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Chronic nicotine treatment decreases striatal α6* nAChR sites and function in mice

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Running title: Chronic nicotine decreases striatal α6* nAChRs

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ABBREVIATIONS: ANOVA, analysis of variance; mAb, monoclonal antibody; nAChR, nicotinic acetylcholine receptor; *, nicotinic receptors containing the indicated α and/or β subunit and possibly also additional undefined subunits.

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ABSTRACT

α-Conotoxin MII-sensitive nicotinic receptors (nAChRs) are distinct from other subtypes in their relatively restricted localization to the striatum and some other brain regions. The effect of nicotine treatment on nAChR subtypes has been extensively investigated, with the exception of changes in α-conotoxin MII-sensitive receptor expression. We therefore determined the consequence of chronic nicotine administration on this subtype and its function. Nicotine was given in drinking water to provide a chronic yet intermittent treatment. Consistent with previous studies, nicotine exposure increased ^{125}I-epibatidine and ^{125}I-A85380, but not ^{125}I-α-bungarotoxin, receptors in cortex and striatum. Unexpectedly, we observed a reduction (30%) in striatal ^{125}I-α-conotoxin MII sites, which was due to a decrease in B_{max}. This decline was more robust in older (>8 month) compared to younger (2-4 month) mice, suggesting age is important for nicotine-induced disruption of nAChR phenotype. Immunoprecipitation experiments using nAChR subunit-directed antibodies indicate that alterations in subunit-immunoreactivity with nicotine treatment agree with those in the receptor binding studies. To determine the relationship between striatal nAChR sites and function, we measured nicotine-evoked ^{3}H-dopamine release. A decline was obtained with nicotine treatment that was due to a selective decrease in α-conotoxin MII-sensitive but not -resistant dopamine release. These results may explain earlier findings that nicotine treatment decreased striatal nAChR-mediated dopamine function, despite an increase in ^{3}H-nicotine (α4*) sites. The present data suggest that the α6* nAChR subtype represents a key factor in the control of dopamine release from striatum, which adapts to chronic nicotine treatment by down-regulation of α6* receptor sites and function.

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Multiple nicotinic receptor (nAChR) subtypes are present in mammalian brain. While some have a widespread distribution others exhibit more select patterns of localization, possibly suggesting they have distinct functions in the area of interest (Dani, 2001; Paterson and Nordberg, 2000; Quik and Kulak, 2002; Wonnacott, 1997). One subtype that exhibits a relatively restricted localization to the nigrostriatal, visual and habenular-interpeduncular pathways is the α-conotoxin MII-sensitive nAChR (Champtiaux et al., 2003; Champtiaux et al., 2002; Quik et al., 2001; Whiteaker et al., 2000; Zoli et al., 2002). Expression studies and work with nAChR subunit null-mutant mice showed that α-conotoxin MII-sensitive sites contain α6 and/or α3 (Champtiaux et al., 2002; McIntosh et al., 1999; Whiteaker et al., 2002), and also β2, β3, and possibly α4 subunits to form pentameric α6α4β2β3 and α6β2β3 receptors (Champtiaux et al., 2003; Cui et al., 2003; Salminen et al., 2004a; Zoli et al., 2002). These nAChRs are not only expressed in the brain but are functional, with the sites in striatum mediating dopamine release (Salminen et al., 2004a). Moreover declines in these sites with nigrostriatal damage result in a corresponding reduction in dopaminergic function in rodent striatum (Quik et al., 2003).

Nicotine is an important modulator of nAChR expression. Numerous studies in animal models have shown that nicotine treatment results in an upregulation of nAChRs that may be attributed to agonist-induced desensitization (Buisson and Bertrand, 2002; Gentry and Lukas, 2002; Wonnacott, 1990). Increases have consistently been observed in radiolabeled epibatidine (identifies multiple nAChR subtypes), nicotine (α4*) and cytisine (α4*) binding sites in numerous brain regions following various treatment regimens including injection, chronic jugular infusion, drinking water, release from minipumps, and self-administration (Flores et al., 1992, 1997; Marks et al., 1992; Parker et al., 2004; Rogers et al., 1998; Ryan et al., 2001; Sparks and Pauly, 1999). In contrast, the results of studies to evaluate effects of chronic nicotine

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exposure on α3* and/or α6* nAChRs are less clear with one study showing an increase in binding in rat striatum, and another no change (Nguyen et al., 2003; Parker et al., 2004). Whether nicotine treatment modulates α-conotoxin MII-sensitive nAChR function has not yet been investigated.

