flupenthixol and gave them 0.35 or 1.75 mg/kg nicotine or saline vehicle in an unbiased place conditioning procedure (Grieder *et al.*, 2010). A two-way ANOVA revealed a dose × genotype interaction ($F_{3,77} = 2.755$, $P = 0.0481$; Fig. 1). α5 +/+ mice given the low dose of acute nicotine (0.35 mg/kg; $n = 8$) and pre-treated with saline showed neither a rewarding or aversive conditioned motivational response, suggesting that this dose is non-motivational in our place conditioning paradigm in wild-type mice. However, α5 −/− mice given the same low dose of acute nicotine and pre-treated with saline ($n = 8$) showed a significant rewarding motivational response ($P = 0.0305$ in comparison to α5 +/+ mice). Previous studies have demonstrated that a rewarding response to a low, apparently non-motivational dose of acute intracerebral nicotine can be revealed if the action of the dopaminergic system is blocked by systemic administration of a dopamine receptor antagonist (Laviolette & van der Kooy, 2003; Tan *et al.*, 2009). When the previously non-motivational dose of acute nicotine (0.35 mg/kg) was given after pre-treatment with α-flupenthixol to α5 +/+ mice ($n = 27$), a rewarding motivational response was revealed ($P = 0.0363$ in comparison to α5 +/+ mice pre-treated with saline). This conditioned rewarding response also was demonstrated by a group of α5 −/− mice after pre-treatment with α-flupenthixol ($n = 12$), but it was not augmented in comparison to the +/+ group treated with α-flupenthixol ($P = 0.9578$; Fig. 1). These results suggest that the deletion of α5 nAChR subunits does not further enhance acute nicotine reward and that the effects of dopaminergic antagonism and α5 nAChR subunit knockout match exactly, but are not additive. These results were not due to a motivational effect of α-flupenthixol on conditioned approach behaviour, as the dose used in this study (0.08 mg/kg) has no conditioned motivational effect on its own (Grieder *et al.*, 2010, 2012).

Similar to our previous studies (Grieder *et al.*, 2010, 2012), nondependent α5 +/+ mice that were given a higher dose (1.75 mg/kg) of nicotine ($n = 7$) showed a conditioned aversive response to the nicotine-paired environment, which was blocked by α-flupenthixol pre-treatment in a separate group of mice ($n = 8$; $P = 0.0217$; Fig. 1). Interestingly, α5 −/− mice ($n = 8$) tested under the same conditions showed no motivational response to this dose of nicotine ($P = 0.0214$ in comparison to α5 +/+ mice), even in the presence of α-flupenthixol pre-treatment ($n = 8$, $P = 0.6583$ comparing α5 −/− and α5 +/+ mice pretreated with α-flupenthixol), providing further support for the hypothesis that knockout of α5 nAChR subunits decreases nicotine aversion, but does not increase reward. These results also are in line with the above results showing that dopamine receptor antagonism and α5 nAChR subunit deletion produce the same, but non-additive, conditioned effects.

We next examined whether mice given acute nicotine would show a conditioned taste aversion (CTA) to nicotine. The CTA paradigm can sample selectively the aversive properties of a drug, as animals will readily acquire aversions to flavours paired with specific drug stimuli (Jorenby *et al.*, 1990; Laviolette & van der Kooy, 2003). Similar to the aversive motivational response in the place conditioning paradigm, CTA for nicotine can be blocked by pre-treatment with α-flupenthixol (Laviolette & van der Kooy, 2003). We first examined the effect of α-flupenthixol pre-treatment on the CTA of our 0.35 and 1.75 mg/kg doses of acute nicotine in +/+ groups of mice. When groups of wild type C57Bl/6 mice were given a two-bottle choice test at the conclusion of CTA conditioning, a two-way ANOVA revealed a dose × pre-treatment interaction ($F_{1,33} = 4.204$, $P = 0.0461$; Fig. 2A). α5 +/+ mice pre-treated with saline ($n = 8$) or α-flupenthixol ($n = 8$) and given 0.35 mg/kg nicotine after flavour pairing showed no CTA to nicotine ($P = 0.7226$). These results are consistent with the above data showing that this dose of nicotine is non-motivational in our place conditioning paradigm. A separate group of C57Bl/6 mice given 1.75 mg/kg nicotine and pre-treated with saline ($n = 7$) showed a significant avoidance of the flavour paired with nicotine ($P = 0.0034$ in comparison to 0.35 mg/kg group), an effect that was blocked in mice ($n = 8$) pretreated with α-flupenthixol ($P = 0.0203$ in comparison to saline pretreated mice), which was again similar to the place conditioning results reported above.

