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Partial and Full Deletion of Nicotinic Acetylcholine Receptor $\alpha 4$ and $\beta 2$ Subunits Reduces Sensitivity to Acute Nicotine Administration and Development of Tolerance following Chronic Nicotine Administration

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Abstract

The diversity of nicotinic cholinergic receptor (nAChR) subunits underlies the complex responses to nicotine. Mice differing in expression of $\alpha 4$ and $\beta 2$ subunits, which are most widely expressed in brain, were evaluated for the responses to acute nicotine administration on Y-Maze Crossings and Rears, Open Field locomotion and Body Temperature following chronic treatment with nicotine (0, 0.25, 1.0 and 4.0 mg/kg/hr). Deletion or partial deletion of the $\alpha 4$ and/or $\beta 2$ nAChR subunits reduced the sensitivity of mice to acute nicotine administration. This reduced sensitivity was gene-dose dependent. Modification of $\alpha 4$ subunit expression elicited a greater reduction in sensitivity than modification of $\beta 2$ subunit expression. No measurable tolerance was observed for mice of any genotype following chronic treatment with 0.25 mg/kg/hr nicotine. Modest tolerance was noted following treatment with 1.0 mg/kg/hr. Greater tolerance was observed following treatment with 4.0 mg/kg/hr. The extent of tolerance differed among the mice depending on genotype: Wild-type ($\alpha 4^{++}$ and $\beta 2^{++}$) developed measurable tolerance for all four tests. Heterozygotes ($\alpha 4^{+-}$, $\beta 2^{+-}$ and $\alpha 4^{+-}/\beta 2^{+-}$) developed tolerance for only Y-Maze Crossings and Body Temperature. Null mutants ($\alpha 4^{-/-}$ and $\beta 2^{-/-}$) did not become tolerant. However, following chronic treatment with 4.0 mg/kg/hr nicotine, wild-type, $\alpha 4^{+-}$ and $\alpha 4^{-/-}$ mice displayed increased Y-Maze Crossings following acute administration of 0.5 mg/kg nicotine that may reflect the activity of $\alpha 6\beta 2^{*}$ -nAChR. These results confirm the importance of the $\alpha 4$ and $\beta 2$ nAChR subunits in mediating acute and chronic effects of nicotine on locomotion and body temperature in the mouse.

Keywords

Null mutant mice; chronic nicotine treatment; tolerance development; locomotor activity; body temperature; $\alpha 4$ nicotinic receptor subunit; $\beta 2$ nicotinic receptor subunit

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The effects of nicotine *in vivo* are mediated primarily by its interaction with neuronal nicotinic cholinergic receptors (nAChR) (Marubio et al., 1999, Tritto et al., 2004, Mineur and Picciotto, 2008, De Biasi and Dani, 2011). Early studies using inbred mouse strains detected significant associations between variability in brain nicotinic receptor expression ($[^3\text{H}]$ -nicotine binding) and sensitivity to nicotine responses measured as locomotor depression and hypothermia (Marks et al., 1989). High affinity $[^3\text{H}]$ -nicotine binding measures $\alpha 4\beta 2^*$ -nAChR, the most widely expressed nAChR subtype in the brain (Picciotto et al., 1995, Marubio et al., 1999, Ross et al., 2000, Baddick and Marks, 2011). The contribution of $\alpha 4\beta 2^*$ -nAChR to behavioral responses to acute nicotine administration has been directly demonstrated with experiments using mice with altered expression of $\alpha 4$ or $\beta 2$ genes: deletion of either the $\alpha 4$ or $\beta 2$ gene reduces sensitivity to nicotine, while introduction of hypersensitive $\alpha 4$ or $\beta 2$ genes increases sensitivity to nicotine (Owens et al., 2003, Tapper et al., 2004, Tritto et al., 2004, Brunzell et al., 2006, Marks, 2013).

Genetic and environmental factors influence smoking behavior and genes encoding nAChRs are among those implicated in aspects of human smoking behavior (Hall et al., 2002, Liu et al., 2019). Individuals differing in receptor expression are likely to respond differently to chronic tobacco use. While the most thoroughly documented genetic variants that mediate responses to nicotine in humans are in the *CHRNA5* gene encoding the nAChR $\alpha 5$, an accessory subunit (Ware et al., 2011, Bierut et al., 2014). The $\alpha 5$ subunit is assembled in receptors with the $\alpha 3$ and $\beta 4$ subunits in the autonomic nervous system and almost exclusively with the $\alpha 4$ and $\beta 2$ subunits in the central nervous system (Brown et al., 2007, Moretti et al., 2018). Genetic studies also support roles for both *CHRNA4* and *CHRNA2* genes in aspects of tobacco use (Feng et al., 2004, Hutchison et al., 2007, Kamens et al., 2013, Esterlis et al., 2016, Melroy-Greif et al., 2016). More than 4000 variants in human *CHRNA4* and 2000 variants in *CHRNA2* have been identified (http://NCBI/SNPdatabase-CHRNA4_and_CHRNA2). The variants are located in many non-coding regions throughout these genes. While variants in non-coding regions would not necessarily alter receptor function, these variants may affect expression of subunit proteins. Indeed, expression for human $\alpha 4\beta 2^*$ -nAChR varies widely among individuals (Breese et al., 1997, Breese et al., 2000, Cosgrove et al., 2007, D'Souza et al., 2012).

Chronic nicotine treatment in rodents (Marks et al., 1983, Schwartz and Kellar, 1983) and tobacco use in humans (Benwell et al., 1988, Breese et al., 1997, Staley et al., 2006) results in up-regulation of high affinity nicotinic binding sites. Up-regulation in mice is dose-dependent and saturable (Marks et al., 2011). Mice that have been chronically treated with nicotine also develop tolerance to the acute effects of the drug (Marks et al., 1983, Sparks and Pauly, 1999, Grabus et al., 2005, Gould et al., 2014). Tolerance development differs markedly among inbred strains, but the strains do not differ significantly in the extent of receptor up-regulation (Marks et al., 1986a, Marks et al., 1991). These findings suggest that up-regulation of nicotinic receptor binding sites is a well-documented consequence of chronic exposure to nicotine.

Genetically modified mice have been studied to address the contribution of the $\alpha 4$ and $\beta 2$ nAChR subunits in tolerance development. The role of the $\beta 2$ subunit in modulating tolerance development has been examined by chronically treating mice differing in

expression of this gene with nicotine (McCallum et al., 2006). As expected, increasing chronic treatment doses resulted in increased tolerance development in wild-type mice, while $\beta 2$ null mutant mice ($\beta 2^{-/-}$ mice) developed little tolerance. The role of the $\alpha 4$ gene has been evaluated using mice expressing hypersensitive receptors ($\alpha 4$ L9'A mice). These mutant mice developed tolerance to the hypothermia producing effects of nicotine at much lower doses than those required for wild-type mice (Tapper et al., 2007). The studies cited above support the contention that $\alpha 4\beta 2^{*}$ -nAChR contribute significantly to nicotine-induced tolerance.

The current study applied the strategy of deletion or partial deletion of the $\alpha 4$ and/or $\beta 2$ nAChR gene to investigate the effects of well-defined differences in receptor expression on acute sensitivity to nicotine as well as on the development of tolerance to the locomotor depressing and hypothermia inducing effects of acute nicotine administration. The expression of $\alpha 4\beta 2^{*}$ -nAChR binding sites and subunit expression is altered by partial deletion of either the $\alpha 4$ and/or $\beta 2$ subunit: receptor expression in $\alpha 4^{+/-}$ and $\beta 2^{+/-}$ mice is approximately 50% that of wildtype mice and expression is further reduced in $\alpha 4^{+/-}/\beta 2^{+/-}$ mice (Whiteaker et al., 2006, Moretti et al., 2018).