Because of the potential importance of α-conotoxin MII-sensitive sites in the nigrostriatal pathway, we investigated the effects of chronic nicotine treatment on α-conotoxin MII-sensitive nAChR-mediated function in synaptosomes prepared from striatal tissue of control and nicotine-treated mice. Nicotine was given in the drinking water because this regimen involves a chronic but intermittent mode of administration. Experiments were also done to determine whether age of the animal modulated the effects of nicotine administration.

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Materials and Methods

Mouse Treatment. Two to 4 month-old and >8 month-old male C57BL/6 mice were purchased from Charles River Laboratories (Gilroy, CA). The >8 month-old mice were used in all studies, unless otherwise indicated. All procedures used conform to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Mice were placed in a temperature-controlled room with an 11/13 h dark/light cycle. The younger and older animals were housed in groups of 3-4 and 1-2 per cage, respectively. All animals had free access to food and water. They were randomly divided into different treatment groups 2 days after arrival.

After acclimatization, mice were given drinking water containing 2% saccharin (Sigma Chemical Co., St. Louis, MO), to mask the bitter taste of nicotine (free base, Sigma). Nicotine was added to the saccharin-containing solution starting at an initial concentration of 25 μg/ml.

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This was increased to 50 μg/ml on days 3-4, 100 μg/ml on days 5-7, 200 μg/ml on days 8-9 to a final dose on day 10 of 300 μg/ml. The animals were maintained on this dose of nicotine for 1, 2, 4, 5, or 6 wk as indicated. The nicotine was then withdrawn for ≥3 h after which time the mice were killed by cervical dislocation.

Cotinine Determinations. As an indirect measure of plasma nicotine levels, the nicotine metabolite cotinine was assayed using an ELISA kit (Orasure Technologies, Bethlehem, PA). Blood samples were collected from the orbital sinus after 1 wk of nicotine (300 μg/ml) treatment, or from trunk blood during sacrifice. Plasma was prepared and a 10 μl aliquot used for assay according to the manufacturer's instructions. A standard curve ranging from 10 to 200 ng/ml cotinine was included in every assay.

Binding Studies

For autoradiographic binding studies, brains were quick frozen in isopentane on dry ice and stored at –80°C until sectioning. Sections (14 μm) were prepared using a cryostat (–20°C), thaw mounted onto poly-L-lysine coated slides, air dried and stored at –80°C.

^{125}I-Epibatidine Autoradiography. Binding of ^{125}I-epibatidine (2200 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA) to mouse brain sections was done as previously described (Quik et al., 2003). Preincubation was at room temperature for 2 x 15 min in Buffer (50 mM Tris buffer, pH 7.0, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$). This was followed by a 40-min incubation in Buffer also containing 0.015 nM ^{125}I-epibatidine, a concentration below the Kd value (0.1 nM) for this radioligand. Nicotine (0.1 mM) was included with some of the sections to define nonspecific binding. Sections were washed (4°C) twice for 5 min with Buffer and once for 10 s in cold deionized water. After drying, slides were exposed to Kodak

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MR film (Perkin Elmer Life Sciences, Boston, MA, USA) for 2-5 days with ^{125}I-standards (Amersham Biosciences, Piscataway, NJ, USA).

^{125}I-A85380 Autoradiography. ^{125}I-A85380 (1450 Ci/mmol, from H.F.) binding was done as previously described (Mukhin et al., 2000; Quik et al., 2003). Sections were preincubated in Buffer for 2 x 15 min and then incubated for 60 min in Buffer with 95 pM ^{125}I-A85385, a concentration at the K_d value for this radioligand. This was followed by washing in Buffer at 4°C twice for 5 min and once for 10 s in cold deionized water. Slides were dried at room temperature, and then exposed to Kodak MR film for 1-2 days with ^{125}I-standards. Nonspecific binding, assayed using 0.1 mM nicotine, was the same as the film blank.