We then conditioned our α5 −/− mice and their +/+ littermates in the CTA paradigm using the 1.75 mg/kg dose of acute nicotine after α-flupenthixol or saline pre-treatment. A two-way ANOVA revealed a genotype × pre-treatment interaction ($F_{1,28} = 5.19$, $P = 0.0211$; Fig. 2B). As expected, α5 +/+ mice pre-treated with saline ($n = 10$) showed a CTA to 1.75 mg/kg nicotine that was blocked in α5 +/+ mice pre-treated with α-flupenthixol ($n = 10$; $P = 0.0001$). However, both groups of α5 −/− mice that were pretreated with either saline ($n = 8$) or α-flupenthixol ($n = 8$) and given 1.75 mg/kg nicotine did not show a CTA, suggesting that functional α5 nAChR subunits as well as signalling at dopamine receptors are required for the expression of a conditioned taste avoidance to acute nicotine. These results were not due to a reduced capacity to drink in α5 −/− mice, as total fluid consumption across conditioning trials and testing was nearly identical between the +/+ groups (mean ± SEM, 1.81 ± 0.09 ml). and −/− groups (mean ± SEM, 1.79 ± 0.1 ml). Further, both groups of mice maintained ~80% of their initial body weight over the course of the experiment: α5 +/+ and −/− mice were 29.5 ± 2.4 g and 31.8 ± 1.8 g at the beginning of experiments and 23.5 ± 2.6 g and 25.3 ± 1.8 g at the end of experiments, respectively (mean ± SD). The block of a CTA for the

higher dose of nicotine in α5 −/− mice differs from that reported in a previous study (Fowler *et al.*, 2013), however an unbiased paradigm employing two equally preferred grape and cherry solutions was utilized in the present study, which may be more likely to sample drug motivation as opposed to neophobic and antianxiety effects.

We next investigated whether genetic deletion of α5 nAChR subunits would prevent nicotine withdrawal-induced aversions in dependent mice that had been chronically treated with nicotine (7 mg/kg/day for 12 days). We have shown previously that dopamine receptor antagonism or knockout of the dopamine D2 receptor prevents the expression of the conditioned aversive response to nicotine withdrawal in nicotine dependent mice without preventing the expression of somatic withdrawal symptoms (Grieder *et al.*, 2010). The results described above show that α5 −/− mice given acute nicotine demonstrate similar motivational responses in the place conditioning and CTA paradigms to +/+ mice pre-treated with a dopamine receptor antagonist. These observations led us to hypothesize that genetic deletion of α5 nAChR subunits also might prevent the expression of conditioned nicotine withdrawal aversions in nicotine dependent mice. We implanted α5 +/+ and −/− mice with osmotic minipumps for 12 days to induce nicotine dependence, and exposed them for 1 h to the place conditioning environment 8 h following pump removal, during peak withdrawal (Grieder *et al.*, 2010), with either saline or α-flupenthixol pre-treatment. They were tested 5 days later in a drug- and withdrawal-free state. A one-way ANOVA revealed a significant group effect ($F_{2,47} = 3.338$, $P = 0.0445$; Fig. 3A). α5 +/+ mice pre-treated with saline ($n = 16$) showed an aversion to the withdrawal-paired environment that was not observed in −/− mice ($n = 16$; $P = 0.0001$) or in α5 +/+ mice pre-treated with the dopamine receptor antagonist ($n = 16$; $P = 0.0003$). These results again show that α5 subunit deletion and dopamine receptor antagonism lead to equivalent, but non-additive, conditioned motivational effects.