Materials and Mice

Mice

All procedures were reviewed and approved by the Institutional Animal Care and Utilization Committee of the University of Colorado Boulder. Mice expressing the $\alpha 4$ nAChR null mutation were originally obtained from John Drago, University of Melbourne Australia (Ross et al., 2000). Mice expressing the $\beta 2$ nAChR null mutation were originally obtained from Marina Picciotto, Yale University (Picciotto et al., 1995). Each line has been backcrossed with C57BL/6J mice for at least ten generations. Wild type ($++$), heterozygote ($+/-$) and null mutant ($---$) mice were generated by mating the appropriate $\alpha 4$ and $\beta 2$ heterozygotes. Offspring were genotyped as described previously (Salminen et al., 2004). Even though C57BL/6 mice served as the background strain for mice differing in both the $\alpha 4$ and $\beta 2$ expression, $\alpha 4^{++}$ and $\beta 2^{++}$ mice, which were littermates of the heterozygote and null mutant mice, were both treated and tested. Double heterozygotic mice ($\alpha 4^{+/-}/\beta 2^{+/-}$) were generated by mating $\alpha 4^{+/-}$ with $\beta 2^{+/-}$ mice. Before surgeries, mice were housed with like sex littermates, maintained on a 12 hr light/12 hr dark cycle (lights on 7 AM) and allowed free access to food and water.

Materials

Liquid nicotine was obtained from Sigma Chemical Co., St. Louis, MO and stored under nitrogen. This reagent was redistilled when the material became yellow. Ketamine, xylazine, acepromazine, buprenorphine and sterile saline were obtained from MWI Veterinary Supply. Silastic tubing, a product of Dow Chemical, was obtained through VWR International. Nylon mesh and 22 gauge stainless steel tubing were obtained from Small Parts, Inc.

Surgery

A cannula constructed of silastic tubing was inserted in the right jugular vein of each mouse (Barr et al., 1979). Briefly, mice were anesthetized with a cocktail composed of ketamine (80 mg/kg), xylazine (20 mg/kg) and acepromazine (1 mg/kg). An incision (approximately 1 cm) was made to expose the superficial right jugular vein. The silastic cannula (0.51 mm I.D. 0.94 mm O.D) was inserted 8 mm into the right jugular vein through a small hole in the vein. The cannula, filled with isotonic saline containing 0.3% citric acid, was anchored to the underlying tissue with surgical thread. The cannula was attached to stainless steel tubing mounted on a nylon disk and from which a section of tubing passed through the back in the midscapular region of the mouse. The disk with the attached tubing was then anchored to the skin with a wound clip. The mouse was injected with buprenorphine (0.1 mg/kg), placed in a clean cage and warmed until waking.

Nicotine Treatment

Following recovery from surgery each mouse was transferred to an individual infusion chamber (15 cm x 15 cm x 25 cm, length x width x height) and its cannula was attached to medical grade Tygon tubing connected to a 1 ml syringe mounted on an infusion pump (Harvard Apparatus, Holliston, MA). Sterile saline was continuously infused at a rate of 35 μ L/hr. After two days of saline infusion, nicotine treatment was begun. Mice were divided into four treatment groups that received the following nicotine doses: 0 mg/kg/hr (saline-infused control), 0.25 mg/kg/hr, 1.0 mg/kg/hr, or 4.0 mg/kg/hr. All doses are free base. Nicotine solutions were prepared from liquid nicotine neutralized with HCl. The nicotine concentration used for the treatment of each mouse was calculated using the weight of each mouse and the infusion rate of 35 μ L/hr. Fresh solutions were supplied every day. All treatment solutions were prepared in sterile saline. Following ten days of treatment with the indicated nicotine dose, the cannula of each mouse was disconnected from the Tygon tubing and checked for free fluid flow.

Tolerance Testing

Behavioral test measures were selected based on previous studies because they provide reliable measures of acute response to nicotine (Marks et al., 1985, Tritto et al., 2004). Furthermore, these measures have proven to be useful measures of tolerance development following chronic nicotine treatment (Marks et al., 1991, McCallum et al., 2006). Chronic nicotine treatment had to be discontinued for at least two hours before testing to allow clearance of nicotine (Petersen et al., 1984). Mice were habituated to the testing room for one hour prior to testing. Following the acclimation period, mice received an intraperitoneal injection of nicotine dissolved in sterile saline (0.01 ml/g). Testing was conducted as described previously (Tritto et al., 2004, McCallum et al., 2006). Mice were placed in a Y-maze apparatus 3 minutes after nicotine injection and both Crossings and Rearings measured for 3 minutes. Each Y-maze is constructed of black acrylic plastic and was fabricated in the instrument shop of the Department of Psychology, University of Colorado Boulder. Each arm of the maze is 5.4 cm wide, 10 cm high and 26.5 cm long. Crossings were monitored by recording beam breaks with infrared detectors mounted 1 cm above the floor placed at the entrance and midpoint of each arm. Rearings were monitored by recording beam breaks with

infrared detectors 6.4 cm above the floor located at the ends of each arm. Immediately following Y-maze assessment, mice were placed in a Med Associates circular open field that was 58 cm diameter and was illuminated by a 23 watt fluorescent bulb 38 cm above the maze that provided 4500 lumens of illumination. Locomotor activity was monitored for 5 minutes. Following completion of the open field test, mice were singly-housed in a cage until temperature was measured with a Thermalert probe inserted 2.5 cm into the mouse's rectum 15 minutes post injection at which maximum hypothermia is observed. Two hours after the initial test, mice were injected with a second dose of nicotine and tested a second time. All mice were tested twice using two different acute nicotine doses. Preliminary experiments were conducted to compare the dose-response curves obtained for mice tested only once to those that had been tested twice. No significant differences were detected between mice receiving one or two injections. Results from both tests were used in the construction of dose-response curves. The following nicotine doses in mg/kg free base were used: 0, 0.5, 1.0, and 1.5.

Supplementary Table 1 enumerates the mice of each genotype chronically treated with nicotine and those receiving each acute challenge dose of nicotine following chronic treatment.

Data Analysis

Results of the tolerance testing were analyzed several ways. Initially, normalized results for the four responses were analyzed using a three-way analysis of variance (ANOVA) using IBM SPSS version 21. Response was the dependent variable and genotype, chronic treatment dose and acute challenge dose as the independent variables. Y-maze Crossings, Y-maze Rearings and open field data were normalized by dividing each value by the average value for the saline-treated mice in each group (a group was composed of mice of the same genotype treated with one of the chronic nicotine doses). Consequently the control (saline-injected) values for each group was 1.0. Change from control body temperature was determined by subtracting the body temperature measured for each condition from the average control body temperature measured for the saline-injected mice. Subsequently two-way ANOVAs were used to analyze the effects of chronic and acute nicotine treatment within mice of each genotype.

In addition, the dose-response curves for each test for mice of each genotype were fit by non-linear least squared curve fitting using the algorithm provided in Sigma Plot 11.0 (Systat Software, San Jose, CA) to the following equation for activity measures:

$$R_{AD} = R_{SAL} / (1 + (NIC_{AD} / ED_{50})^{NH})$$

Where R_{AD} is the response measured after the acute nicotine challenge dose (NIC_{AD}), R_{SAL} is the activity following saline administration (the initial response), ED_{50} is the acute nicotine dose eliciting half-maximal response and NH is the slope of the dose-response curve. The equation used for these calculations is analogous to the Hill equation. Several genotypes of mice displayed locomotor activation following treatment with the highest nicotine dose (4.0 mg/kg/hr). ED_{50} values for locomotor depression in these mice were

calculated using the maximal measured activity as the starting activity rather than that of the saline-treated mice.

The equation used for body temperature was:

$$BT_{AD} = (BT_{SAL} - BT_{FIN}) / (1 + (NIC_{AD}/ED_{50})^{NH}) + BT_{FIN}$$

Where BT_{AD} is the body temperature after the acute nicotine challenge dose, BT_{SAL} is the body temperature following saline injection and BT_{FIN} is the body temperature following administration of the maximally effective nicotine dose (set at 32°C). This value was obtained from the initial curve fits described as follows.

Initial curve fits were done using all of the data for mice of each genotype that had been treated chronically with the same nicotine dose without constraint of the variables R_{SAL} , ED_{50} and NH for activity measures and BT_{SAL} , BT_{FIN} , ED_{50} and NH for body temperature. No statistically significant differences in NH were observed among the groups for each test. However, each NH was significantly greater than 1 and could not be disregarded. Subsequently, calculations were made using the average value for NH for each test: Y-maze Crossings, $NH = 4.72$; Y-maze Rearings, $NH = 5.56$; Open-field distance, $NH = 3.97$; Body temperature, $NH = 3.38$. This constraint of NH assured that the dose-response curves for an individual test were parallel and allowed the calculation of potency ratio. $ED_{50} \pm SEM$ for each treatment group was calculated from the non-linear curve fits for all of the data for that group.

