



Published in final edited form as:

*Pharmacol Biochem Behav.* 2015 March ; 130: 1–8. doi:10.1016/j.pbb.2014.12.013.

## Differential expression of the beta4 neuronal nicotinic receptor subunit affects tolerance development and nicotinic binding sites following chronic nicotine treatment

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### Abstract

The role of neuronal nicotinic acetylcholine receptors (nAChR) containing the  $\beta 4$  subunit in tolerance development and nicotinic binding site levels following chronic nicotine treatment was investigated. Mice differing in expression of the  $\beta 4$  nAChR subunit [wild-type ( $\beta 4^{++}$ ), heterozygote ( $\beta 4^{+-}$ ) and null mutant ( $\beta 4^{-/-}$ )] were chronically treated for 10 days with nicotine (0, 0.5, 1.0, 2.0 or 4.0 mg/kg/hr) by constant intravenous infusion. Chronic nicotine treatment elicited dose-dependent tolerance development.  $\beta 4^{-/-}$  mice developed significantly more tolerance than either  $\beta 4^{++}$  or  $\beta 4^{+-}$  mice which was most evident following treatment with 4.0 mg/kg/hr nicotine. Subsets of [<sup>125</sup>I]-epibatidine binding were measured in several brain regions. Deletion of the  $\beta 4$  subunit had little effect on initial levels of cytosine-sensitive [<sup>125</sup>I]-epibatidine binding (primarily  $\alpha 4\beta 2$ -nAChR sites) or their response (generally increased binding) to chronic nicotine treatment. In contrast,  $\beta 4$  gene-dose-dependent decreases in expression 5IA-85380 resistant [<sup>125</sup>I]-epibatidine binding sites (primarily  $\beta 4^*$ -nAChR) were observed. While these  $\beta 4^*$ -nAChR sites were generally resistant to regulation by chronic nicotine treatment, significant increases in binding were noted for habenula and hindbrain. Comparison of previously published tolerance development in  $\beta 2^{-/-}$  mice (less tolerance) to that of  $\beta 4^{-/-}$  mice (more tolerance) supports a differential role for these receptor subtypes in regulating tolerance following chronic nicotine treatment.

### Keywords

nicotinic acetylcholine receptor; beta4 null mutant mice; chronic nicotine treatment; epibatidine binding; nicotine tolerance; 5I-85380; cytosine

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Several lines of evidence indicate that  $\beta 4^*$ -nAChR [nicotinic acetylcholine receptor, \* indicates the presence of additional subunits (Lukas et al., 1999)] are important mediators of responses to both acute and chronic nicotine administration. For example, deletion of the  $\beta 4$  nAChR subunit virtually eliminated somatic signs of nicotine withdrawal in mice that had been chronically treated with nicotine (Salas et al., 2004b). In addition,  $\beta 4$  null mutant mice ( $\beta 4^{-/-}$ ) have greatly reduced seizures following acute administration of nicotine (Salas et al., 2004a). The  $\beta 4$  subunit is densely expressed in the medial habenula (Duvoisin et al., 1989), a brain region that has been demonstrated to be an important modulator of nicotinic responses ((Fowler and Kenny, 2012; Fowler et al., 2011)). The gene encoding the  $\beta 4$  subunit is part of a gene cluster that also includes the genes encoding the  $\alpha 3$  and  $\alpha 5$  subunits (Boulter et al., 1990), and both of these genes also are important regulators of nicotine-induced seizures (Salas et al., 2004a; Salas et al., 2003). Furthermore, the genome wide association studies have repeatedly demonstrated that variants in the  $\alpha 3\alpha 5\beta 4$  gene cluster affect many phenotypes related to nicotine use in humans (Stevens et al., 2008). Although most of the attention has focused on polymorphisms in the  $\alpha 5$  gene, polymorphisms in the  $\beta 4$  gene also influence tobacco-related phenotypes (Kapoor et al., 2012).

We previously examined the effect of deletion of the  $\beta 2$  gene on tolerance development and nAChR binding sites following chronic nicotine treatment (McCallum et al., 2006). This study confirmed the deletion of the  $\beta 2$  subunit eliminated cytosine-sensitive epibatidine binding sites, which are the sites most responsive to chronic nicotine treatment. Furthermore, little tolerance, and even evidence for increased sensitivity, to the acute effects of nicotine were noted for  $\beta 2^{-/-}$  mice following chronic nicotine treatment. This result suggests that tolerance may not develop to the responses mediated by residual, non $\beta 2^*$ -nAChR following chronic nicotine treatment. In order to investigate the role of  $\beta 4^*$ -nAChR in mediating response to chronic nicotine treatment, mice differing in expression of this subunit [ $\beta 4^{+/+}$  (wild-type),  $\beta 4^{+/-}$  (heterozygote) and  $\beta 4^{-/-}$  (null mutant)] were chronically treated with one of several doses of nicotine. Response to the administration of acute nicotine on locomotion and body temperature was measured. In addition, several subsets of high-affinity [ $^{125}$ I]-epibatidine binding were measured. The results indicate that  $\beta 4^{-/-}$  mice develop tolerance more readily than do wild-type mice and that deletion of the  $\beta 4$  subunit has little effect on the regulation of the expression of cytosine-sensitive [ $^{125}$ I]-epibatidine binding sites (primarily  $\alpha 4\beta 2$ -nAChR sites).

## 2. Materials and Methods

### 2.1. Materials

[ $^{125}$ I]-Epibatidine was obtained from Perkin-Elmer NEN, Boston, MA. NaCl, KCl, MgSO<sub>4</sub>, CaCl<sub>2</sub>, Na<sub>2</sub>HPO<sub>4</sub>, NaH<sub>2</sub>PO<sub>4</sub>, bovine serum albumin, polyethyleneglycol, polyethylenimine, nicotine, and cytosine were obtained from Sigma Chemical Co., St. Louis, MO. 5I-A-85380 was purchased from Tocris Bioscience, Bristol, UK. Ketamine, xylazine, acepromazine and buprenorphine were obtained from MWI Veterinary Supply, Nampa, ID. Sucrose was obtained from Roche Diagnostics, Indianapolis, IN. HEPES and NaHEPES (products of BDH) and silastic tubing (a product of Dow Chemical) were obtained through VWR International, Denver, CO. Glass filters Type B were products of MicroFiltration Systems,

Dublin, CA and glass fiber filters Type A/E were products of Pall Life Sciences, Port Washington, NY. Nylon mesh and 22 gauge stainless steel tubing were obtained from AmazonSupply.com.

## 2.2. Mice

Animal production methods and experimental procedures using mice were reviewed and approved by the Animal Care and Utilization Committee at the University of Colorado, Boulder.

$\beta 4$  null mutant mice (Xu et al., 1999) were originally obtained from Richard Paylor, Baylor College of Medicine, Houston, TX. Mice were bred in the Specific Pathogen Free Mouse Colony at the Institute for Behavioral Genetics, University of Colorado, Boulder. Heterozygous mice were mated to yield wild type (+/+), heterozygous (+/-) and homozygous (-/-) mutant mice. Mice were weaned at 25 days of age. Genotypes were determined from tail clippings as described previously (Salminen et al., 2004). Mice were housed in a vivarium maintained at 22°C and allowed free access to food and water. Lights were on from 7 AM to 7 PM.

## 2.3. Surgery and nicotine treatment

**2.3.1. Surgery**—Chronic nicotine treatment was performed by intravenous infusion through a cannula inserted in the right jugular vein of each mouse to assure careful control of dosing. A cannula constructed of silastic tubing was inserted in the right jugular vein of each mouse (Barr et al., 1979) and used successfully to treat mice with nicotine (Marks et al., 1983; McCallum et al., 2006). 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. 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.