Separate groups of α5 +/+ and −/− mice were observed for somatic withdrawal symptoms after chronic nicotine treatment, with a student's *t* test showing no difference between the groups ($t_{17} = 0.3423$, $P = 0.7363$). Nicotine-dependent and -withdrawn α5 +/+ mice ($n = 8$) exhibited 38.22 ± 5.243 (mean ± SEM) abstinence signs, while α5 −/− mice ($n = 10$) averaged 36.40 ± 1.833 (mean ± SEM), both scores that are similar to our previously published results in dopamine D2 receptor knockout mice (Grieder *et al.*, 2010). Taken together, these results suggest that both dopamine receptors and α5 subunit-containing nAChRs are involved in mediating the aversive motivational effects of nicotine withdrawal, but both do not effect the expression of a somatic withdrawal syndrome.

Finally, to investigate whether α5 −/− can show conditioned motivational responses to non-nicotine stimuli, we conditioned separate groups of α5 −/− and +/+ mice after administration of a rewarding dose of morphine (5 mg/kg; Vargas-Perez *et al.*, 2014) or of the opioid receptor antagonist naloxone (0.6 mg/kg). We also conditioned additional groups of α5 −/− and +/+ mice after administration of a non-motivational dose of morphine (0.5 mg/kg; Laviolette *et al.*, 2004) to investigate whether α5 nAChR subunit deletion induced a general potentiation of sub-threshold rewarding effects. A two-way ANOVA revealed no significant effect of genotype ($F_{1,46} = 0.1211$, $P = 0.7294$), but a significant effect of treatment ($F_{2,46} = 63.42$, $P < 0.0001$; Fig. 3B). α5 +/+ ($n = 8$–10 per group) and α5 −/− ($n = 8$–10 per group)

groups both showed a conditioned rewarding response to 5 mg/kg morphine ($P = 0.6081$) and a conditioned aversive response to naloxone ($P = 0.8197$), suggesting that α5 nAChR subunit deletion does not prevent conditioning of an aversive or rewarding response in general. Importantly, both α5 +/+ ($n = 8$) and α5 −/− ($n = 8$) groups did not find 0.5 mg/kg morphine rewarding ($P = 0.9669$), which suggests that α5 nAChR subunit deletion does not potentiate the rewarding effects of morphine.

Discussion

Genetic deletion of α5 nAChR subunits abolished the aversive conditioned motivational effects of acute nicotine and prevented the expression of the aversive response to withdrawal from chronic nicotine, but did not prevent somatic withdrawal in dependent subjects. These conditioned motivational effects were equivalent to those observed after nonspecific dopamine receptor antagonism, or D2 receptor knockout (Grieder *et al.*, 2010, 2012). Further, acute nicotine and morphine reward in nondependent animals was neither blocked nor potentiated in α5 −/− mice, and α5 deletion did not prevent conditioned aversive responses to aversive stimuli in general. Taken together, these results reveal that α5 nAChR subunits are necessary for specifically signalling nicotine's aversive, but not rewarding, conditioned motivational effects in both the nondependent and nicotine-dependent and -withdrawn motivational states. The conditioned aversive effects of nicotine observed after α5 deletion and dopamine receptor antagonism matched precisely, but were not additive, suggesting that activation by nicotine of nAChRs containing the α5 subunit may modulate dopaminergic neuronal firing in brain motivational systems.

Genetic deletion of α5 subunits may lead to three potential effects on nAChRs in the adult: (i) deletion of α5 subunits may produce developmental changes, (ii) absence of the α5 subunit may lead to the complete absence of functional, cell surface nAChRs that would have included this subunit, and (iii) nAChRs in −/− mice may function similarly to those in the +/+ mice except that nAChRs do not contain α5 subunits. We cannot rule out the first possibility of developmental changes in reward circuitry or in the pattern of nAChR expression, however α5-deficient mice have normal brain anatomy and normal levels of mRNA for other nAChR subunits, including α4 and β2 (Salas *et al.*, 2003). The second possibility can be ruled out because we and others have observed sensitive nicotine-mediated behavioural responses in the α5-deficient mice. Instead, we suggest that the third possibility (nAChRs can function without α5 subunits) is most likely, due to the fact that the ability of a null α5 nAChR deletion to prevent the aversive motivational conditioned place response and conditioned taste avoidance to acute nicotine in nondependent mice, and to withdrawal from chronic nicotine in dependent mice, is strikingly similar to the results seen after dopamine receptor antagonism or D2 receptor deletion (Laviolette & van der Kooy, 2003; Tan *et al.*, 2009; Grieder *et al.*, 2010, 2012). We previously have demonstrated that separate specific patterns of ventral tegmental area (VTA) dopaminergic activity signal acute nicotine aversions and the aversions due to withdrawal from chronic nicotine (Grieder *et al.*, 2012). Indeed, the α5 nAChR subunit is known to regulate nicotine-induced dopamine release probability in the striatum (Exley *et al.*, 2012) and their presence in the VTA blunts desensitization after nicotine exposure (Chatterjee *et al.*, 2013). We therefore suggest that α5 nAChR subunits modify VTA dopaminergic signalling patterns, and their deletion prevents