^{125}I-α-Conotoxin MII Autoradiography. ^{125}I-α-Conotoxin MII (2200 Ci/mmol) was synthesized and binding was performed as detailed earlier (Quik et al., 2001; Whiteaker et al., 2000). Thawed sections were preincubated at room temperature for 2 x 15 min in 20 mM HEPES buffer (pH 7.5, 144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 0.1% BSA and 1 mM phenylmethylsulfonyl fluoride). This was followed by a 1-h incubation with 0.5 nM ^{125}I-α-conotoxin MII, a concentration below the K_d value (0.9 nM) for this radioligand. Incubation was at room temperature in the same HEPES buffer but now also containing 0.2% BSA, 5 mM EDTA, 5 mM EGTA, and 10 μg/ml each of aprotinin, leupeptin and pepstatin A, rather than 0.1% BSA and 1 mM phenylmethylsulfonyl fluoride. Epibatidine (0.1 μM) was included with some of the sections to determine nonspecific binding. Slides were washed for 10 min in the HEPES salt buffer at room temperature, 10 min in ice-cold buffer, 2 x 10 min in 0.1× buffer (0°C), and 2 x 10 s at 4°C in deionized water. Sections were dried and exposed to Kodak MR film for 2-5 days with ^{125}I-standards.

^{125}I-α-Bungarotoxin Autoradiography. Thawed sections were preincubated in 50 mM

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Tris HCl, pH 7.0 for 2 x 15 min at room temperature (Quik et al., 2003). This was followed by a 1 h incubation in the same buffer plus 3 nM ^{125}I-α-bungarotoxin (128 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA. USA). Nicotine (0.1 mM) was added to consecutive sections to measure blank binding. The sections were then rinsed 4 x 15 min in ice-cold buffer, once in cold water, air-dried and placed against Kodak MR film for 2-5 days with ^{125}I-standards.

Quantitation and Data Analyses. A mouse brain atlas (Franklin and Paxinos, 1997) was used to identify brain regions. Optical densities from the different brain regions were quantitated using an ImageQuant system (Molecular Dynamics, Sunnyvale, CA). After background subtraction, the optical density values for the different brain areas were converted to fmol/mg tissue using standard curves generated from ^{125}I-standards. Results are expressed as mean $\pm$ S.E.M. of the indicated number of animals. Statistical analyses were done with GraphPad Prism (San Diego, CA) using one-way ANOVA followed by Newman-Keuls multiple comparison test or Student's t-test. A level of $p \leq 0.05$ was considered significant.

Dopamine Release Assay

Brains were removed, and the striatum dissected and placed into 0.5 ml of 0.32 M sucrose buffered with 5 mM HEPES, pH 7.5. The tissue (8-10 mg) was homogenized (16-20 strokes by hand), diluted to 2 ml with buffered sucrose, and divided into two aliquots, which were centrifuged for 20 min at 12,000*g*.

^{3}H-Dopamine Release. The release assay was performed according to the method of Grady et al. (2001). An aliquot of the striatal synaptosomal preparation was resuspended in 0.8 ml of uptake buffer (128 mM NaCl, 2.4 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES, pH 7.5, 10 mM glucose, 1 mM ascorbic acid, and 0.01 mM pargyline).

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The synaptosomes were incubated for 10 min at 37°C, followed by addition of 4 μCi of ^{3}H-dopamine (3,4-(ring-2,5,6)-^{3}H) at 30 to 60 Ci/mmol (Perkin Elmer Life Sciences), and a further 5 min incubation. Aliquots of labeled synaptosomes were distributed onto eight filters, which were each perfused at 1 ml/min with perfusion buffer (uptake buffer with 0.1% BSA and 10 μM nomifensine added) for 10 min before fraction collection. Release was initiated with an 18 s exposure to 20 mM K^{+} or to varying concentrations of nicotine (0.03, 0.1, 0.3, 1, 3, 10, and 30 μM). A second set of filters was pre-treated with 50 nM α-conotoxin MII for 3 min immediately prior to nicotine exposure. Fifteen fractions were collected per filter at 18 s intervals, which included fractions of basal release before and after the stimulated release.