**2.3.2. Nicotine treatment**—Following recovery from surgery each mouse was transferred to an individual infusion chamber (15 cm × 15 cm × 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 $\mu$ L/hr. After two days of saline infusion, nicotine treatment was begun. Mice were divided into five treatment groups that received the following nicotine doses: 0 mg/kg/hr (saline-infused control), 0.5 mg/kg/hr, 1.0 mg/kg/hr, 2.0 mg/kg/hr or 4.0 mg/kg/hr. All doses are free base. Nicotine solutions were prepared from liquid nicotine neutralized with HCl. 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. The total number of mice of each genotype and the number of mice treated with saline, 0.5

mg/kg/hr, 1.0 mg/kg/hr, 2.0 mg/kg/hr and 4.0 mg/kg/hr nicotine were:  $\beta 4^{++}$ : 84 mice total, 16, 15, 16, 19 and 18 in the five treatment groups, respectively;  $\beta 4^{+-}$ : 86 mice total, 16, 19, 18, 18 and 15 in the five treatment groups, respectively; and  $\beta 4^{--}$ : 87 mice total, 15, 16, 17, 16 and 23 in the five treatment groups, respectively. Few mice died during the treatments and there was no significant difference in mortality among the mice differing in  $\beta 4$  expression.

**2.3.3. Tolerance testing**—Behavioral test measures were selected based on previous studies that have demonstrated dose-dependent responses to nicotine that can be altered by altering nAChR expression (Marks et al., 1985; O'Neill et al., 2013; Tritto et al., 2004). Chronic nicotine treatment had been discontinued for at least two hr 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 (McCallum et al., 2006; Tritto et al., 2004). Mice were placed in a Y-maze apparatus 3 minutes after nicotine injection and both crosses and rears measured for 3 minutes. Immediately following Y-maze assessment, mice were placed in a bright light Med Associates circular open field (58 cm diameter) and locomotor activity was monitored for 5 minutes. Following completion of the open field test, mice were singly housed until temperature was measured 15 minutes post injection. Two hr after the initial test mice were injected with a second dose of nicotine and tested a second time. Preliminary experiments indicated little differences in test results provided the initial test was conducted with a lower nicotine dose. 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, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0.

#### 2.4. Assessment of nAChR binding sites with [ $^{125}$ I]-epibatidine binding

**2.4.1. Sample preparation**—Two hr following the second tolerance test each mouse was euthanized by cervical dislocation. The brain was removed and placed on an ice cold surface and the following brain regions were dissected: olfactory bulb, cerebral cortex, thalamus, habenula, superior colliculus, inferior colliculus and hindbrain (pons and medulla not including the interpeduncular nucleus). Previous studies indicated that these regions differ in expression of nAChR subtypes. Each brain region was placed in a polypropylene test tube containing hypotonic salt solution (NaCl = 14.4 mM; KCl = 0.15 mM; CaCl<sub>2</sub> = 0.2 mM, MgSO<sub>4</sub> = 0.1 mM, HEPES = 2.5 mM, pH = 7.5) and homogenized using a Teflon pestle fitted to the test tube. The resulting homogenate was centrifuged at 20,000 × g for 15 min. The supernatant was discarded, the pellet suspended in hypotonic buffer and centrifuged again. This process was repeated twice more. Fresh hypotonic buffer was added to each tube and the samples were stored frozen until assay.

On the assay day, the samples were thawed, the pellets suspended in the overlying buffer and the samples were centrifuged at 20,000 × g for 15 min. The resulting pellet was suspended in water for use in the binding assay.

**2.4.2. [<sup>125</sup>I]-Epibatidine binding**—The binding was measured essentially as described previously (Whiteaker et al., 2000a). The binding assay was conducted in 96 well polyethylene microtiter plates in a final volume of 30  $\mu$ L of isotonic buffer (NaCl = 144 mM, KCl = 1.5 mM, CaCl<sub>2</sub> = 2 mM, MgSO<sub>4</sub> = 1 mM, HEPES = 25 mM, pH = 7.5) containing 200 pM [<sup>125</sup>I]-epibatidine. In order to determine the cytosine-resistant sites, some samples included either 50 mM or 150 nM cytosine (Marks et al., 1998). In order to determine 5I-A85380-resistant sites other samples included either 10 nM or 50 nM 5I-A85380 (Whiteaker et al., 2000b). Blanks were established by including 10  $\mu$ M nicotine in the incubation. Protein was adjusted to assure that less than 10% of the [<sup>125</sup>I]-epibatidine was bound. Following an incubation of 2 hr at 22°C, samples were filtered at 4°C using an Inotech Cell Harvester on holding two glass fiber filters that had been soaked in isotonic binding buffer containing 0.5% polyethylenimine (top filter MFS type B, bottom filter Pell type A/E). Filters were washed 4 times with ice-cold isotonic binding buffer. Filters were transferred to 96 well sample plates and 150  $\mu$ L of Optiphase Supermix Scintillation Cocktail was added to each well. Samples were counted at 55% efficiency using a Perkin-Elmer Trilux Wallac 1500 Trilux scintillation counter.

**2.4.3. Protein**—Protein was measured using the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard.

## 2.5. Data analysis

**2.5.1. Analysis of tolerance data**—Results of the tolerance testing were analyzed several ways. Each test was initially analyzed using a three-way analysis of variance using IBM SPSS version 21 with the responses as the dependent variable and genotype, chronic treatment dose and acute challenge dose as the independent variables. In addition, the dose-response curves for each test for mice of each  $\beta$ 4 genotype were fit to the following equations 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.

And for body temperature:

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

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 (approximately 31°C).

Initial curve fits for each genotype and each chronic treatment dose were constructed allowing all variables to float. No significant difference in  $NH$  was observed among the

groups for each test, so subsequent calculations were made with a fixed value for NH: Y-maze crosses, NH = 2.66; Y-maze rears, NH = 2.14; Open-field distance, NH = 2.66; Body temperature, NH = 2.17.

**2.5.2. Analysis of [<sup>125</sup>I]-epibatidine binding data—**[<sup>125</sup>I]-Epibatidine binding to subsets of sites determined by differential inhibition by cytosine or 5I-A85380 was calculated using the following equation:

$$EPI_{\text{Bound}} = EPI_{\text{Sen}} / (1 + \text{INH} / IC_{50-1}) + EPI_{\text{Res}} / (1 + \text{INH} / IC_{50-2}),$$

Where  $EPI_{\text{Bound}}$  is the measured binding of [<sup>125</sup>I]-epibatidine at each concentration of either cytosine or 5I-A85380 (INH),  $EPI_{\text{Sen}}$  and  $EPI_{\text{Res}}$  are the densities of [<sup>125</sup>I]-epibatidine binding sites that are more and less sensitive to inhibition with effective inhibition constants of  $IC_{50-1}$  and  $IC_{50-2}$ , respectively. Inhibition constants were calculated in preliminary experiments using a wide range of inhibitor concentrations. Under the conditions of the experiments described in the current study  $IC_{50-1}$  and  $IC_{50-2}$  were 3 nM and 300 nM for cytosine and 0.3 nM and 300 nM for 5I-A85380. Two-way ANOVA with IBM SPSS version 21 was subsequently used to analyze the [<sup>125</sup>I]-epibatidine binding data with genotype and chronic treatment dose as the independent variables.