aversive motivational responses to acute nicotine and withdrawal from chronic nicotine. α5 deletion from the habenula-interpeduncular nucleus pathway leads to increases in nicotine self-administration (Fowler *et al.*, 2011) and decreases in ICSS thresholds (Fowler *et al.*, 2013). Although previous studies have suggested that the α5 nAChR subunit is expressed by the medial, but not lateral, habenula (Fowler *et al.*, 2011), recent studies have shown that α5 mRNA levels are low in the medial habenula (Hsu *et al.*, 2013), and that α5-containing receptors on GABAergic midbrain neurons may play a role in mediating medial habenula neuronal activity (Hsu *et al.*, 2013). Given that the medial and lateral habenula have direct projections to both the interpeduncular nucleus and VTA, respectively (Herkenham & Nauta, 1979), we suggest that deletion of the α5 nAChR subunit may obscure the specific patterns of downstream VTA dopaminergic activity that signal the aversive motivational response in both dependent and nondependent animals. Therefore nicotine may be activating α5 nAChRs in the habenula or interpeduncular nucleus, or on the VTA neurons directly. Each of these sites of action is possible and would be upstream of the specific pattern of dopaminergic activity that signals the conditioned aversive response to nicotine (Grieder *et al.*, 2012). In the present study, the effects of α5 −/− and dopamine receptor antagonism after acute nicotine treatment were similar but not additive. If these manipulations were modulating separate pathways, the effects would have been additive. Similar to previous studies that demonstrated the importance of α5 nAChR subunits in regulating dopaminergic neurons (Morel *et al.*, 2014) and the function of nicotinic receptors in the VTA (Chatterjee *et al.*, 2013), the present results support the hypothesis that α5 subunit deletion modifies downstream VTA dopaminergic transmission.

α5 −/− mice did not show the conditioned aversive motivational response to spontaneous withdrawal from chronic nicotine shown by +/+ mice, although both groups showed the same somatic withdrawal syndrome, suggesting that they did not find the experience of affective or somatic nicotine withdrawal aversive. A previous study using antagonist-precipitated withdrawal in a biased conditioned place preference paradigm suggested that α5 −/− mice experienced affective but not somatic withdrawal (Jackson *et al.*, 2008), however the methods for place conditioning and eliciting withdrawal differed from the present study. We suggest that an anxiogenic response may have accounted for the observation of affective withdrawal in the α5 −/− mice in their biased place preference paradigm, since the mice continued to show anxiety-related responses in the elevated plus maze in the previous study (Jackson *et al.*, 2008). Further, these authors reported a block of somatic withdrawal signs, however they measured these signs after approximately 18 h of withdrawal (Jackson *et al.*, 2008), which is a time point that is 10 or more hours after peak affective and somatic nicotine withdrawal (Grieder *et al.*, 2010). We suggest that somatic withdrawal signs would have been largely dampened 18 h into withdrawal and therefore could have been more easily disrupted. Considering the present results with previous results showing that dopamine D2 receptor knockout mice show a robust somatic withdrawal syndrome (Grieder *et al.*, 2010), it appears that somatic withdrawal and affective nicotine withdrawal are mediated by separate neural substrates.