Potency ratios were calculated for each measure by dividing the ED_{50} measured following chronic treatment with nicotine by the ED_{50} following chronic treatment with saline. SEM for the potency ratios were calculated using Taylor's expansion.

Results

Responses to acute nicotine injection for saline treated mice

Results for mice that had been chronically treated with saline were evaluated to determine the effects of deletion or partial deletion of the $\alpha 4$ and/or $\beta 2$ nAChR genes on each of the four tests.

The results from the two-way ANOVAs evaluating the effects of genotype and acute nicotine dose are summarized in Table 1. These ANOVA results revealed significant main effects of genotype and acute nicotine dose for all four tests, as well as significant genotype by dose interactions for Y-Maze Crossings and body temperature. Since these ANOVAs demonstrated significant differences among the mice expressing different $\alpha 4$ and/or $\beta 2$ expression (the main effects of genotype) the dose-response curves for mice of each genotype were analyzed. Dose-response curves for mice differing in expression of the $\alpha 4$ gene are shown in Figure 1 and curves for mice differing in expression for the $\beta 2$ gene are shown in Figure 2. The effects of deletion or partial deletion of these genes on ED_{50} values for nicotine are shown in Figure 3.

Effects of differential $\alpha 4$ expression

The effects of differential expression of the $\alpha 4$ nAChR gene expression on the acute effects of nicotine are quite similar for the four tests as illustrated in Figures 1 and 3. Significant increases in ED₅₀ values were noted following partial deletion of the $\alpha 4$ gene ($\alpha 4^{+-}$ mice) for Y-maze Crossings ($38 \pm 10.8\%$), Y-maze Rearings ($42 \pm 19.3\%$), open field distance ($47 \pm 12.0\%$) and body temperature ($20 \pm 8.1\%$). Deletion of the $\alpha 4$ gene ($\alpha 4^{-}$ mice) elicited even more pronounced increases in the ED₅₀ values for Y-maze Crossings ($208 \pm 4.4\%$), Y-maze Rearings ($126 \pm 8.6\%$), open field distance ($89 \pm 6.9\%$) and body temperature ($184 \pm 3.8\%$).

Effects of differential $\beta 2$ expression

Deletion of the $\beta 2$ nAChR gene reduced responses to acute nicotine administration as shown in Figures 2 and 3. These reductions were smaller than those noted following deletion of the $\alpha 4$ gene. Partial deletion of the $\beta 2$ gene ($\beta 2^{+-}$ mice) resulted in modest increases in ED₅₀ values for Y-maze Crossings ($18.4 \pm 10.0\%$), Y-maze Rearings ($8 \pm 14.9\%$), and open field distance ($29 \pm 9.1\%$) but not body temperature ($0 \pm 9.0\%$). Deletion of the $\beta 2$ gene ($\beta 2^{-}$ mice) elicited significant increases in the ED₅₀ values for Y-maze Crossings ($47 \pm 11.1\%$), Y-maze Rearings ($21 \pm 11.3\%$), open field distance ($43 \pm 8.6\%$) and body temperature ($45 \pm 6.7\%$).

Effects of partial deletion of both $\alpha 4$ and $\beta 2$

Partial deletion of both the $\alpha 4$ and $\beta 2$ genes ($\alpha 4^{+-}/\beta 2^{+-}$ mice) elicited increased ED₅₀ values for Y-maze Crossings ($33 \pm 10.4\%$), Y-maze Rearings ($47 \pm 12.1\%$), open field distance ($22 \pm 10.4\%$) and body temperature ($37 \pm 7.7\%$). These changes were similar to those observed for the $\alpha 4^{+-}$ mice.

Tolerance development following chronic nicotine treatment

Mice differing in expression of the $\alpha 4$ and/or $\beta 2$ nAChR genes were chronically treated with one of four nicotine doses: 0, 0.25, 1.0 or 4.0 mg/kg/hr nicotine and subsequently tested for their responses to acute injections of nicotine. Results were evaluated by ANOVA and by calculation of ED₅₀ values. Dose-response curves for the four tests for each genotype are provided in Supplemental Figures 1–7. ANOVA results are presented in Tables 2 and 3. The calculated ED₅₀ values and potency ratios following chronic nicotine treatment are presented in Table 4. In addition potency ratios following chronic treatment with 4.0 mg/kg/hr nicotine for mice of each genotype are plotted as a function of the initial expression of $\alpha 4$ and $\beta 2$ nAChR subunits (Figure 4). Supplemental figure 8 provides a similar comparison between subunit expression and potency ratios calculated following treatment with 1.0 mg/kg/hr nicotine.

Three-way ANOVAs provided an overall comparison of the effects of genotype, chronic nicotine treatment dose and acute nicotine challenge dose for the four tests (Table 2). These analyses revealed significant main effects of genotype, chronic nicotine treatment dose and acute challenge nicotine dose for the four tests. Furthermore, a significant genotype by chronic treatment dose interaction was observed for body temperature, significant genotype by acute nicotine dose interactions and chronic treatment dose by acute treatment dose

interactions were observed for all four measures. No significant three-way interactions were noted.

Results were subsequently analyzed by two-way ANOVA to evaluate the effects of chronic nicotine treatment and acute nicotine test dose in mice of each of the seven genotypes separately (Table 3). In addition, ED₅₀ values for the responses to the acute nicotine challenge were calculated following chronic treatment with each nicotine dose in each genotype (Table 4).

Wild-type mice ($\alpha 4^{++}$ and $\beta 2^{++}$)

The dose-response curves for Y-Maze Crossings, Y-Maze Rearings, open field distance and body temperature for $\alpha 4^{++}$ mice are shown in Supplemental Figure 1 and for $\beta 2^{++}$ mice in Supplemental Figure 2. The results of the two-way ANOVAs for these wild-type mice are given in Table 3. Inspection of these figures indicates that a notable shift to the right in the dose-response curves followed chronic treatment with 1.0 mg/kg/hr nicotine with a more pronounced rightward shift following treatment with 4.0 mg/kg/hr nicotine.

Two-way ANOVAs for both the $\alpha 4^{++}$ and $\beta 2^{++}$ mice reveal highly significant main effects of acute nicotine administration and chronic nicotine treatment as well as significant chronic by acute interactions for the four tests indicative of tolerance development (Table 3). The ANOVA results are supported by the chronic dose dependent increases in ED₅₀ values with resulting increases in potency ratios for both wild-type strains ($\alpha 4^{++}$ and $\beta 2^{++}$ mice) following treatment with 4.0 mg/kg/hr nicotine for all four tests (Table 4) and for Y-Maze Crossings and body temperature following treatment with 1.0 mg/kg/hr nicotine. No significant tolerance was observed following chronic treatment with 0.25 mg/kg/hr nicotine.

$\alpha 4^{+-}$ mice

The dose-response curves for Y-Maze Crossing, Y-Maze Rearings, open field distance and body temperature for $\alpha 4^{+-}$ mice are shown in Supplemental Figure 3. The results of the two-way ANOVAs are given in Table 3. Inspection of Supplemental Figure 3 indicates that the degree of tolerance development varied among the four tests with measureable tolerance observed for Y-Maze Crossings and body temperature following chronic treatment with 4.0 mg/kg/hr nicotine.

The results of two-way ANOVAs provided in Table 3 are consistent with the impressions noted by visual inspection of the dose-response curves: significant main effects of both chronic nicotine treatment and acute nicotine test as well as significant chronic by acute interactions were obtained for Y-Maze Crossings and body temperature indicating that chronic nicotine treatment elicited tolerance to acute nicotine administration for these two measures. In contrast, no significant main effects of chronic nicotine treatment or chronic by acute interactions were found for Y-Maze Rearings and Open Field Distance.