### 3. Results

#### 3.1. Tolerance measurements

Mice differing in the expression of the  $\beta 4$  nAChR subunit were chronically treated for 10 days with 0, 0.5, 1.0, 2.0 or 4.0 mg/kg/hr nicotine by intravenous infusion. Mice were subsequently tested for tolerance to the acute effects of nicotine to evaluate the effects of nicotine treatment and the influence of differences in expression of the  $\beta 4$  subunit. Appendices A-D (results for Y-maze crosses, Y-maze rears, open field activity and body temperature, respectively) provide dose-response curves for responses of  $\beta 4^{++}$ ,  $\beta 4^{+-}$ , and  $\beta 4^{-}$  mice to acute nicotine administration following chronic nicotine treatment following chronic treatment with each nicotine dose. Results in Figure 1 provide an abbreviated illustration of these data.

**3.1.1. Effect of  $\beta 4$  genotype on tolerance development following chronic nicotine treatment: Representative tolerance test results—**The results presented in Figure 1 for Y-maze crosses and body temperature illustrate the responses of  $\beta 4^{++}$  (wild-type),  $\beta 4^{+-}$  (heterozygote) and  $\beta 4^{-}$  (null mutant) mice to acute injections of nicotine following chronic treatment with saline (control), 1.0 or 4.0 mg/kg/hr nicotine.

**3.1.1. a. Y-Maze crosses:** Acute nicotine injection elicited a dose-dependent decrease in locomotor activity in the Y-maze. The responses of  $\beta 4^{++}$ ,  $\beta 4^{+-}$  and  $\beta 4^{-}$  mice that had been chronically infused with saline (controls) exhibited very similar responses to acute nicotine injection (Figure 1A) as indicated by the results of a two-way ANOVA (main effect of genotype,  $F_{2,77} = 2.66$ , NS; main effect of acute nicotine dose,  $F_{5,77} = 22.41$ ,  $P < 0.001$ ) and comparison of the  $ED_{50}$  values for nicotine also indicated that the response to acute nicotine

for this test was very similar among the mice differing in  $\beta 4$  genotype ( $0.25 \pm 0.04$ ,  $0.40 \pm 0.09$  and  $0.39 \pm 0.08$  mg/kg for the  $\beta 4^{++}$ ,  $\beta 4^{+-}$  and  $\beta 4^{- -}$  mice, respectively).

Some tolerance development was noted following treatment with 1.0 mg/kg/hr nicotine (Figure 1B,  $ED_{50}$  values:  $0.47 \pm 0.07$ ,  $0.73 \pm 0.14$  and  $0.80 \pm 0.15$  mg/kg for the  $\beta 4^{++}$ ,  $\beta 4^{+-}$  and  $\beta 4^{- -}$  mice, respectively). As was the case with the saline-treated mice, the overall responses of the mice differing in  $\beta 4$  genotype following treatment with 1.0 mg/kg/hr nicotine were similar (two-way ANOVA, main effect of genotype  $F_{2,82} = 0.88$ ).

Chronic treatment with a dose of 4.0 mg/kg/hr nicotine (Figure 1C) elicited greater tolerance that was particularly noteworthy for the  $\beta 4^{- -}$  mice ( $ED_{50}$  values:  $0.58 \pm 0.10$ ,  $0.71 \pm 0.14$  and  $1.45 \pm 0.23$  mg/kg for the  $\beta 4^{++}$ ,  $\beta 4^{+-}$  and  $\beta 4^{- -}$  mice, respectively). The differences among the genotypes were also greater than those observed following treatment with the lower dose (two-way ANOVA, main effect of genotype  $F_{2,86} = 15.76$ ,  $P < 0.001$ ).  $\beta 4^{- -}$  mice developed significantly more tolerance than either  $\beta 4^{++}$  or  $\beta 4^{+-}$  mice.

**3.1.1.b. Body temperature:** Acute nicotine injection elicited a dose-dependent decrease in body temperature. The responses of  $\beta 4^{++}$ ,  $\beta 4^{+-}$  and  $\beta 4^{- -}$  mice that had been chronically infused with saline (controls) exhibited virtually identical responses to acute nicotine injection (Figure 1D) as indicated both by the results of a two-way ANOVA (main effect of genotype,  $F_{2,76} = 0.28$ ; main effect of acute nicotine dose,  $F_{5,76} = 21.94$ ,  $P < 0.001$ ) and by comparison of the  $ED_{50}$  values ( $0.98 \pm 0.08$ ,  $1.01 \pm 0.12$  and  $0.96 \pm 0.12$  mg/kg for the  $\beta 4^{++}$ ,  $\beta 4^{+-}$  and  $\beta 4^{- -}$  mice, respectively).

Some tolerance was noted following treatment with 1.0 mg/kg/hr nicotine (Figure 1E,  $ED_{50}$  values:  $1.17 \pm 0.08$ ,  $1.38 \pm 0.10$  and  $1.96 \pm 0.24$  mg/kg for the  $\beta 4^{++}$ ,  $\beta 4^{+-}$  and  $\beta 4^{- -}$  mice, respectively). The extent of tolerance differed among the mice (two-way ANOVA, main effect of genotype  $F_{2,81} = 4.24$ ,  $P = 0.018$ ).

Chronic treatment with a dose of 4.0 mg/kg/hr nicotine (Figure 1F) elicited greater tolerance than that elicited by treatment with 1.0 mg/kg/hr nicotine. This difference was particularly noteworthy for the  $\beta 4^{- -}$  mice ( $ED_{50}$  values:  $1.34 \pm 0.08$ ,  $1.64 \pm 0.15$  and  $4.12 \pm 0.57$  mg/kg for the  $\beta 4^{++}$ ,  $\beta 4^{+-}$  and  $\beta 4^{- -}$  mice, respectively). The differences among the genotypes was also greater than that observed following treatment with the 1.0 mg/kg/hr dose of nicotine (two-way ANOVA, main effect of genotype  $F_{2,86} = 15.41$ ,  $P < 0.001$ ; and genotype by acute nicotine dose interaction  $F_{10,86} = 2.23$ ,  $P = 0.023$ ). As was the case for Y-maze crosses,  $\beta 4^{- -}$  mice developed significantly more tolerance to the hypothermic effects of acute nicotine than did either  $\beta 4^{++}$  or  $\beta 4^{+-}$  mice.

**3.1.2. Effect of  $\beta 4$  genotype on tolerance development following chronic nicotine treatment: Four tests**—In order to provide a more complete picture of the effects of deletion of the  $\beta 4$  subunit on tolerance development after chronic nicotine treatment, data for all four tests (shown in Appendices AD) were analyzed and the results of the three-way ANOVAs (independent variables were genotype, chronic treatment dose and acute challenge dose) are compiled in Appendix E. Significant main effects for response to acute nicotine administration were obtained for all four tests. In addition, significant main

effects of chronic treatment dose indicated that tolerance had developed for each test. Furthermore, a significant main effect of  $\beta 4$  genotype was also obtained. While none of the three-way interactions were statistically significant, a variable pattern of significant two-way interactions was noted. For the measurement of body temperature, all three two-way interactions were statistically significant. In contrast, no two-way interactions achieved significance for open field activity.

The results presented in Figure 2 summarize the effects of chronic nicotine treatment on  $ED_{50}$  values for each of the four tests and three genotypes. The overall pattern of the effects of chronic nicotine treatment is very similar for all four tests. The increases in  $ED_{50}$  values with increasing chronic nicotine dose provide further demonstration of tolerance development. However, significantly more tolerance development was observed for the  $\beta 4^{-/-}$  mice, particularly following treatment with the highest nicotine dose (4 mg/kg/hr). The response of  $\beta 4^{+/+}$  and  $\beta 4^{+/-}$  mice to chronic nicotine treatment was quite similar, although the  $\beta 4^{+/-}$  mice tended to develop somewhat more tolerance than did the  $\beta 4^{+/+}$  mice.