Similar to most drugs of abuse, nicotine produces both rewarding and aversive motivational effects in nondependent animals. The effect of α5 nAChR knockout has been studied previously using the self-administration and ICSS paradigms, where increases in nicotine

intake or in self-stimulation were attributed to a decrease in nicotine's acute aversive effects (Fowler *et al.*, 2011, 2013). However, since increases in self-administered nicotine or self-stimulation after nicotine administration can be interpreted as either an increase in the appetitive properties or as a decrease in the aversive properties of the drug, we suggest that the use of a place conditioning paradigm that can demonstrate separate rewarding and aversive conditioned motivational responses (Mucha *et al.*, 1982) is more suitable for the study of α5 nAChR subunit deletion. The present results showed that a low dose (0.35 mg/kg) of acute nicotine that was non-motivational in +/+ mice produced a rewarding motivational response in α5 −/− mice, and that −/− mice did not show the conditioned aversive response to nicotine withdrawal shown by +/+ mice. Most important, the findings that α5 nAChR subunit deletion and dopamine receptor antagonism were not additive in producing rewarding nicotine effects at any dose of nicotine or in the CTA paradigm suggests that neither dopamine receptor antagonism or α5 nAChR subunit deletion increased specifically the primary rewarding effects of nicotine. Instead, we suggest that genetic deletion of the α5 nAChR subunit phenocopies dopamine receptor antagonism, making nicotine less aversive in both nondependent and nicotine-dependent and -withdrawn animals and revealing rewarding effects that are due to a TPP-mediated mechanism (Laviolette *et al.*, 2002; Laviolette & van der Kooy, 2003). The block of nicotine's aversive effects in α5 −/− mice was specific to nicotine, as the aversive effects of the opioid receptor antagonist naloxone were observed in both α5 +/+ and α5 −/− mice. Further, α5 nAChR subunit deletion did not potentiate the rewarding effects of either a sub-threshold or a rewarding dose of morphine, which support the hypothesis that α5 −/− mice are not experiencing a potentiation of the rewarding motivational effects of drugs in general.

These results potentially may help explain the initiation of smoking in some humans, despite known health risks and the fact that acute nicotine in nondependent subjects often is aversive (Grieder *et al.*, 2010). Indeed, a single nucleotide polymorphism (SNP) in the neuronal α5 nAChR subunit (CHRNA5) is associated with heavy smoking behaviour (Berrettini *et al.*, 2008) and enhanced pleasurable responses to the first daily cigarette in regular smokers (Sherva *et al.*, 2008). Although global α5 knockdown in mice does not equate directly to human SNPs, as not all SNPs are deletions, our hypothesis that α5 nAChR subunit deletion makes both acute nicotine and nicotine withdrawal less aversive due to modulation of downstream dopamine is in line with these human studies. Indeed, the human CHRNA5 D398N polymorphism causes a change in the α5 nAChR subunit gene, which leads to a reduced response to nicotine and may lead to distinct downstream cellular signalling (Tammimäki *et al.*, 2012).

In conclusion, the present results reveal that the presence of α5 nAChR subunits affecting downstream dopamine signalling are critical for the experience of nicotine's conditioned aversive motivational effects in both a nondependent and nicotine-dependent and -withdrawn motivational state. These data provide some insight into the motivation behind initial smoking behaviour and indicate that increased activity at α5 subunit-containing nAChRs may make nicotine more aversive. Since the ability to relieve or prevent withdrawal has been suggested to be the driving force of relapse to smoking during quit attempts (Baker *et al.*, 2004), drugs that increase the ability of nicotine to activate nicotinic receptors containing the α5 subunit, and therefore modify dopaminergic transmission (Exley *et al.*, 2012; Chatterjee

et al., 2013; Morel *et al.*, 2014) indirectly, may be a viable avenue for smoking cessation therapies.

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Conflict of interest

This work was supported by Canadian Institutes of Health Research grants. The authors declare no competing financial interests.