The ANOVA results are supported by the analysis of ED₅₀ values and potency ratios for the various measures presented in Table 4. Modest tolerance development was indicated following chronic treatment with 1.0 mg/kg/hr nicotine (a potency ratio of 1.42 ± 0.12 for Y-Maze Crossings). In contrast to mice chronically treated with saline, which show an acute

dose-dependent reduction in Y-Maze, mice chronically treated with 4.0 mg/kg/hr nicotine display significantly increased Y-Maze Crossings (a 45.0 ± 15.8 % increase compared to acute saline) following acute administration of 0.5 mg/kg nicotine. A decrease in activity was observed following acute administration of either 1.0 or 1.5 mg/kg nicotine (See Supplemental Figure 3). Chronic treatment with 4.0 mg/kg/hr nicotine resulted in an increase in potency ratio for Y-Maze Crossings, Y-Maze Rearings and Body Temperature (Table 4).

$\beta 2^{+-}$ mice

The dose-response curves for Y-Maze Crossing, Y-Maze Rearings, open field distance and body temperature for $\beta 2^{+-}$ mice are shown in Supplemental Figure 4. Inspection of the figure indicates that the degree of tolerance development appeared to be similar to that observed for $\alpha 4^{+-}$ mice.

The results of two-way ANOVAs for $\beta 2^{+-}$ mice are provided in Table 3. Significant main effects of both chronic nicotine treatment and acute nicotine test dose were obtained as for Y-Maze Crossings and body temperature. However, no main effects of chronic treatment or chronic by acute interactions were found for Y-Maze Rearings or Open Field Distance. The ANOVA results are supported by the observed increase in ED_{50} values and increased potency ratios presented in Table 4. Tolerance development was indicated following chronic treatment with 1.0 mg/kg/hr nicotine for Y-Maze Crossings and body temperature. Tolerance development was greater following chronic treatment with 4.0 mg/kg/hr nicotine for both Y-Maze Crossings and body temperature.

$\alpha 4^{+-}/\beta 2^{+-}$ mice

The dose-response curves for Y-Maze Crossing, Y-Maze Rearings, open field distance and body temperature for $\alpha 4^{+-}/\beta 2^{+-}$ mice are shown in Supplemental Figure 5. Inspection of the figure suggests a pattern similar to those noted for both $\alpha 4^{+-}$ and $\beta 2^{+-}$ mice. The results of two-way ANOVAs for $\alpha 4^{+-}/\beta 2^{+-}$ mice provide insight to the visual inspection of the dose-response curves: significant main effects of both chronic nicotine treatment and acute nicotine test Y-Maze Crossings, Y-Maze Rearings and body temperature (Table 3). However, the main effect of chronic nicotine treatment for open field distance was not significant. Significant increases in ED_{50} values and potency ratios were only noted after treatment with 4.0 mg/kg/hr nicotine for each test.

$\alpha 4^{-/-}$ mice

The dose-response curves for Y-Maze Crossings, Y-Maze Rearings, open field distance and body temperature for $\alpha 4^{-/-}$ mice are shown in Supplemental Figure 6. Inspection of the figure indicates that a relatively little tolerance developed for Y-Maze Rearings, open field distance, and body temperature. In addition, little tolerance was observed for Y-Maze Crossings following treatment with 0.25 or 1.0 mg/kg/hr nicotine. However, a change in response for Y-Maze Crossings following treatment with 4.0 mg/kg/hr nicotine occurred: an apparent increase in activity following acute administration of 0.5 mg/kg nicotine and a modest shift to the right at the higher doses.

Two-way ANOVAs (Table 3) revealed highly significant main effects of acute nicotine administration for all four tests, but found no significant effects of chronic nicotine treatment for Y-Maze Rearings, open field distance, and body temperature. However, consistent with the pattern noted graphically for Y-Maze Crossings, a significant main effect of chronic nicotine treatment dose was noted. ED₅₀ values and potency ratios for the various measures are presented in Table 4. ED₅₀ values for Y-Maze Rearings and open field distance showed no significant changes as a function of chronic nicotine treatment reflected by potency ratios that did not differ significantly from 1. It should be noted that the temperature decrease observed for the $\alpha 4^{-/-}$ with the acute nicotine doses used in the current study was not as large as that observed for the other genotypes, consequently the values calculated for ED₅₀ and potency ratios were obtained by extrapolation and need to be treated with caution.

While mice chronically treated with saline show a dose-dependent reduction in Y-Maze Crossings following acute nicotine administration, the ED₅₀ for this response is significantly higher than that for mice of any other genotype tested. Furthermore, $\alpha 4^{-/-}$ mice chronically treated with 4.0 mg/kg/hr nicotine display significantly increased Y-Maze Crossings (a 58.8 ± 12.7 % increase) following acute administration of 0.5 mg/kg nicotine (See Supplemental Figure 6). This increase in activity is greater than the increase observed for mice of any other genotype. Decreased activity was noted following acute administration of either 1.0 or 1.5 mg/kg nicotine.

$\beta 2^{-/-}$ mice

The dose-response curves for Y-Maze Crossings, Y-Maze Rearings, open field distance and body temperature for $\beta 2^{-/-}$ mice are shown in Supplemental Figure 7. Inspection of the figure indicates that relatively little tolerance developed for Y-Maze Crossings, Y-Maze Rearings, open field activity, and body temperature.

Two-way ANOVAs (Table 3) showed highly significant main effects of acute nicotine dose for all four tests, but found no significant effect of chronic nicotine treatment. In addition, results presented in Table 4, chronic nicotine treatment elicited no significant changes in ED₅₀ values or potency ratios for three of the tests.

Unlike wild-type, $\alpha 4^{+/+}$ and $\alpha 4^{-/-}$ mice, $\beta 2^{-/-}$ mice chronically treated with 4.0 mg/kg/hr nicotine display no increased Y-Maze Crossings following acute administration of 0.5 mg/kg nicotine (See Supplemental Figure 7). Chronic treatment resulted in a modest increase in ED₅₀ and potency ratio for Y-Maze Crossings following chronic treatment with 4.0 mg/kg/hr nicotine.

Comparison of Potency Ratios to $\alpha 4$ and $\beta 2$ Subunit Expression

Inasmuch as the expression of the $\alpha 4$ and $\beta 2$ nAChR subunit proteins were measured in the mice used in the current study, a comparison of $\alpha 4\beta 2^*$ -nAChR levels for saline-treated mice and potency ratios is possible. Figure 4 displays the relationship between potency ratios following treatment with 4.0 mg/kg/hr nicotine for the four tests. A similar comparison is shown for potency ratios following treatment with 1.0 mg/kg/hr nicotine in Supplemental Figure 8. As shown in Figure 4 significant linear relationships between initial expression of

$\alpha 4$ and $\beta 2$ nAChR subunit proteins measured immunochemically for a subset of mice tested in the current study (Moretti et al., 2018) and potency ratios were observed for all four tests.

Discussion

The $\alpha 4$ and $\beta 2$ nAChR subunits are required for the expression of the high affinity receptor measured with [3 H]-nicotine (Picciotto et al., 1997, Marubio et al., 1999, Ross et al., 2000), [3 H]-cytisine (Flores et al., 1992) or cytisine-sensitive [3 H]- or [125 I]-epibatidine binding (Marks et al., 2010, Baddick and Marks, 2011). The results presented here demonstrate that deletion or partial deletion of the genes that encode the $\alpha 4$ and $\beta 2$ nAChR subunits reduces responses to an acute challenge dose of nicotine (termed here: acute sensitivity) and also reduces the development of tolerance to the acute effects of nicotine following chronic nicotine treatment (termed here: tolerance).

As noted in the introduction, studies with inbred mouse strains demonstrated variability among the strains in response to nicotine measured using a test battery that included Y-maze activities and body temperature (responses that were also measured in the experiments reported here). Differences in acute sensitivity among the strains were significantly correlated with differences in brain [3 H]-nicotine binding ($\alpha 4\beta 2^*$ -nAChR expression) (Marks et al., 1989). These results suggested that mice expressing lower levels of [3 H]-nicotine binding exhibited less acute sensitivity to nicotine than mice with higher levels of [3 H]-nicotine binding. However, many factors other than receptor number such as the existence of receptor polymorphisms and of nAChR mediation of neurotransmitter and hormone release can contribute to the strain differences (Picciotto, 2003, Portugal and Gould, 2008, Wilking and Stitzel, 2015). The studies reported here used genetically engineered mice that have been bred onto the same inbred mouse background (C57BL/6). Thus, variation in genetic background is unlikely to contribute significantly to the differences in acute sensitivity to the effects of nicotine. The current results are consistent with the proposal that the level of $\alpha 4\beta 2^*$ -nAChR expression, which have been published previously for the mice examined here (Moretti et al., 2018), mediates variability in the effects of nicotine on locomotion or body temperature.