### 3.2. Nicotinic binding sites

In order to examine the effects of chronic nicotine treatment on nAChR expression, the binding of [ $^{125}$ I]-epibatidine was measured. [ $^{125}$ I]-Epibatidine binds with relatively equal affinity to heteromeric nAChR subtypes in mouse brain (Marks et al., 1998; Whiteaker et al., 2000b). However, nAChR subtypes display different affinities for ligands other than epibatidine: Cytisine displays higher affinity for  $\alpha 4\beta 2^*$ -nAChR (Baddick and Marks, 2011; Marks et al., 1998) and A85380 (or 5I-A85380) displays higher affinity for  $\beta 2^*$ -nAChR than these ligands do for other subtypes (Mukhin et al., 2000; Whiteaker et al., 2000b). Consequently, assay conditions were selected to measure several [ $^{125}$ I]-epibatidine subtypes using differential inhibition by cytisine or A85380. Levels of nAChR binding sites in seven brain regions of mice differing in  $\beta 4$  subunit expression as well as the results of the two-way ANOVAs are presented in Appendix F. Graphical presentation of results for cytisine-sensitive [ $^{125}$ I]-epibatidine binding and 5I-A85380-resistant [ $^{125}$ I]-epibatidine are shown in Figures 3 and 4 for visual presentation of the results.

**3.2.1. Cytisine-sensitive [ $^{125}$ I]-epibatidine binding (primarily  $\alpha 4\beta 2^*$ -nAChR sites)**—The effects of chronic nicotine treatment on the levels of cytisine-sensitive [ $^{125}$ I]-epibatidine binding are shown in Figure 3. Chronic nicotine treatment elicited significant, saturable, dose-dependent increases in cytisine-sensitive [ $^{125}$ I]-epibatidine binding sites (primarily  $\alpha 4\beta 2^*$ -nAChR sites) in hindbrain (Figure 3A,  $F_{4,174} = 5.14$ ,  $P < 0.001$ ), inferior colliculus (Figure 3C,  $F_{4,104} = 4.51$ ,  $P < 0.01$ ) and olfactory bulb (Figure 3D,  $F_{4,148} = 11.46$ ,  $P < 0.001$ ). The pattern noted for the response to chronic nicotine treatment of each  $\beta 4$  genotype was very similar (there were no significant genotype by nicotine treatment interactions). However, modest overall differences among the genotypes were noted for hindbrain ( $F_{2,174} = 3.33$ ,  $P = 0.033$ ) and olfactory bulb ( $F_{2,148} = 3.23$ ,  $P = 0.042$ ). In contrast to the increased binding noted for hindbrain, inferior colliculus and olfactory bulb, cytisine-sensitive [ $^{125}$ I]-epibatidine binding in habenula was unaffected by chronic nicotine treatment (Figure 3B,  $F_{4,64} = 1.76$ ,  $P > 0.05$ ).



Cytisine-sensitive [<sup>125</sup>I]-epibatidine binding was also measured in cortex, thalamus and superior colliculus and values for these regions are included in Appendix F. Significant nicotine dose-dependent increases in these sites were for each region. However, the average maximal increases measure in thalamus (29%) and superior colliculus (27%) were relatively modest compared to the increase in cortex (47%).

**3.2.2. Cytisine-resistant [<sup>125</sup>I]-epibatidine binding (mostly non  $\alpha 4\beta 2^*$ -nAChR sites)**—The effects of chronic nicotine treatment on cytisine-resistant [<sup>125</sup>I]-epibatidine binding sites are summarized in Appendix F. Effects of  $\beta 4$  gene deletion and chronic nicotine treatment provide general information about the response of this diverse set of nAChR binding sites to chronic nicotine treatment.

Deletion of the  $\beta 4$  subunit did not significantly affect cytisine-resistant [<sup>125</sup>I]-epibatidine binding in cortex, thalamus or superior colliculus. While these sites are relatively minor components of total [<sup>125</sup>I]-epibatidine binding in cortex and thalamus, they do comprise a significant fraction of binding in superior colliculus. No statistically significant effects of chronic nicotine treatment were observed in these three regions as well.

Deletion of the  $\beta 4$  subunit significantly reduced cytisine-resistant [<sup>125</sup>I]-epibatidine binding in habenula ( $F_{2,64} = 184.71$ ,  $P < 0.001$ ), hindbrain ( $F_{2,174} = 30.23$ ,  $P < 0.001$ ), inferior colliculus ( $F_{2,104} = 77.30$ ,  $P < 0.001$ ) and olfactory bulb ( $F_{2,148} = 188.39$ ,  $P < 0.001$ ) demonstrating that  $\beta 4^*$ -nAChR comprise a significant fraction of these sites in these four brain regions. Furthermore, chronic nicotine treatment elicited significant changes in habenula ( $F_{4,64} = 2.91$ ,  $P < 0.05$ ), hindbrain ( $F_{4,174} = 4.51$ ,  $P < 0.01$ ) and olfactory bulb ( $F_{4,148} = 4.03$ ,  $P < 0.01$ ). The significant genotype by chronic treatment dose interactions observed in habenula ( $F_{8,64} = 2.23$ ,  $P < 0.05$ ) and olfactory bulb ( $F_{8,148}$ ,  $P < 0.05$ ) suggests that response to chronic nicotine treatment varied among the mice differing in expression of the  $\beta 4$  subunit. Consequently, subsets of cytisine-resistant [<sup>125</sup>I]-epibatidine binding were measured as described below.

**3.2.3. 5I-A85380-resistant [<sup>125</sup>I]-epibatidine binding (primarily  $\beta 4^*$ -nAChR sites)**—The effects of chronic nicotine treatment on the levels of 5I-A85380-resistant [<sup>125</sup>I]-epibatidine binding are shown in Figure 4. In contrast to the modest effects on cytisine-sensitive [<sup>125</sup>I]-epibatidine binding, deletion of the  $\beta 4$  nAChR subunit significantly reduced 5I-A-85380-resistant [<sup>125</sup>I]-epibatidine binding in each of the brain regions shown in Figure 4 (main effects of genotype: 4A, hindbrain,  $F_{2,174} = 49.32$ ,  $P < 0.001$ ; 4B, habenula,  $F_{2,64} = 308.59$ ,  $P < 0.001$ ; 4C, inferior colliculus,  $F_{2,104} = 212.49$ ,  $P < 0.001$ ; 4D, olfactory bulb,  $F_{2,148} = 122.71$ ,  $P < 0.001$ ). 5I-A85380-resistant [<sup>125</sup>I]-epibatidine binding was reduced by more than 90% in habenula, inferior colliculus and olfactory bulb and by more than 70% in hindbrain following deletion of the  $\beta 4$  nAChR subunit. Chronic nicotine treatment had less effect on 5I-A85380-resistant [<sup>125</sup>I]-epibatidine binding sites ( $\beta 4^*$ -nAChR sites) than on the  $\alpha 4\beta 2$ -nAChR sites. However, significant effects of nicotine treatment were observed for the  $\beta 4^*$ -nAChR sites in hindbrain (Figure 4A,  $F_{4,174} = 2.98$ ,  $P < 0.05$ ) and habenula (Figure 4B,  $F_{4,64} = 4.60$ ,  $P < 0.01$ ). The increases were most evident for the  $\beta 4^{++}$  mice following treatment with 4.0 mg/kg/hr nicotine. Deletion of the  $\beta 4$  subunit eliminated these nicotine-induced increases.