Data Analysis. Fractions preceding and after the stimulated release were used to calculate basal release with SigmaPlot (Jandel Scientific, San Rafael, CA) using the first-order equation $R_t = R_0 (e^{-kt})$, where R_t is release at time t, R_0 is initial basal release, and k is the rate of decline of basal release. Theoretical basal release for fractions with stimulated release was calculated and subtracted to give the amount of stimulated release in each fraction. Fractions with significant stimulated release were summed to obtain nicotine-evoked ^{3}H-dopamine release. GraphPad Prism was used to generate dose response curves and perform statistical comparisons, which were done using one- or two-way ANOVA followed by Newman-Keuls multiple comparison test or Student's unpaired or paired t-test. A level of $p \leq 0.05$ was considered significant.

Immunoprecipitation of nAChR Subunits

Immunoabsorption of ^{125}I-epibatidine sites with nAChR subunit-specific monoclonal antibodies (mAbs) was done as described (Parker et al., 2004). Striata (20 mg tissue/ml) were homogenized in assay buffer pH 7.4 (containing 50 mM NaCl, 50 mM Na phosphate, 2 mM

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EDTA, 2 mM EGTA, and 2 mM phenylmethanesulfonyl fluoride) and centrifuged for 12 min at 20,000*g*. Pellets were resuspended in assay buffer supplemented with 10 μg/ml each of leupeptin, pepstatin A, and aprotinin, and then solubilized at 2% Triton X-100 for 90 min on ice. This was followed by centrifugation for 15 min at 20,000*g*. ^{125}I-Epibatidine (0.2 nM) was added to the supernatant and the mixture incubated at 4°C for 16-18 h. Rat mAbs (6 μg) against rat α4 (mAb 299) and β2 (mAb 295) subunits (from J.M.L) were added, as well as protein A/G agarose (Santa Cruz Biochemicals, Santa Cruz, CA), to each sample and the mixture rotated for 8 h at 4°C. The assays were terminated by dilution and sedimentation for 1 min at 1000*g* followed by three re-sedimentations in fresh assay buffer. Pellets were dispersed in buffer and the radioactivity measured by liquid scintillation counting. Nonspecific absorption was measured in the absence of the mAb.

The specificity of mAb 295 and mAb 299 for their target subunits (β2 and α4, respectively) was tested using the corresponding subunit-null mutant mice. Brains from 6 wild-type mice and 3 each of $\beta2^{-/-}$ and $\alpha4^{-/-}$ genotypes were dissected into 14 regions on an ice-cold platform. MAb 295 and 299 immunoabsorption assays were then done as described above, except that reactions were terminated by centrifugation and collection of the supernatant before pellets were washed. Both the supernatant and pellet were then assayed for ^{125}I-epibatidine binding sites. Pellet ^{125}I-epibatidine binding determinations were performed as detailed above. ^{125}I-Epibatidine binding sites in the supernatant were first precipitated by addition of polyethylene glycol (average molecular mass 8000; PEG-8000) to a final concentration of 20%, then collected by filtration onto polyethyleneimine-soaked (0.5% w.v) Gelman GF/F filter paper (Gelman Sciences, Ann Arbor, MI) using a 48-well format Inotech filtration apparatus (Inotech, Rockville, MD). Non-specific binding was defined using 1 mM nicotine. Control (no mAb)

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conditions were tested in parallel for each sample and in all cases the sum of supernatant and pellet binding matched the supernatant binding in the control samples. No ^{125}I-epibatidine binding was detected in any of the no-mAb control pellets.

Protein Determination. Protein was determined using the method of Lowry et al. (1951), with bovine serum albumin as standard.