Deletion of either the $\alpha 4$ or $\beta 2$ subunit gene elicited rightward shifts in the dose-response curves (increased ED_{50} values) for all three locomotor tests and for hypothermia. Reduced acute sensitivity was also noted for the $\alpha 4^{+/-}$, $\beta 2^{+/-}$ and $\alpha 4^{+/-}/\beta 2^{+/-}$ mice, but the reductions were smaller than those observed for null mutants. The residual effect seen in the $\beta 2^{-/-}$ mice was totally blocked by pretreatment with the nAChR antagonist, mecamylamine (Tritto et al., 2004). Thus, nAChRs that do not include a $\beta 2$ subunit (for example $\alpha 7$ -nAChR and $\alpha 3\beta 4^*$ -nAChR) regulate effects elicited by higher doses of nicotine in the $\beta 2^{-/-}$ mice. These increases in ED_{50} values measured for $\alpha 4^{-/-}$ mice were nearly twice those observed following deletion of $\beta 2$. The increase in ED_{50} values for the $\alpha 4^{+/-}$ mice for all of the measures were significantly less than those noted for $\alpha 4^{-/-}$ mice. ED_{50} values measured for $\alpha 4^{+/-}$ mice were similar to those obtained for locomotor effects with the $\beta 2^{-/-}$ mice. In as much as virtually all $\alpha 4^*$ -nAChR are $\alpha 4\beta 2^*$ -nAChR (Flores et al., 1992, Picciotto et al., 1995, Marubio et al., 1999, Baddick and Marks, 2011, Fasoli et al., 2016) the observation that deletion of the $\alpha 4$ subunit elicited a larger effect than did deletion of the $\beta 2$ subunit

seems counterintuitive. Unlike $\beta 2^*$ -nAChR for which $\beta 4^*$ -nAChR provide an alternative functional pathway, particularly in the autonomic nervous system (De Biasi, 2002), there may not be similar redundancy for the $\alpha 4\beta 2^*$ -nAChR in the brain. Residual response to nicotine in $\alpha 4^{-/-}$ mice are likely to be mediated by nAChR with lower affinity for nicotine. For example, it has been shown that the EC_{50} for striatal dopamine release for $\alpha 6\beta 2\beta 3$ -nAChR remaining following deletion of the $\alpha 4$ subunit is higher than the EC_{50} for the $\alpha 4\alpha 6\beta 2\beta 3$ -nAChR eliminated by the $\alpha 4$ deletion (Salminen et al., 2007). Alternatively it may be possible that $\alpha 4\beta 2^*$ -nAChR mediate a subset of responses separate from those of other $\beta 2^*$ -nAChR.

It has been reported that deletion of either the $\alpha 4$ or $\beta 2$ subunits alters the behavioral and/or physiological responses to acutely administered nicotine including locomotor activity, body temperature, nociception, self-administration, conditioned place preference and anhedonia (Marubio et al., 1999, Ross et al., 2000, Marubio and Paylor, 2004, Tritto et al., 2004, Jackson et al., 2008, Portugal et al., 2008, Marks, 2013, Stoker et al., 2015). The role of nAChR including $\alpha 4$ and $\beta 2$ subunits in mediating several of these measures is supported by observations of increased sensitivity to nicotine in mice expressing hypersensitive receptors (Fonck et al., 2003, Tapper et al., 2004, Fonck et al., 2005, Damaj et al., 2007, Tapper et al., 2007, Marks, 2013, O'Neill et al., 2013).

Fewer studies have examined the effects of modification $\alpha 4$ or $\beta 2$ subunit expression on response to chronic nicotine treatment. We have previously reported that $\beta 2^{-/-}$ mice developed relatively little tolerance to the acute effects of nicotine following chronic nicotine treatment (McCallum et al., 2006). The current study confirms and extends that observation: mice with partial deletion of $\alpha 4$ ($\alpha 4^{+/-}$ mice) or $\beta 2$ ($\beta 2^{+/-}$) or partial deletion of both subunits ($\alpha 4^{+/-}/\beta 2^{+/-}$ mice) tended to develop less tolerance to the acute effects of nicotine than did wild-type mice. In addition, mice that had either the $\alpha 4$ or $\beta 2$ subunit fully deleted (null mutants: $\alpha 4^{-/-}$ mice and $\beta 2^{-/-}$ mice) developed little or no tolerance. Inasmuch as the expression of $\alpha 4\beta 2^*$ -nAChR has been measured autoradiographically and immunochemically for the same mice used in the current study (Moretti et al., 2018), potency ratios measured for mice of each genotype were compared to the initial expression of the receptors. Significant correlations between potency ratios for the four tests and $\alpha 4/\beta 2$ subunit expression were obtained for mice treated with either 1.0 mg/kg/hr nicotine (Supplemental Figure 8) or 4.0 mg/kg/hr nicotine (Figure 4). This analysis indicates that expression of either the $\alpha 4$ or $\beta 2$ subunit is essential for the development of tolerance to the locomotor depressing and hypothermia inducing effects of acute nicotine administration. While deletion of either subunit essentially eliminated tolerance development, it remains to be determined whether chronic treatment with higher nicotine doses would elicit tolerance. Owing to the substantial reduction in initial sensitivity exhibited by the $\alpha 4^{-/-}$ mice, it is possible that no further decrease in sensitivity is possible, that is no tolerance will be observed.

The $\alpha 4$ subunit has been reported to contribute to tolerance to the acute effects of nicotine. Mice expressing a gain of function mutation of the $\alpha 4$ subunit (L9'A mice) developed tolerance to the hypothermia inducing effects of nicotine at much lower chronic treatment doses than did wild-type mice (Tapper et al., 2004, Tapper et al., 2007). Similarly $\beta 2^{-/-}$ mice

showed significantly less severe withdrawal following chronic nicotine treatment when assessed by the measurement of anhedonia (Brady et al., 2008), fear conditioning (Davis and Gould, 2007), or conditioned place aversion (Jackson et al., 2008). However, expression of the $\beta 2$ subunit does not appear essential for the appearance of somatic signs of withdrawal, which are mediated by the function of $\beta 4$, $\alpha 2$ and $\alpha 5$ nAChR subunits (Salas et al., 2004b). The current study is consistent with these reports: deletion of either the $\alpha 4$ or $\beta 2$ nAChR subunit virtually eliminates tolerance development to the acute effect of nicotine on locomotion and body temperature following treatment with as high a dose of 4.0 mg/kg/hr. It should be noted that this chronic treatment dose yields a plasma nicotine level of approximately 1 μ M (Marks et al., 2004).