5I-A85380-resistant [<sup>125</sup>I]-epibatidine binding was also measured in three other regions (Appendix F). However, these sites are sparsely expressed in these regions as indicated by the percentage of total epibatidine binding sites: cortex (2.5%), superior colliculus (3.3%) and thalamus (2.0%).

**3.2.4. Cytisine-resistant, 5I-A85380-sensitive [<sup>125</sup>I]-epibatidine binding sites (mostly non- $\alpha 4\beta 2$ -nAChR,  $\beta 2^*$ -nAChR sites)**—The effects of chronic nicotine treatment on cytisine-resistant, A-85380-sensitive [<sup>125</sup>I]-epibatidine binding sites are also compiled in Appendix F. These binding sites comprise a relatively large fraction of total [<sup>125</sup>I]-epibatidine binding sites in superior colliculus, where neither  $\beta 4$  gene deletion nor chronic nicotine treatment elicited significant changes in binding site density. However, in olfactory bulb where these sites also comprise a significant fraction of total [<sup>125</sup>I]-epibatidine binding sites deletion of the  $\beta 4$  subunit significantly reduced binding. The diversity of responses for these sites that represent a mixed population of nAChR subtypes is further indicated in hindbrain where deletion of  $\beta 4$  reduced the density of these sites and also eliminated the increased binding elicited by nicotine treatment in the wild-type mice. This subset of sites represents less than 5% of total epibatidine binding in the other five brain regions that were assayed.

## 4. Discussion

Cytisine-sensitive [<sup>125</sup>I]-epibatidine binding sites (primarily  $\alpha 4\beta 2$ -nAChR sites) are relatively unaffected by deletion of the  $\beta 4$  nAChR subunit. In addition, deletion of  $\beta 4$  has virtually no effect on the nicotine-induced changes in cytisine-sensitive [<sup>125</sup>I]-epibatidine binding sites in most brain regions. However, somewhat less up-regulation of these sites was observed in the hindbrain and olfactory bulb of  $\beta 4^{-/-}$  mice than in  $\beta 4^{+/+}$  mice suggesting that a subset of these binding sites in hindbrain may express a complex nAChR containing both  $\beta 2$  and  $\beta 4$  subunits. Since mRNA encoding both of these subunits is expressed in these brain regions, assembly of complex receptor subtypes is possible. Selective immunoprecipitation studies in other brain areas have identified nAChR subtypes that co-express  $\beta 2$  and  $\beta 4$  subunits (Gotti et al., 2007; Mao et al., 2006; Mineur et al., 2009; Turner and Kellar, 2005).

Heteromeric A85380-resistant [<sup>125</sup>I]-epibatidine binding sites have been considered to be primarily  $\beta 4^*$ -nAChR since A85380 is highly selective for  $\beta 2^*$ -nAChR (Mukhin et al., 2000). The expression of this subset of binding sites is relatively high in olfactory bulb, habenula and inferior colliculus. These sites are reduced by at least 90% following deletion of the  $\beta 4$  subunit. Most of the  $\beta 4^*$ -nAChR binding sites are likely  $\alpha 3\beta 4^*$ -nAChR, but although the presence of receptors assembled from more than two types of subunits (including  $\alpha 4$ ,  $\alpha 5$ ,  $\beta 2$  and  $\beta 3$ ) has been demonstrated in several neuronal preparations (Grady et al., 2009; Mao et al., 2006; Turner and Kellar, 2005). While deletion of the  $\beta 4$  subunit significantly reduced the levels of 5I-A-85380 resistant [<sup>125</sup>I]-epibatidine binding sites in hindbrain, superior colliculus and thalamus, substantial residual binding remained in each of these regions suggesting that a subset of these binding sites do not fit the normal classification as strictly  $\beta 4^*$ -nAChR sites. Chronic nicotine treatment elicited significant increases in 5I-A85380-resistant [<sup>125</sup>I]-epibatidine binding sites only in habenula and hindbrain. These increases were most apparent in the samples from mice treated with the 4

mg/kg/hr nicotine dose. Treatment with this dose of nicotine produces plasma concentrations over 1  $\mu$ M (Marks et al., 2004), suggesting that some  $\beta 4^*$ -nAChR binding sites respond to exposure to high nicotine concentrations. The fact that no up-regulation was observed following deletion of the  $\beta 4$  subunit indicates brain region selective  $\beta 4^*$ -nAChR up-regulation occurred. This selective increase may reflect the region specific expression of  $\beta 4^*$ -nAChR with complex pentameric composition. Only a few studies have examined the effect of chronic nicotine treatment *in vivo* on the expression of [ $^{125}$ I]-epibatidine binding to putative  $\alpha 3\beta 4$ -nAChR sites and these studies indicated that the  $\alpha 3\beta 4^*$ -nAChR are resistant to nicotine-induced upregulation (Marks et al., 2004; McCallum et al., 2006; Nguyen et al., 2003). However, upregulation of  $\alpha 3\beta 4^*$ -nAChR has been observed in cultured cells after exposure to relatively high concentrations of nicotine (Peng et al., 1997). nAChR in habenula have been identified as being important regulators of nicotine self-administration and withdrawal (Fowler and Kenny, 2012; McCallum et al., 2012; Salas et al., 2009), so alteration of their expression by chronic nicotine treatment, if confirmed, could have important implications for the regulation of responsiveness to the drug as suggested previously (Olale et al., 1997). Several compounds that appear to act relatively selectively on  $\alpha 3\beta 4^*$ -nAChR are being actively investigated as useful pharmacological agents (Glick et al., 2000; Glick et al., 2011; Toll et al., 2012).

The [ $^{125}$ I]-epibatidine binding sites that are resistant to inhibition by cytisine, but sensitive to inhibition by 5I-A85380 comprise a diverse set of receptors that may include nAChR assembled with  $\alpha 2$ ,  $\alpha 3$  and/or  $\alpha 6$  subunits the expression of which varies among brain regions (Baddick and Marks, 2011). The fact that these sites are sensitive to inhibition by 5I-A85380 implies the presence of the  $\beta 2$  subunit, as well (Mukhin et al., 2000). Significant expression of these sites was found in inferior colliculus, olfactory bulb and, particularly, superior colliculus. However, response following deletion of the  $\beta 4$  subunit indicates that the receptor composition in these regions differs. Deletion of the  $\beta 4$  subunit had little effect on these sites in either superior or inferior colliculus, but reduced them in olfactory bulb, indicating the presence of a relatively complex set of receptors in this region. Such complexity could be expected owing to the diversity of nAChR subunit mRNA expression in olfactory bulb a brain region that includes relatively high levels of mRNA encoding  $\alpha 2$ ,  $\alpha 3$ ,  $\beta 2$  and  $\beta 4$  nAChR subunits (Dineley-Miller and Patrick, 1992; Wada et al., 1989) and the expression of  $\alpha 2^*$ -nAChR in olfactory bulb has been demonstrated (Whiteaker et al., 2009). Characterization of nAChR expression in superior colliculus has revealed the existence of significant receptor populations that include  $\alpha 3$  and  $\alpha 6$  subunits (McClure-Begley et al., 2014). Chronic nicotine treatment had no significant effect on the expression of this subset of receptors in any brain region, except hindbrain, where a dose-dependent up-regulation was observed for wild-type mice that was lost in both the  $\beta 4^{+/+}$  and  $\beta 4^{-/-}$  mice. The observation that chronic nicotine treatment elicits up-regulation of all three major subsets of high affinity [ $^{125}$ I]-epibatidine binding sites in hindbrain suggests that the receptors in this brain region may be uniquely responsive to chronic nicotine treatment.