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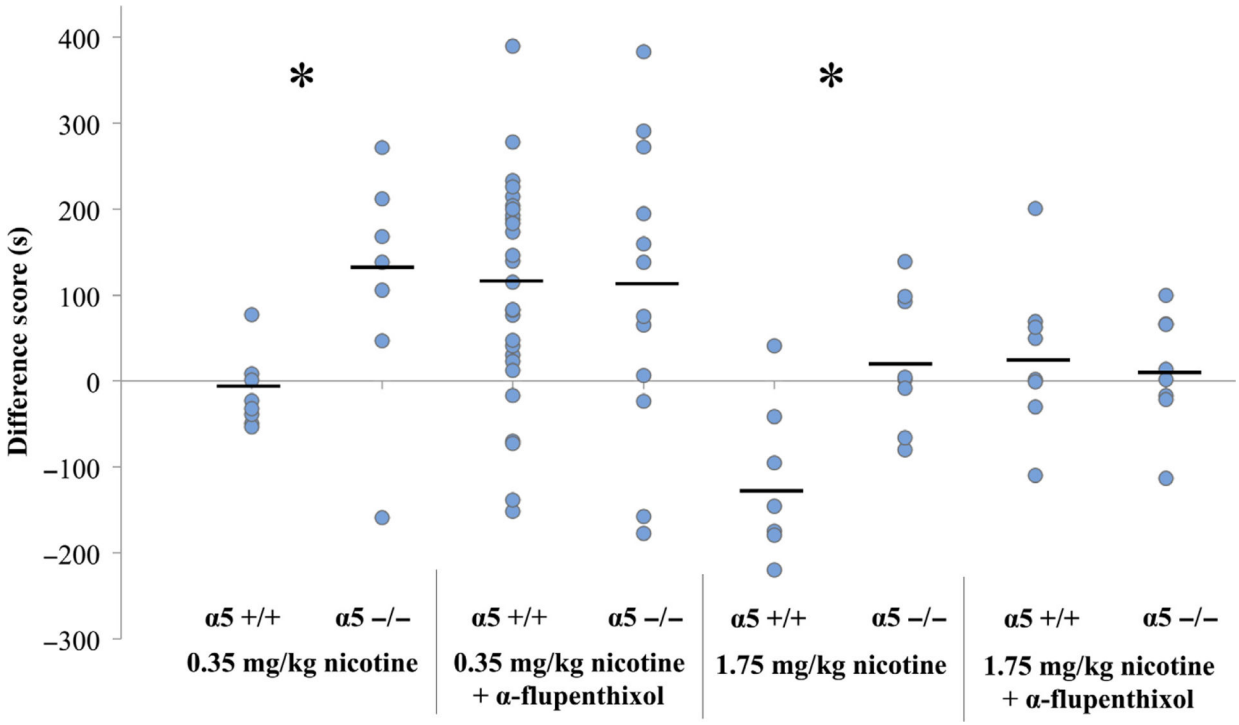

Fig. 1.

Knockout of the α5 nAChR subunit makes nicotine less aversive and not more rewarding. A low dose of acute nicotine (0.35 mg/kg) is non-motivational in +/+ mice ($n = 8$), but is rewarding in α5 −/− mice ($n = 8$; $P < 0.05$). Blocking activity at dopaminergic receptors with α-flupenthixol (α-flu) makes the low dose of acute nicotine rewarding in +/+ mice ($n = 27$), but does not augment the rewarding response in −/− mice ($n = 12$). A higher dose of 1.75 mg/kg nicotine is aversive in +/+ mice ($n = 7$), but not in −/− mice ($n = 8$; $P < 0.05$). Pre-treatment with α-flu blocks the aversive effect of 1.75 mg/kg nicotine in +/+ mice ($n = 8$), an effect that is not augmented in −/− mice ($n = 8$). *$P < 0.05$ comparing α5 +/+ and α5 −/− mice given the same treatment.