Data Analysis. Radioactive counts were calculated by subtracting blank samples containing no mAb and normalizing to protein concentration. All values are expressed as the mean $\pm$ S.E.M. of the indicated number of animals.

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Results

Plasma Cotinine Levels with Nicotine Administration in the Drinking Water. Plasma levels of the nicotine metabolite cotinine were measured as an indirect marker of nicotine intake. Cotinine levels were assayed in blood taken from the orbital sinus 1 wk after the mice were on the maximal concentration of nicotine (300 μg/ml) in the drinking water. Plasma levels were 254 $\pm$ 29 (n = 11) ng/ml cotinine (Table 1) similar to those previously obtained by others (Sparks and Pauly, 1999). Plasma cotinine levels were not detectable when nicotine was removed from the drinking water 18 h prior to blood collection from the trunk during sacrifice, indicating that nicotine is fully metabolized during that time period in mice. Nicotine administration did not affect body weight (Table 1).

^{125}I-α-Conotoxin MII Binding in the Striatum is Selectively Decreased in Nicotine-Treated Mice. Fig. 1 depicts changes in nAChR binding in >8 month-old mice after 300 μg/ml nicotine treatment for 1, 2, 4, 5, and 6 wk. We observed a 30 $\pm$ 7.2% (n = 11) reduction in ^{125}I-α-

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conotoxin MII binding (α6*) in the striatum with 1 to 6 wk nicotine treatment (Fig. 1A). To determine whether this decrease was due to a change in affinity (K_d) or maximal number (B_{max}) of sites, saturation studies were done (Fig. 2). Saturation analyses done using striatal sections from four control and four nicotine-treated mice, respectively, yielded a significant decline ($p < 0.01$) in B_{max} from 6.45± 0.42 ($n = 4$) to 3.77 ± 0.49 ($n = 4$), with no change in K_d 1.25 ± 0.29 ($n = 4$) and 1.47 ± 0.52 ($n = 4$).

This contrasts with an increase in ^{125}I-epibatidine (multiple subtypes) and ^{125}I-A85380 (β2*) binding in both the striatum and cortex of nicotine-treated mice (Figs. 1B, 1D). The increases in both ^{125}I-epibatidine and ^{125}I-A85380 binding sites tended to be smaller in striatum than cortex with nicotine treatment. For instance, ^{125}I-A85380 binding in the cortex was maximally increased after two wk to 147 ± 5.2% ($n = 11$) of control whereas in the striatum it was increased to 127 ± 3.9% ($n = 11$) of control. It was also noted that the magnitude of the increases with nicotine treatment were generally larger for ^{125}I-A85380 compared to ^{125}I-epibatidine binding sites in both cortex and striatum. ^{125}I-α-Bungarotoxin binding (α7*) was not significantly affected by nicotine administration in either the striatum or cortex (Figs. 1C, 1E).

Age-Related Effect of Nicotinic Treatment in Younger Animals as Compared to Older Mice. The experiments described above were done in >8 month old mice. To determine whether age may affect drug responses, the experiments were also done in 2-4 month-old mice using a similar treatment regimen, that is, 300 μg/ml nicotine in the drinking water for 1, 2, 4, or 6 wk (Table 2). An increase was observed in ^{125}I-epibatidine and ^{125}I-A85380 binding in cortex and striatum with nicotine treatment at most time points. Again, a somewhat greater increase in binding was found for ^{125}I-A85380 than for ^{125}I-epibatidine binding in both the striatum and

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cortex, as observed in the older mice. However, although there was a trend for a decrease in ^{125}I-α-conotoxin MII binding in striatum in the younger mice, the differences were not statistically significant. ^{125}I-α-Bungarotoxin binding was unchanged in both brain areas in the younger animals after nicotine administration. Since we found more pronounced receptor changes in older mice, further studies were done using only older animals.