Deletion of the  $\beta 4$  nAChR subunit has relatively little effect on the nicotine-elicited responses of saline-infused (control) mice for any of the four tests used in the current study. This observation is consistent with previous reports that  $\beta 2^*$ -nAChR appear to modulate

responses to relatively low doses of nicotine for these tests (Tritto et al., 2004). However, deletion of the  $\beta 4$  subunit significantly affects the extent of tolerance development following chronic nicotine treatment that was particularly evident in mice treated with 4 mg/kg/hr nicotine, the highest dose used in the current study. This substantial tolerance contrasts with the relatively feeble tolerance observed for  $\beta 2^{-/-}$  mice treated with nicotine using the same paradigm (McCallum et al., 2006). These results suggest that functional changes mediated by  $\beta 2^*$ -nAChR responses observed following chronic nicotine treatment are primarily responsible for the observed tolerance, and that these effects are more apparent in the absence of expression of  $\beta 4^*$ -nAChR. In contrast, functional changes mediated by  $\beta 4^*$ -nAChR do not seem to be significant contributors to tolerance development, at least for the four tests used in the current study. These changes may actually render mice slightly more sensitive to nicotine following chronic treatment with this drug. This observation is consistent with the demonstration that  $\beta 4^*$ -nAChR are important in mediating somatic signs of nicotine withdrawal (Salas et al., 2004b).

The relative contribution of responses mediated by  $\beta 4$ - and  $\beta 2$ -nAChR was evaluated by comparing the effects of deletion of either of these genes on tolerance development. The changes in  $ED_{50}$  values for the combined responses following chronic nicotine treatment of either  $\beta 2^{-/-}$  (McCallum et al., 2006) or  $\beta 4^{-/-}$  (current study) are illustrated in Figure 5A. As noted previously for the individual tests, significant, dose-dependent tolerance (likely mediated by  $\beta 2^*$ -nAChR elicited responses) develops in  $\beta 4^{-/-}$  mice, while relatively little tolerance and perhaps even some super-sensitivity (likely mediated by  $\beta 4^*$ -nAChR elicited responses) develops in  $\beta 2^{-/-}$  mice. In order to test whether the alteration of the acute responses to nicotine by chronic nicotine treatment of wild-type mice (tolerance to the effects of nicotine) can be approximated by averaging the responses of the two knockout mouse lines, changes in  $ED_{50}$  values of wild-type mice were compared to the average of extent of tolerance development mediated by  $\beta 2^*$ - and  $\beta 4^*$ -nAChR and are illustrated in Figure 5B. In general, this analysis is consistent with the hypothesis that development of tolerance to chronic nicotine treatment in wild-type mice is relatively well approximated by the contributions of  $\beta 2^*$ -nAChR (dose-dependent tolerance observed in  $\beta 4^{-/-}$  mice) and  $\beta 4^*$ -nAChR (modest super-sensitivity at lower nicotine doses observed in  $\beta 2^{-/-}$  mice). Given that many of the effects of these nAChR subtypes are mediated by their effects on neurotransmitter and/or hormone release, the exact mechanisms for the interactions between these two populations of nAChRs remains to be determined.

## Conclusions

Chronic nicotine treatment elicits more tolerance in  $\beta 4^{-/-}$  mice than that in either  $\beta 4^{+/+}$  or  $\beta 4^{+/-}$  mice suggesting that responses mediated by nAChR remaining in the  $\beta 4^{-/-}$  mice, probably  $\beta 2^*$ -nAChR, contribute significantly to tolerance development following chronic nicotine treatment. In contrast, responses mediated by  $\beta 4^*$ -nAChR don't substantially contribute to the tolerance of the behaviors measured here. Deletion of the  $\beta 4$  subunit has relatively little effect on the subset of [ $^{125}$ I]-epibatidine binding sites (cytisine-sensitive sites) thought to be primarily  $\alpha 4\beta 2$ -nAChR sites. However, the subset of [ $^{125}$ I]-epibatidine binding sites thought to have a significant contribution of  $\beta 4^*$ -nAChR (A-85380-resistant sites) were indeed reduced in the brains of  $\beta 4^{-/-}$  mice. The significant and brain-region

selective increase in 5I-A85380 resistant [<sup>125</sup>I]-epibatidine binding sites in several brain regions of wild-type mice indicates that some  $\beta 4^*$ -nAChR, perhaps those also containing the  $\beta 2$  subunit, indicates that the  $\beta 4^*$ -nAChR are not universally resistant to increases elicited by chronic nicotine treatment.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by the following grants to Michael J. Marks from the National Institutes of Health, National Institute on Drug Abuse: R01 DA003194 and P30 DA015663. Jill Miyamoto-Ditman provided some technical assistance. We also wish to thank Sharon R. Grady and Allan C. Collins for critical reading of the manuscript.

## Abbreviations

<b>nAChR</b>	nicotinic acetylcholine receptor
<b>ED<sub>50</sub></b>	effective dose giving 50% maximal response
<b>5I-A85380</b>	3-[(2S)-2-azetidylmethoxy]-5-iodopyridine
<b>HEPES</b>	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