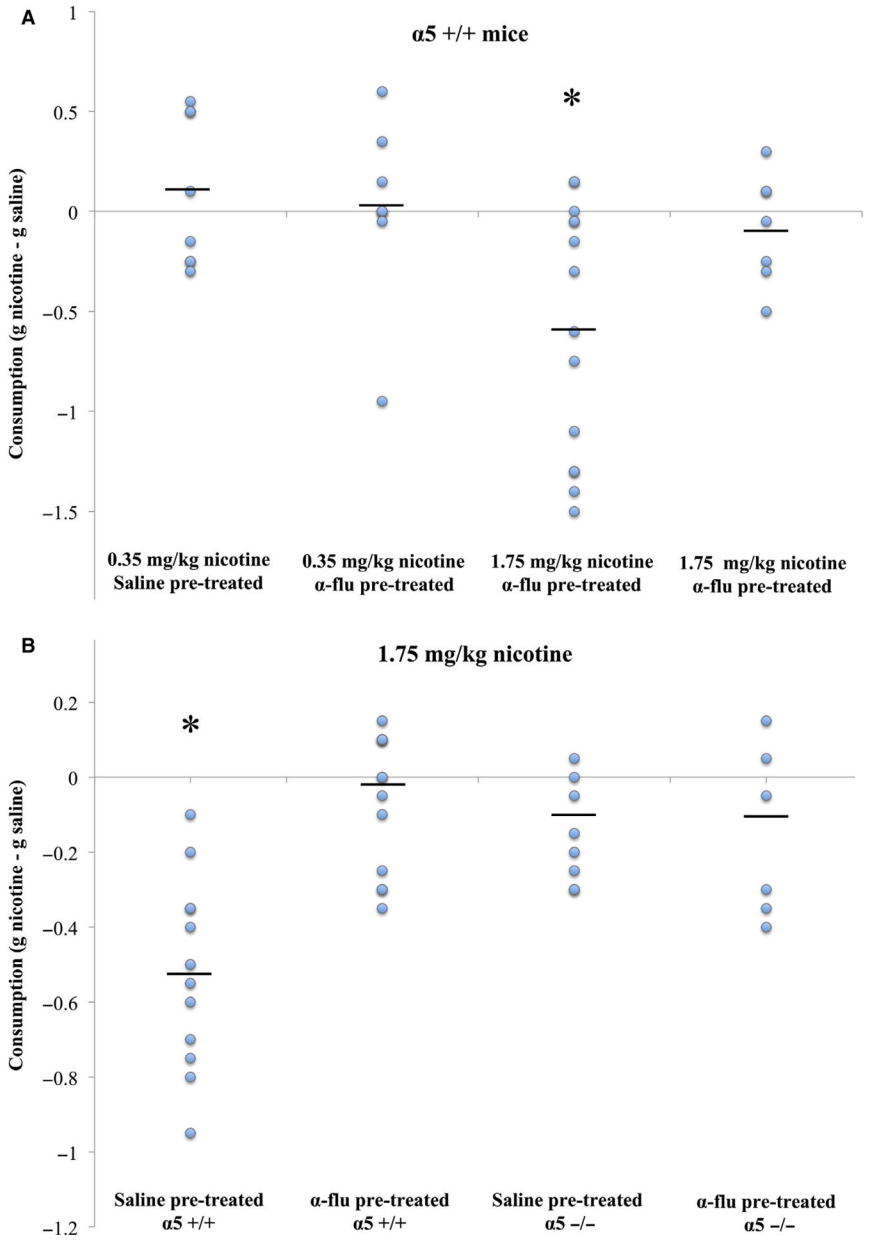

Fig. 2.

A high but not low dose of acute nicotine elicits conditioned taste avoidance (CTA) in α5 +/+ mice that is blocked by dopaminergic antagonism and in α5 −/− mice. (A) A low dose of acute nicotine (0.35 mg/kg) did not elicit a CTA in C57Bl/6 control mice pre-treated with saline ($n = 8$) or α-flu ($n = 8$). A higher dose of acute nicotine (1.75 mg/kg) led to a significant CTA in control mice pre-treated with saline ($n = 7$; $P < 0.05$), which was blocked with α-flu pre-treatment ($n = 8$). *$P < 0.05$ in comparison with all other groups. (B) α5 +/+ mice pre-treated with saline ($n = 10$) showed a CTA to 1.75 mg/kg nicotine ($P < 0.05$), but not when pre-treated with α-flu ($n = 10$). α5 −/− mice did not show a CTA to 1.75 mg/kg nicotine when pre-treated with saline ($n = 8$) or the dopamine receptor antagonist ($n = 8$).

Consumption was calculated as the amount of nicotine-paired solution minus the amount of saline-paired solution, in ml. *$P < 0.05$ in comparison with all other groups.