Subunit-Selective Immunoprecipitation Demonstrates an Increase in α4* and β2* nAChRs After Nicotine Treatment. As a complementary approach to the radioreceptor assays, immunoprecipitation experiments were also done to identify the striatal nAChR subtypes altered with nicotine treatment in older mice. These involved immunoabsorption of solubilized ^{125}I-epibatidine-bound receptors with mAbs against β2 (mAb 295) and α4 (mAb 299).

The specificity of mAbs 295 and 299 was tested using striatum and cortex, as well as other brain regions from wild-type and null-mutant mice (Fig. 3). In wildtype mice, both mAbs (6 μg per sample) immunoabsorbed the majority of ^{125}I-epibatidine binding sites from the regions tested, except for the interpeduncular nucleus, medial habenula, and olfactory bulbs, where they immunoabsorbed ~50% of sites (Fig. 3A, 3B). This outcome confirms the dominant expression of α4β2* nAChRs in most regions, with other subtypes such as α3β4* expressed in the interpeduncular nucleus, medial habenula, and olfactory bulbs (Whiteaker et al., 2002). Two-way ANOVA demonstrated that both region ($F(13,56) = 278$, $p < 0.001$) and mAb ($F(1,56) = 12.1$, $p < 0.002$) strongly influenced the amount of binding immunoabsorbed. There was also a strong interaction between the factors ($F(13,56) = 5.194$, $p < 0.001$), indicating that in some regions mAb 295 and 299 immunoabsorbed different amounts of ^{125}I-epibatidine binding sites. Subsequent t-tests in individual regions demonstrated that mAb 295 immunoprecipitation was

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more effective than that using mAb 299 in four regions (striatum ($p = 0.002$); cerebellum ($p = 0.050$); superior colliculus ($p = 0.015$); thalamus ($p = 0.001$), while no significant differences were measured in other regions. Of these regions, only superior colliculus exhibited a large absolute difference in binding following immunoprecipitation (supernatant ^{125}I-epibatidine binding after mAb 295 = 17.3 ± 1.9 fmol/mg protein; after mAb 299 = 118 ± 43 fmol/mg protein; difference = 101 fmol mg/protein), the maximum difference seen in other regions being that in striatum (supernatant ^{125}I-epibatidine binding after mAb 295 = 7.6 ± 0.5 fmol/mg protein; after mAb 299 = 14.1 ± 1.5 fmol/mg protein; difference = 6.5 fmol/mg protein). These regions would appear to express populations of non-α4-containing β2* nAChRs. In both superior colliculus and striatum, α-conotoxin MII-sensitive nAChRs would be likely candidates (Champtiaux et al., 2003; Champtiaux et al., 2002; Quik et al., 2001; Whiteaker et al., 2000; Zoli et al., 2002). In contrast to the extensive immunoprecipitation seen in wild-type preparations, neither mAb was able to immunoabsorb significant amounts of ^{125}I-epibatidine binding sites from regions lacking expression of the target subunit ($\beta2^{-/-}$ regions for mAb 295; $\alpha4^{-/-}$ regions for mAb 299). This demonstrates that both mAbs exhibit specificity for their target nAChR subunits. Experiments were also done with mAb 350, which interacts with α6* nAChR in rat brain (Parker et al., 2004). However, no specific signal was obtained using mouse control brain tissue, most likely due to a lower level of α6* nAChRs in this species (Parker et al., 2004).

Immunoprecipitation assays done using solubilized striatal membranes from control mice showed that maximal immunoabsorption occurred between 3 and 10 μg of each of the mAbs (data not shown), similar to previous results (Parker et al., 2004). Six μg of either mAb 295 or 299 was therefore used per sample. Studies were next done using solubilized striatal receptors prepared from mice receiving 300 μg/ml nicotine in the drinking water for 1.5 wk and the results

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compared to those with control animals (Table 3). Significant increases in immunoabsorption were observed with both mAb 295 (β2*) and mAb 299 (α4*). These results demonstrate that changes in subunit-specific immunoprecipitation were consistent with the binding studies.