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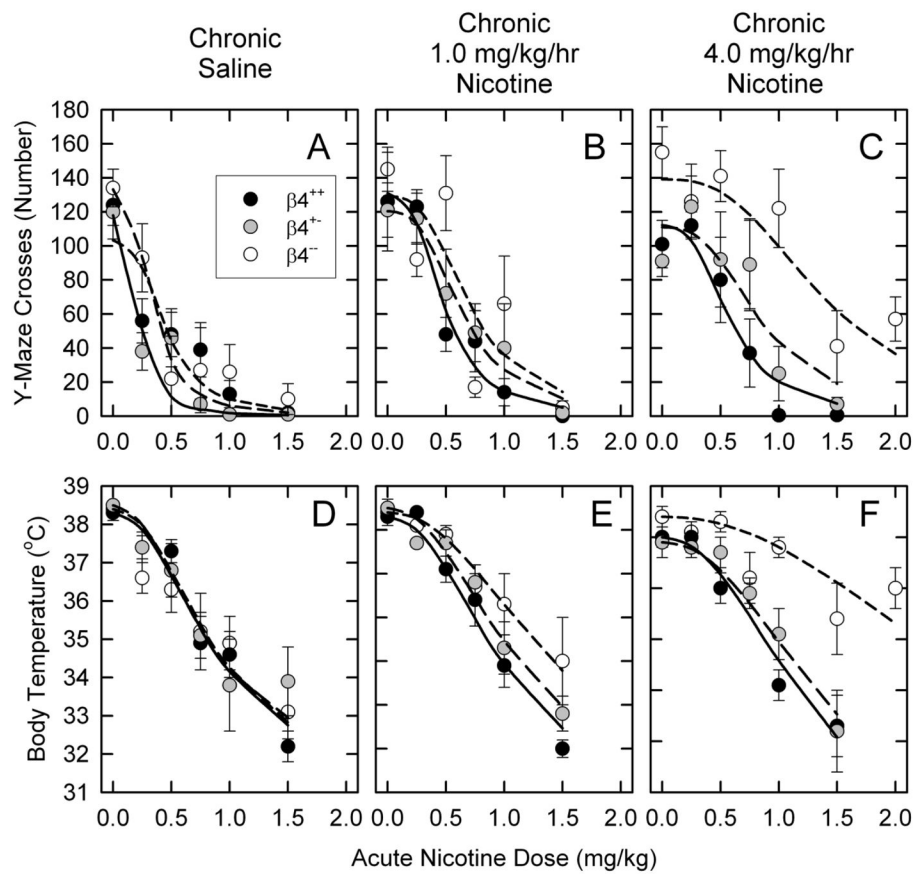
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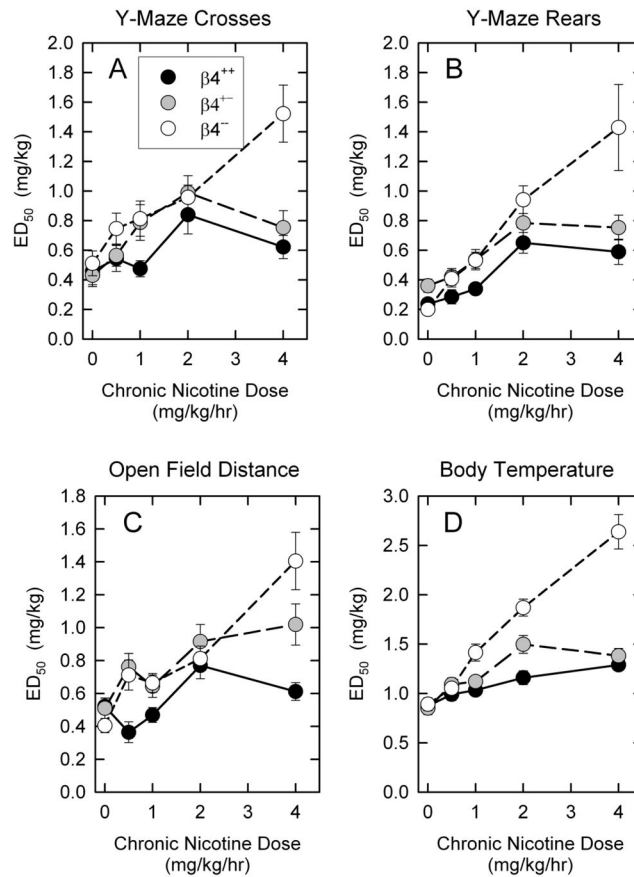
### Highlights

- Mice differing in beta4 nicotinic subunit expression received nicotine chronically
- Beta4 null mutants develop more nicotine tolerance than wild-types or heterozygotes
- Deletion of beta4 has selective effects on subsets of epibatidine binding sites
- Responses to chronic nicotine of receptors with beta4 differ among brain regions
- Both beta2 and beta4 receptors appear to influence nicotine tolerance development

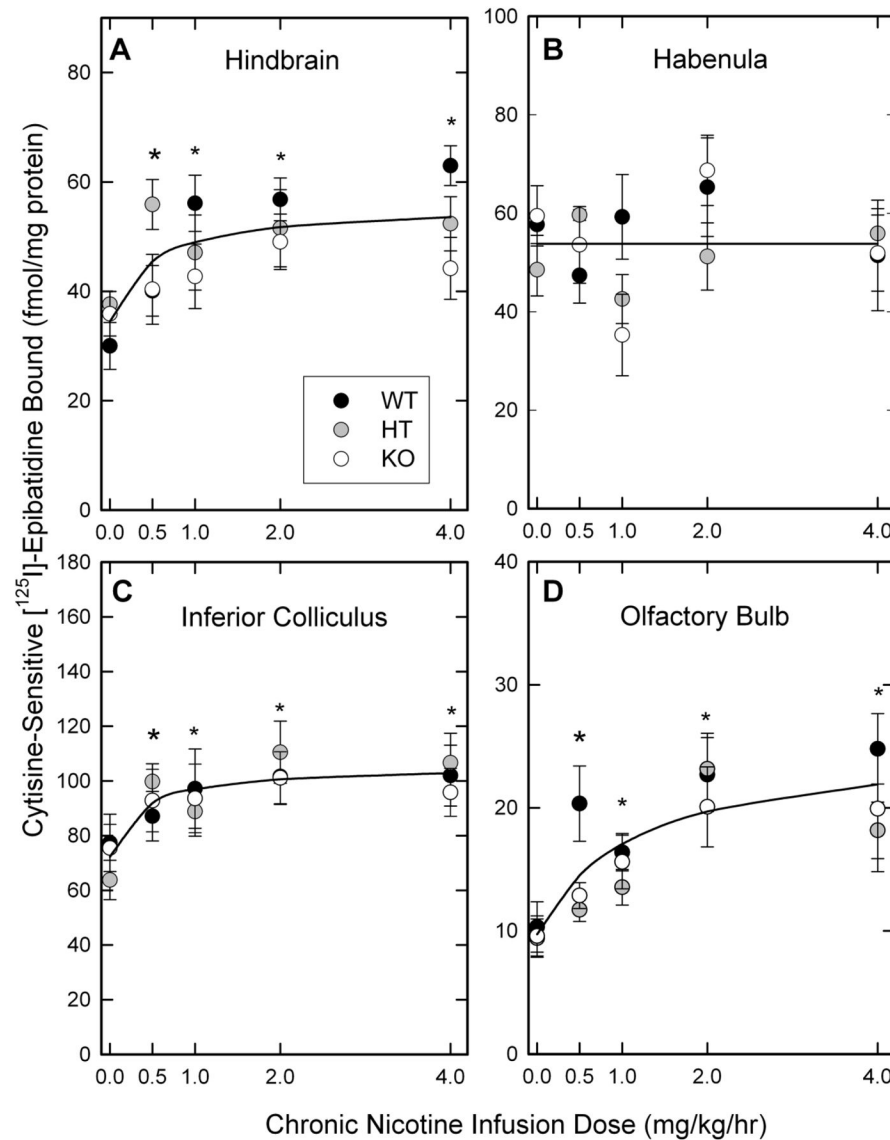


**Figure 1. Dose-response curves for the effects of chronic nicotine treatment of mice differing in  $\beta 4$  genotype for select tests**

Mean  $\pm$  SEM (N =4-9) for responses of  $\beta 4^{+/+}$ ,  $\beta 4^{+/-}$  and  $\beta 4^{-/-}$  mice treated with saline, 1.0 mg/kg/hr nicotine or 4.0 mg/kg/hr nicotine as indicated and acutely injected with the indicated nicotine dose. The lines are least squares curve fits of the results as described in the Methods.