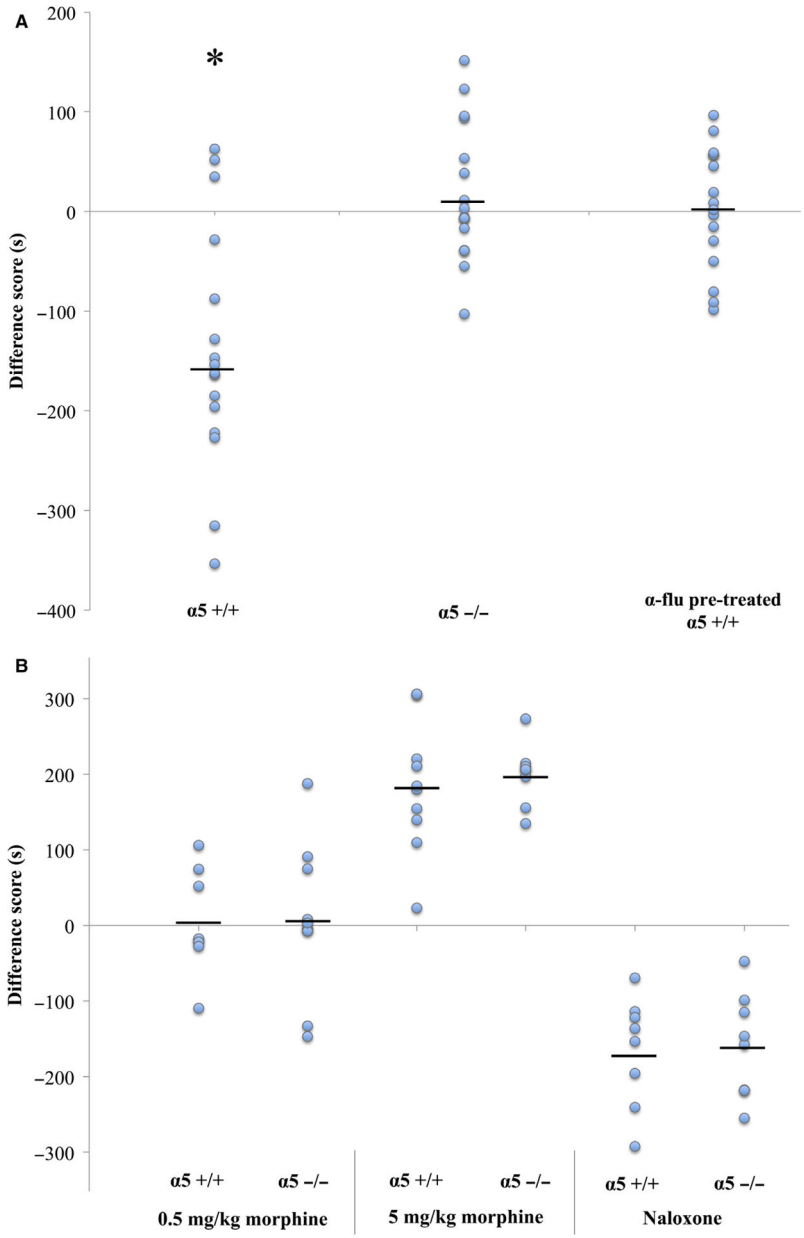

Fig. 3.

α5 nAChR subunit deletion prevents the aversive motivational response to chronic nicotine withdrawal, but not motivational responses in general. (A) Knockout of the α5 nAChR subunit (α5 −/−) or dopamine receptor antagonism in wild-type mice (α-flu pre-treated α5 +/+ mice) prevents the aversive motivational response to withdrawal from chronic nicotine. α5 +/+ mice ($n = 16$) in peak withdrawal from chronic nicotine showed an aversive motivational response to the withdrawal-paired environment ($P < 0.05$). This conditioned aversive response to nicotine withdrawal was not observed in α5 −/− mice ($n = 16$) or mice that were pre-treated with the dopamine receptor antagonist α-flupenthixol ($n = 16$). *$P <$ 0.05 in comparison with α5 −/− and α-flupenthixol pre-treated groups of mice. (B) A low dose of acute morphine (0.5 mg/kg) was non-motivational in both α5 +/+ ($n = 8$) and −/−

mice ($n = 8$; $P > 0.05$). A higher dose of acute morphine (5 mg/kg) was rewarding in both α5 +/+ ($n = 10$) and −/− mice ($n = 10$; $P > 0.05$). The opiate receptor antagonist naloxone (0.6 mg/kg) was aversive in both α5 +/+ ($n = 8$) and −/− mice ($n = 8$; $P > 0.05$).