Wild-type, $\alpha 4^{+/-}$ and $\alpha 4^{-/-}$ mice treated chronically with 4.0 mg/kg/hr nicotine exhibited increased activity in the Y-maze following an acute injection of 0.5 mg/kg nicotine. The magnitude of this increase in nicotine activation of Y-Maze Activity tended to increase with the extent of deletion of the $\alpha 4$ subunit from $31 \pm 11.2\%$ for wild-type to $45.0 \pm 15.8\%$ for $\alpha 4^{+/-}$ to $58.8 \pm 12.7\%$ for $\alpha 4^{-/-}$ mice. Perhaps this increase reflects the activity of $\alpha 6\beta 2^{*}$ -nAChR, which are highly expressed in the nigro-striatal pathway and for which a role in locomotor activation has been demonstrated for mice expressing a hyperactive $\alpha 6$ subunit ($\alpha 6$ L9'S mice) (Drenan et al., 2008, Drenan et al., 2010). It has been postulated that the absence of measurable locomotor activation in wild-type mice reflected the greater effect of locomotor depression mediated by $\alpha 4\beta 2^{*}$ -nAChR than activation mediated by $\alpha 6\beta 2^{*}$ -nAChR. (Drenan et al., 2008, Drenan et al., 2010). The increased activity may become measureable as a result of the development of tolerance to locomotor depression elicited by chronic nicotine treatment and/or $\alpha 4$ subunit deletion

The studies described above for the null mutant mice indicate that both $\alpha 4$ and $\beta 2$ subunits contribute to aspects of nicotine tolerance and withdrawal. The heterozygotes show a gene dose dependent decrease in expression of the $\alpha 4\beta 2^{*}$ -nAChR subtype (Moretti et al., 2018) and measurable decrease in sensitivity to the acute administration of nicotine. These heterozygotes may provide a model with which to explore the influence of differences in gene expression and initial sensitivity to acute nicotine on the development of tolerance to chronically administered nicotine. The fact that C57BL/6 is the background strain for all of the mice tested allows the focus on the differential receptor expression. The $\alpha 4^{+/-}$, $\beta 2^{+/-}$, and $\alpha 4^{+/-}/\beta 2^{+/-}$ mice all developed tolerance measured with Y-Maze Crossings and Body Temperature following chronic nicotine treatment, but the tolerance was less than that exhibited by wild-type mice. No significant tolerance was observed for Y-Maze Crossings or Open Field Distance. These results are consistent with our previous studies that reported that inbred mouse strains that were initially less sensitive to the acute effects of nicotine required higher treatment doses to elicit tolerance (Marks et al., 1986a, Marks et al., 1991). However, those studies also indicated that the increases in [3 H]-nicotine binding [a measure of $\alpha 4\beta 2^{*}$ -nAChR sites (Picciotto et al., 1995, Marubio et al., 1999, Ross et al., 2000)] elicited by chronic nicotine treatment were similar in the inbred strains tested.

While the results presented in the current study establish that expression of the $\alpha 4$ and $\beta 2$ nAChR subunits is critical for both the acute and chronic effects of nicotine on locomotor behavior and body temperature, other nAChR subunits have been demonstrated to be

important for different behaviors. For example, somatic signs of nicotine withdrawal require the express of $\beta 4^*$ -nAChR rather than $\beta 2^*$ -nAChR (Salas et al., 2004b) and withdrawal signs are also mediated by $\alpha 2$ and $\alpha 5$ nAChR subunits expressed in the medial habenula (Salas et al., 2009). Furthermore, nicotine-induced seizures are mediated primarily by $\alpha 3\beta 4^*$ -nAChR (Salas et al., 2004a). The $\alpha 5$ nAChR subunit expressed in the medial habenula also is important in regulating nicotine self-administration (Fowler et al., 2011). $\alpha 5$ is an auxiliary subunit that can be a component of $\beta 2^*$ -nAChR and $\beta 4^*$ -nAChR. Since deletion of either the $\alpha 4$ or $\beta 2$ nAChR genes virtually eliminates the expression of the $\alpha 5$ subunit in mouse brain (Moretti et al., 2018), it seems likely that most receptors in whole mouse brain that include this subunit are the $\alpha 4\beta 2\alpha 5$ -nAChR subtype.

Changes in the density of binding sites and the extent of tolerance development do not show the same pattern of response to chronic nicotine treatment; even though expression of the $\alpha 4$ and $\beta 2$ nAChR subunits are essential for aspects of tolerance development and for expression of the major nicotinic receptor subtype in the brain as previously reported for inbred strains (Marks et al., 1986b, Marks et al., 1991) and for mice differing in expression of the $\beta 2$ subunit (McCallum et al., 2006). Chronic nicotine treatment elicited increases in $\alpha 4\beta 2^*$ -nAChR expression (measured with ligand binding) at lower doses than the doses that elicit measurable tolerance development. A similar discordance occurs between tolerance development reported here and $\alpha 4\beta 2^*$ -nAChR expression measured both autoradiographically and immunochemically for a subset of mice differing in expression of the $\alpha 4$ and $\beta 2$ nAChR subunits that were evaluated in the current study (Moretti et al., 2018). In that study significant increases in receptor expression were noted following treatment with 0.25 mg/kg/hr nicotine and were near maximal following treatment with 1.0 mg/kg/hr nicotine. The current study reports that no significant tolerance or modest tolerance was observed following treatment with chronic 0.25 or 1.0 mg/kg/hr nicotine doses, respectively.

It is not established why a clear relationship between tolerance development and $\alpha 4\beta 2^*$ -nAChR expression was not observed. The pharmacology of nicotine is complex and elicits diverse biochemical and physiological responses that are affected by chronic nicotine exposure that mediate behavioral responses indicative of tolerance and/or withdrawal (De Biasi and Dani, 2011, Jackson et al., 2015). Furthermore, the receptors may not be fully functional (Marks et al., 1993, Marks et al., 2004). Another contributing factor to this discordance may reflect the role of $\beta 4^*$ -nAChR in influencing nicotine effects on locomotion and body temperature. In a study of the responses of mice differing in expression of the $\beta 4$ subunit following chronic nicotine treatment, a much closer correspondence between $\alpha 4\beta 2^*$ -nAChR binding sites and tolerance development was observed for $\beta 4^-$ mice than for $\beta 4^{++}$ mice (Meyers et al., 2015). This result suggests that responses mediated by $\beta 4^*$ -nAChR contribute to the dissociation between tolerance development and receptor changes. Indeed, when the responses observed for $\beta 2^-$ mice and $\beta 4^-$ mice were averaged the estimated tolerance development was very similar to the tolerance development observed for wild-type mice (Meyers et al., 2015).

Conclusions

Deletion or partial deletion of either the $\alpha 4$ and/or $\beta 2$ nAChR subunits in mice reduced the sensitivity of the mice to the acute effects of nicotine on locomotion and body temperature. Furthermore, partial deletion of either the $\alpha 4$ and/or $\beta 2$ nAChR subunits decreased the extent of tolerance development following chronic nicotine administration. Deletion of either subunit virtually eliminated tolerance development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Dose-response curves for acute nicotine effects on saline infused mice differing in $\alpha 4$ genotype.

$\alpha 4^{+/+}$, $\alpha 4^{+/-}$, $\alpha 4^{-/-}$ and $\alpha 4^{+/}\beta 2^{+/+}$ mice that had been chronically treated with saline were injected with either saline, 0.5 mg/kg, 1.0 mg/kg or 1.5 mg/kg nicotine. Y-maze crossings, Y-maze rearings, open field activity and body temperature were measured as described in the methods. Each point represents the mean $\pm$ SEM for the following numbers of mice: $\alpha 4^{+/+}$ 11–15, $\alpha 4^{+/-}$ 7–10, $\alpha 4^{-/-}$ 8–10 and $\alpha 4^{+/}\beta 2^{+/+}$ 12–13. The spline curves were determined by non-linear least squares fits of the data as described in the methods. ED_{50} values obtained from these curve fits are displayed graphically in Figure 3.

Figure 2. Dose-response curves for acute nicotine effects on saline infused mice differing in $\beta 2$ genotype.

$\beta 2^{++}$, $\beta 2^{+-}$, $\beta 2^{-/-}$ and $\alpha 4^{+/-}/\beta 2^{+/-}$ mice that had been chronically treated with saline were injected with either saline, 0.5 mg/kg, 1.0 mg/kg or 1.5 mg/kg nicotine. Y-maze crossings, Y-maze rearings, open field activity and body temperature were measured as described in the methods. Each point represents the mean $\pm$ SEM for the following numbers of mice: $\beta 2^{++}$ 10–12, $\beta 2^{+-}$ 9–11, $\beta 2^{-/-}$ 8–10 and $\alpha 4^{+/-}/\beta 2^{+/-}$ 9–11 (the $\alpha 4^{+/-}/\beta 2^{+/-}$ mice are the same as those shown in Figure 1). The spline curves were determined by non-linear least squares fits

of the data as described in the methods. ED_{50} values obtained from these curve fits are displayed graphically in Figure 3.

Figure 3. Comparison of ED₅₀ values for mice differing in $\alpha 4$ and $\beta 2$ genotype.
The ED₅₀ values (mean $\pm$ SEM) determined by non-linear fitting of the curves shown in Figures 1 and 2 were calculated as described in the methods.