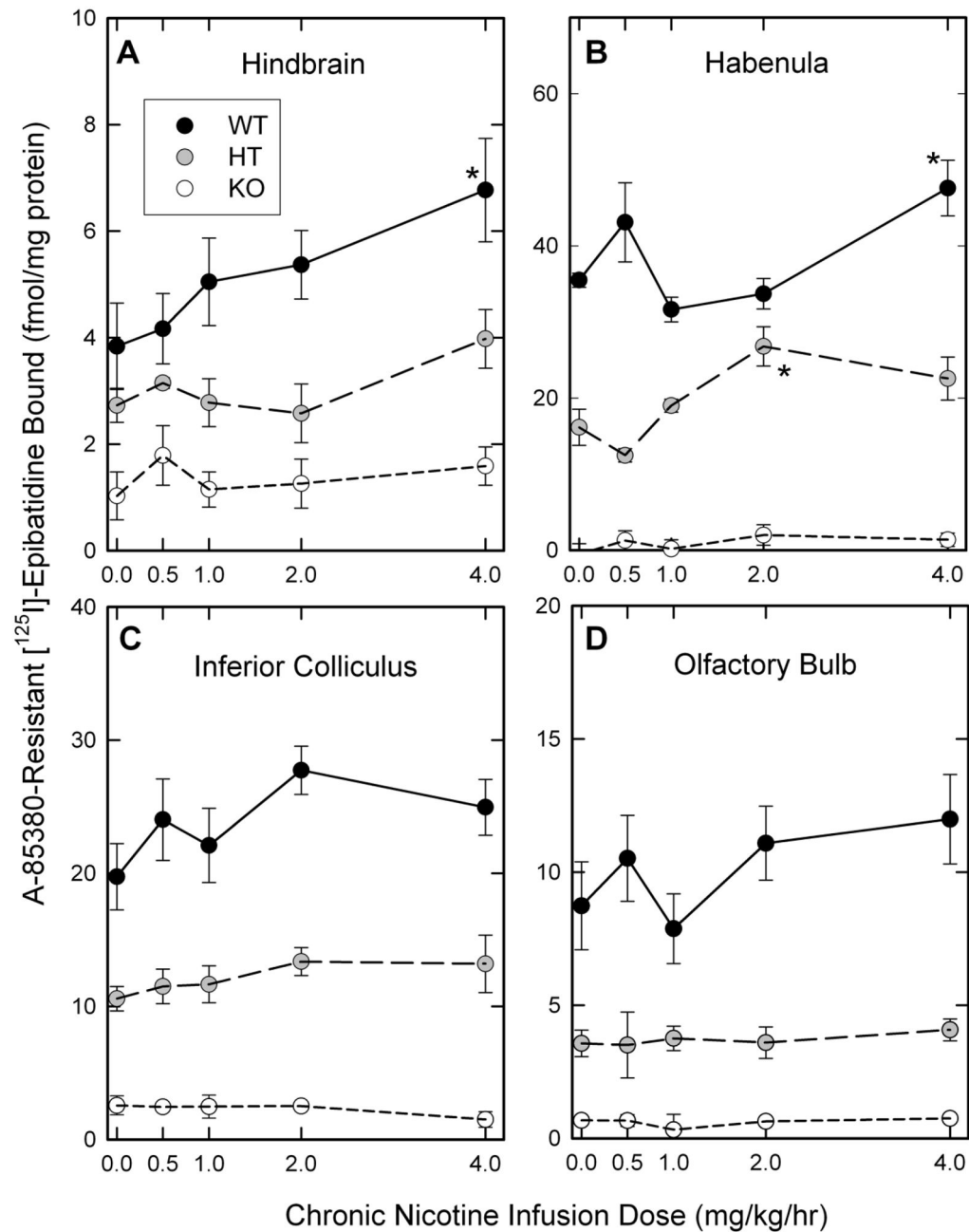


**Figure 2. ED<sub>50</sub> values following chronic nicotine treatment for mice differing in  $\beta 4$  genotype**  
 Mean  $\pm$  SEM of ED<sub>50</sub> values calculated by non-linear least squares curve fits for dose-response curves from each of the four tests for  $\beta 4^{+/+}$ ,  $\beta 4^{+/-}$  and  $\beta 4^{-/-}$  that had been chronically treated with the indicated doses of nicotine.



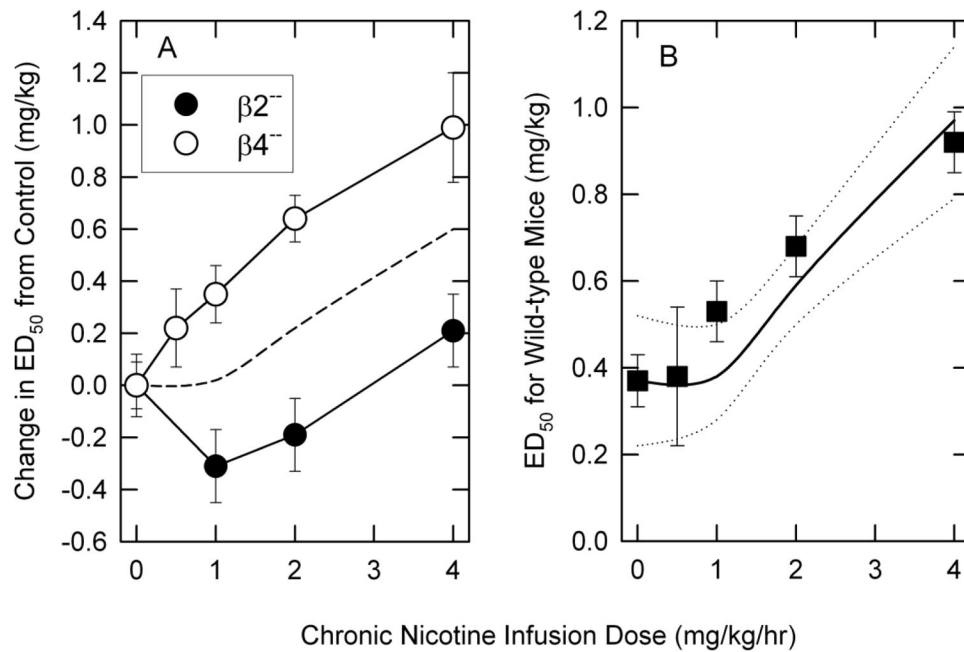
**Figure 3. Effects of chronic nicotine treatment on expression of cytosine-sensitive  $[^{125}\text{I}]$ -epibatidine binding sites**

The expression of cytosine-sensitive  $[^{125}\text{I}]$ -epibatidine binding sites in membranes prepared from four brain regions of  $\beta 4^{+/+}$ ,  $\beta 4^{+/-}$  and  $\beta 4^{-/-}$  mice treated with the indicated chronic doses of nicotine was calculated as described in the methods. Points represent the mean  $\pm$  SEM of 5-11 independent samples. The lines in each panel are nonlinear least squares curve fits of the data. Cytosine-sensitive  $[^{125}\text{I}]$ -epibatidine binding in each brain region except habenula was significantly higher than that of saline treated mice after nicotine treatment as indicated by the asterisks (\*).



**Figure 4. Effects of chronic nicotine treatment on expression of 5I-A-85380 resistant [<sup>125</sup>I]-epibatidine binding sites**

The expression of 5I-A-85380-resistant [<sup>125</sup>I]-epibatidine binding sites in membranes prepared from four brain regions of  $\beta 4^{+/+}$ ,  $\beta 4^{+/-}$  and  $\beta 4^{-/-}$  mice treated with the indicated chronic doses of nicotine as described in the methods. Points represent the mean  $\pm$  SEM of 5-11 independent samples. Lines in all panels merely connect the data points. Values significantly different from saline-treated mice of the same genotype are indicated by asterisks (\*).



**Figure 5. Comparison of the effects of deletion of the  $\beta 2$  or  $\beta 4$  nAChR on the development of tolerance to nicotine to that of wild-type mice**  
 The results in Panel A compare the change in ED<sub>50</sub> values (mean  $\pm$  SEM) for nicotine of  $\beta 2^{-/-}$  and  $\beta 4^{-/-}$  mice following chronic treatment with the indicated doses of nicotine. The dotted line is the average of the ED<sub>50</sub> values for the two genotypes at each dose. The results in panel B compare ED<sub>50</sub> values for wild-type C57BL/6 mice to the average change in ED<sub>50</sub> values for  $\beta 2^{-/-}$  and  $\beta 4^{-/-}$  mice added to the ED<sub>50</sub> for saline-infused mice (solid line). The dotted lines are one SEM above and below the average line.