Figure 4. Relationship between initial expression of $\alpha 4$ and $\beta 2$ nAChR subunits and potency ratios following chronic treatment with 4.0 mg/kg/hr nicotine. Potency ratios calculated for Y-Maze Crossing, Y-Maze Rearings, open field activity and body temperature following chronic treatment with 4.0 mg/kg/hr nicotine are graphed as a function of the expression of the $\alpha 4$ and $\beta 2$ nAChR subunits measured by immunoprecipitation of [3 H]-epibatidine binding sites in saline-treated mice by antibodies raised against the $\alpha 4$ and $\beta 2$ nAChR subunits and published previously (Moretti et al, 2018).

The regression lines are solid, the 95% confidence intervals are dashed. Correlation coefficients are included in each panel.

Table 1.

AVOVA results for evaluation of the effects of genotype and nicotine dose on responses of mice differing in nAChR genotype to acute nicotine administration.

Test	Main effect of genotype	Main effect of dose	Geno x dose interaction
Y-Maze Crossings	$F_{6,265} = 10.45$, $P < 0.001$	$F_{3,265} = 160.41$, $P < 0.001$	$F_{18,265} = 2.67$, $P < 0.001$
Y-Maze Rearings	$F_{6,265} = 3.38$, $P = 0.003$	$F_{3,265} = 161.21$, $P < 0.001$	$F_{18,265} = 1.44$, NS
Open Field Distance	$F_{6,264} = 2.84$, $P = 0.011$	$F_{3,264} = 153.87$, $P < 0.001$	$F_{18,264} = 1.01$, NS
Body Temperature	$F_{6,266} = 27.44$, $P < 0.001$	$F_{3,266} = 220.46$, $P < 0.001$	$F_{18,266} = 4.38$, $P < 0.001$

Three-way ANOVAs for the effect of acute nicotine challenge dose, chronic nicotine treatment dose and mouse genotype on Y-Maze Crossings, Y-Maze Rearings, Open Field distance travelled and body temperature.

Table 2.

Test	Main Effect Genotype	Main Effect Chronic	Main Effect Acute	Geno x chronic	Geno x Acute	Chronic x Acute	Geno x Chronic X Acute
Y-maze Crossings	$F_{6,854}=21.10$ $P<0.001$	$F_{3,855}=45.27$ $P<0.001$	$F_{3,854}=367.28$ $P<0.001$	$F_{18,854}=1.51$ NS	$F_{18,855}=4.82$ $P<0.001$	$F_{9,854}=11.98$ $P<0.001$	$F_{54,854}=0.74$ NS
Y-Maze Rearings	$F_{6,854}=6.02$ $P<0.001$	$F_{3,854}=18.93$ $P<0.001$	$F_{3,854}=409.31$ $P<0.001$	$F_{18,854}=1.78$ $P=0.023$	$F_{18,854}=2.83$ $P<0.001$	$F_{9,854}=8.54$ $P<0.001$	$F_{54,854}=1.00$ NS
Open Field Distance	$F_{6,847}=6.16$ $P<0.001$	$F_{3,847}=13.45$ $P<0.001$	$F_{3,847}=532.73$ $P<0.001$	$F_{18,847}=0.85$ NS	$F_{18,847}=1.65$ $P=0.044$	$F_{9,847}=5.57$ $P<0.001$	$F_{54,847}=0.67$ NS
Body Temperature	$F_{6,855}=36.34$ $P<0.001$	$F_{3,855}=56.91$ $P<0.001$	$F_{3,855}=689.93$ $P<0.001$	$F_{18,855}=4.79$ $P<0.001$	$F_{18,855}=6.86$ $P<0.001$	$F_{9,855}=8.55$ $P<0.001$	$F_{54,855}=1.45$ $P=0.021$

Table 3.

Two-way ANOVAs for the effect of acute nicotine challenge dose and chronic nicotine treatment dose on Y-Maze Crossings, Y-Maze Rearings, Open Field Distance and Body Temperature.

Genotype	Test	Chronic Nicotine	Acute Nicotine	Chronic x Acute
$\alpha 4^{++}$	Y-Maze Crossings	$F_{3,122} = 12.93, P < 0.001$	$F_{3,122} = 47.37, P < 0.001$	$F_{9,122} = 3.71, P < 0.001$
	Y-Maze Rearings	$F_{3,122} = 12.39, P < 0.001$	$F_{3,122} = 54.73, P < 0.001$	$F_{9,122} = 6.08, P < 0.001$
	Open Field Distance	$F_{3,117} = 5.38, P < 0.001$	$F_{3,117} = 71.76, P < 0.001$	$F_{9,117} = 2.82, P = 0.005$
	Body Temperature	$F_{3,122} = 22.23, P < 0.001$	$F_{3,122} = 77.74, P < 0.001$	$F_{9,122} = 6.78, P < 0.001$
$\beta 2^{++}$	Y-Maze Crossings	$F_{3,123} = 15.28, P < 0.001$	$F_{3,123} = 87.21, P < 0.001$	$F_{9,123} = 7.27, P < 0.001$
	Y-Maze Rearings	$F_{3,123} = 8.23, P < 0.001$	$F_{3,123} = 93.18, P < 0.001$	$F_{9,123} = 4.65, P < 0.001$
	Open Field Distance	$F_{3,123} = 7.65, P < 0.001$	$F_{3,123} = 128.28, P < 0.001$	$F_{9,123} = 4.70, P < 0.001$
	Body Temperature	$F_{3,123} = 34.86, P < 0.001$	$F_{3,123} = 197.00, P < 0.001$	$F_{9,123} = 6.00, P < 0.001$
$\alpha 4^{+-}$	Y-Maze Crossings	$F_{3,150} = 9.96, P < 0.001$	$F_{3,150} = 66.60, P < 0.001$	$F_{9,150} = 2.13, P = 0.03$
	Y-Maze Rearings	$F_{3,150} = 2.65, NS$	$F_{3,150} = 76.20, P < 0.001$	$F_{9,150} = 1.42, NS$
	Open Field Distance	$F_{3,150} = 1.05, NS$	$F_{3,150} = 82.48, P < 0.001$	$F_{9,150} = 0.77, NS$
	Body Temperature	$F_{3,150} = 8.20, P < 0.001$	$F_{3,150} = 122.89, P < 0.001$	$F_{9,150} = 2.49, P = 0.01$
$\beta 2^{+-}$	Y-Maze Crossings	$F_{3,110} = 4.16, P = 0.008$	$F_{3,110} = 57.94, P < 0.001$	$F_{9,110} = 1.71, NS$
	Y-Maze Rearings	$F_{3,110} = 1.12, NS$	$F_{3,110} = 46.06, P < 0.001$	$F_{9,110} = 0.86, NS$
	Open Field Distance	$F_{3,108} = 1.91, NS$	$F_{3,108} = 58.89, P < 0.001$	$F_{9,108} = 0.52, NS$
	Body Temperature	$F_{3,110} = 10.25, P < 0.001$	$F_{3,110} = 145.70, P < 0.001$	$F_{9,110} = 2.83, P = 0.005$
$\alpha 4^{+-}/\beta 2^{+-}$	Y-Maze Crossings	$F_{3,123} = 3.26, P = 0.024$	$F_{3,123} = 48.19, P < 0.001$	$F_{9,123} = 1.11, NS$
	Y-Maze Rearings	$F_{3,123} = 3.54, P = 0.017$	$F_{3,123} = 46.85, P < 0.001$	$F_{9,123} = 1.52, NS$
	Open Field Distance	$F_{3,123} = 1.39, NS$	$F_{3,123} = 78.15, P < 0.001$	$F_{9,123} = 0.64, NS$
	Body Temperature	$F_{3,123} = 8.51, P < 0.001$	$F_{3,123} = 53.62, P < 0.001$	$F_{9,123} = 1.63, NS$
$\alpha 4^{--}$	Y-Maze Crossings	$F_{3,88} = 7.14, P < 0.001$	$F_{3,88} = 22.75, P < 0.001$	$F_{9,88} = 1.06, NS$
	Y-Maze Rearings	$F_{3,88} = 2.13, NS$	$F_{3,88} = 46.69, P < 0.001$	$F_{9,88} = 0.45, NS$
	Open Field Distance	$F_{3,88} = 1.67, NS$	$F_{3,88} = 46.77, P < 0.001$	$F_{9,88} = 0.68, NS$
	Body Temperature	$F_{3,88} = 2.45, NS$	$F_{3,88} = 28.31, P < 0.001$	$F_{9,88} = 1.18, NS$
$\beta 2^{--}$	Y-Maze Crossings	$F_{3,138} = 1.27, NS$	$F_{3,138} = 122.78, P < 0.001$	$F_{9,138} = 1.03, NS$
	Y-Maze Rearings	$F_{3,138} = 0.20, NS$	$F_{3,138} = 102.56, P < 0.001$	$F_{9,138} = 0.17, NS$
	Open Field Distance	$F_{3,138} = 1.24, NS$	$F_{3,138} = 133.80, P < 0.001$	$F_{9,138} = 0.63, NS$
	Body Temperature	$F_{3,138} = 1.94, NS$	$F_{3,138} = 261.30, P < 0.001$	$F_{9,138} = 1.07, NS$

Genotype	Test	Chronic Nicotine	Acute Nicotine	Chronic x Acute

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Table 4.

ED₅₀ values and potency ratios for four tests for mice differing in $\alpha 4$ and/or $\beta 2$ nAChR expression.

Genotype	Chronic Nicotine Dose (mg/kg/hr)	ED ₅₀ Y-Maze Crossings (mg/kg)	ED ₅₀ Y-Maze Rearings (mg/kg)	ED ₅₀ Open Field Distance (mg/kg)	ED ₅₀ Body Temperature (mg/kg)	Potency Ratio Y-Maze Crossings	Potency Ratio Y-Maze Rearings	Potency Ratio Open Field Distance	Potency Ratio Body Temperature
$\alpha 4^{++}$	0.0	0.41±0.04	0.39±0.04	0.44±0.04	0.61±0.05	1.00±0.13	1.00±0.14	1.00±0.13	1.00±0.12
	0.25	0.39±0.05	0.35±0.03	0.44±0.05	0.61±0.07	0.95±0.10	0.90±0.12	1.00±0.14	1.00±0.09
	1.0	0.63±0.06	0.77±0.15	0.66±0.07	0.84±0.09	1.53±0.15 [*]	1.97±0.43	1.50±0.21	1.38±0.13 [*]
	4.0	0.97±0.10	0.98±0.10	0.87±0.08	1.58±0.09	2.37±0.23 [*]	2.51±0.36 [*]	1.98±0.26 [*]	2.59±0.22 [*]
$\beta 2^{++}$	0.0	0.39±0.03	0.40±0.03	0.42±0.03	0.45±0.03	1.00±0.11	1.00±0.11	1.00±0.10	1.00±0.09
	0.25	0.28±0.04	0.31±0.08	0.37±0.03	0.45±0.03	0.72±0.06	0.78±0.21	0.88±0.10	1.00±0.07
	1.0	0.61±0.09	0.59±0.06	0.60±0.08	0.77±0.06	1.56±0.12 [*]	1.48±0.19	1.43±0.21	1.71±0.12 [*]
	4.0	0.85±0.07	0.83±0.07	0.78±0.07	1.15±0.05	2.18±0.17 [*]	2.07±0.23 [*]	1.86±0.21 [*]	2.56±0.18 [*]
$\alpha 4^{+-}$	0.0	0.55±0.04	0.51±0.04	0.66±0.06	0.81±0.05	1.00±0.10	1.00±0.08	1.00±0.13	1.00±0.09
	0.25	0.63±0.05	0.70±0.11	0.64±0.06	0.75±0.05	1.15±0.09	1.37±0.24	0.97±0.13	0.93±0.08
	1.0	0.78±0.10	0.71±0.10	0.72±0.08	0.96±0.06	1.42±0.12 [*]	1.39±0.22	1.09±0.16	1.19±0.10
	4.0	0.94±0.08	0.83±0.09	0.84±0.10	1.34±0.09	1.79±0.13 [*]	1.63±0.22	1.27±0.19	1.65±0.13 [*]
$\beta 2^{+-}$	0.0	0.44±0.04	0.43±0.04	0.56±0.06	0.54±0.04	1.00±0.13	1.00±0.13	1.00±0.15	1.00±0.10
	0.25	0.55±0.05	0.54±0.07	0.51±0.05	0.62±0.04	1.25±0.11	1.25±0.20	0.91±0.13	1.20±0.09
	1.0	0.63±0.05	0.62±0.07	0.60±0.06	0.73±0.06	1.43±0.13 [*]	1.42±0.21	1.07±0.16	1.35±0.11 [*]
	4.0	0.84±0.11	0.68±0.10	0.77±0.10	1.00±0.07	1.89±0.18 [*]	1.58±0.28	1.38±0.23	1.85±0.14 [*]
$\alpha 4^{+-}/\beta 2^{+-}$	0.0	0.53±0.04	0.52±0.04	0.55±0.04	0.92±0.07	1.00±0.11	1.00±0.11	1.00±0.10	1.00±0.11
	0.25	0.52±0.04	0.49±0.04	0.57±0.04	0.77±0.08	0.98±0.11	0.94±0.11	1.04±0.10	0.84±0.10
	1.0	0.71±0.12	0.49±0.05	0.58±0.06	0.91±0.10	1.34±0.12	0.94±0.12	1.05±0.13	0.99±0.12
	4.0	0.92±0.10	0.87±0.10	0.83±0.08	1.58±0.13	1.74±0.14 [*]	1.67±0.23 [*]	1.51±0.18 [*]	1.72±0.18 [*]
$\alpha 4^{-}$	0.0	1.26±0.09	0.85±0.06	0.84±0.07	1.87±0.15	1.00±0.10	1.00±0.10	1.00±0.12	1.00±0.11
	0.25	1.27±0.12	0.97±0.14	0.64±0.08	1.49±0.09	1.01±0.17	1.14±0.18	0.76±0.11	0.80±0.18
	1.0	1.20±0.14	0.70±0.09	0.76±0.09	1.46±0.10	0.95±0.19	0.82±0.11	0.90±0.13	0.78±0.20

Genotype	Chronic Nicotine Dose (mg/kg/hr)	ED ₅₀ Y-Maze Crossings (mg/kg)	ED ₅₀ Y-Maze Rearings (mg/kg)	ED ₅₀ Open Field Distance (mg/kg)	ED ₅₀ Body Temperature (mg/kg)	Potency Ratio Y-Maze Crossings	Potency Ratio Y-Maze Rearings	Potency Ratio Open Field Distance	Potency Ratio Body Temperature
	4.0	1.38±0.11	0.92±0.09	0.97±0.12	2.05±0.21	1.09±0.16	1.08±0.13	1.15±0.17	1.10±0.40
β ²⁻⁻⁻	0.0	0.53±0.03	0.47±0.03	0.60±0.05	0.87±0.05	1.00±0.08	1.00±0.09	1.00±0.12	1.00±0.08
	0.25	0.56±0.04	0.47±0.05	0.46±0.05	0.68±0.05	1.06±0.07	1.00±0.12	0.77±0.11	0.78±0.06
	1.0	0.51±0.04	0.49±0.04	0.45±0.03	0.73±0.03	0.96±0.06	1.04±0.11	0.75±0.08	0.84±0.05
	4.0	0.69±0.05	0.51±0.03	0.55±0.03	0.81±0.05	1.30±0.08 [*]	1.09±0.09	0.92±0.09	0.93±0.07

Values for ED₅₀ represent mean ± SEM calculated by non-linear curve fits of the data to modified Hill equations as described in the Methods. Values for potency ratios of ED₅₀ values of mice treated chronically with the indicated nicotine doses represent mean ± SEM. Potency ratios were calculated as the ratio of ED₅₀ for each chronic nicotine dose relative to control (saline-treated) ED₅₀ value for each genotype. SEM for the potency ratios were calculated using Taylor's expansion. Potency ratios marked with

^{*} are significantly greater than 1.0. t-tests using the Holm-Bonferroni correction were used to evaluate statistical significance.