Conotoxin MII-Sensitive But Not Resistant-Nicotine-Evoked ^{3}H-Dopamine Release in Striatum is Decreased by Chronic Nicotine Administration. To ascertain if chronic nicotine treatment altered nAChR function, we measured nicotine-evoked ^{3}H-dopamine release from synaptosomes prepared from striatum of >8 month old mice. A dose response curve (Fig. 4A) shows that receptor-stimulated release was significantly lower in synaptosomes prepared from nicotine-treated mice as compared to control, with a decline in the V_{max} value from 11.75 $\pm$ 0.8 (n = 8) to 9.43 $\pm$ 0.58 (n = 9), but similar EC_{50} values (1.19 μM in control mice versus 1.24 μM in nicotine mice). This effect was selective with no change in potassium-evoked or basal release following nicotine-treatment (Fig. 4B). To determine the contribution of α-conotoxin MII-sensitive and resistant nAChR subtypes, ^{3}H-dopamine release was measured in the absence and presence of 50 nM α-conotoxin MII. The results in Fig. 4 (C, D) show there was a significant decrease in total ^{3}H-dopamine release (28.1 $\pm$ 4.9%, n = 9) at a maximal (10^{-5} M) but not submaximal (10^{-6} M) nicotine concentration, in agreement with the results of the dose response curve. Moreover, they indicate that the decline was due to a decrease in α-conotoxin MII-sensitive (65.2 $\pm$ 15.9%, n = 9) and not α-conotoxin MII-resistant ^{3}H-dopamine release.

Discussion

The present results are the first to show that chronic nicotine administration in the drinking water results in a reduction in ^{125}I-α-conotoxin MII-sensitive nAChRs in mouse striatum. Moreover,

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this decline in binding is associated with a decrease in α-conotoxin MII-sensitive nicotine-evoked ^{3}H-dopamine release from striatal synaptosomes, suggesting that the receptor changes are of functional significance. Since studies using nAChR subunit null mutant mice show that striatal α-conotoxin MII-sensitive nAChR in mice express the α6 but not the α3 subunit (Champtiaux et al., 2003; Whiteaker et al., 2002), these data suggest that α6* nAChR sites and function are decreased in nicotine-treated mice.

The declines in striatal nAChRs with chronic nicotine exposure were selective for ^{125}I-α-conotoxin MII binding sites. There was no change in striatal α7 receptors, and an increase in ^{125}I-epibatidine and ^{125}I-A85380 binding sites, in agreement with previous studies (Nguyen et al., 2003; Parker et al., 2004; Pauly et al., 1991). One point of note is that the nicotine-induced increases in striatal ^{125}I-epibatidine binding sites were generally smaller than those in cortex consistent with earlier reports (Nguyen et al., 2003). The present results provide a possible explanation for this finding. Binding of ^{125}I-epibatidine represents an interaction with multiple nAChR subtypes (α2* to α6*). In striatum, this would consist of α-conotoxin MII-sensitive and α-conotoxin MII-resistant sites with the former decreased or not changed (present data) but the latter enhanced, as previously demonstrated using ^{3}H-nicotine or ^{3}H-cytisine binding (Collins et al., 1994; Marks et al., 1992; Pauly et al., 1991; Pauly et al., 1996). These combined changes would result in a smaller increase in overall striatal ^{125}I-epibatidine binding. In contrast, in cortex ^{125}I-epibatidine binds only to α-conotoxin MII-resistant sites, which are enhanced with nicotine treatment (Collins et al., 1994; Marks et al., 1992; Pauly et al., 1991; Pauly et al., 1996), yielding a comparatively larger increase in ^{125}I-epibatidine sites. A similar explanation would extend to the results with ^{125}I-A85380 which labels multiple β2* nAChR populations (Mukhin et al., 2000).

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Previous work had shown that nicotine administration led to a decrease in nAChR-mediated ^{3}H-dopamine release from striatal synaptosomes (Marks et al., 1993), despite an increase in high affinity ^{3}H-nicotine binding sites (α4β2* nAChRs) under the same conditions (Marks et al., 1992; Pauly et al., 1996). These combined findings had led to the hypothesis that the decline in nAChR-mediated activity in striatum was due to receptor (presumably α4β2*) desensitization in response to chronic agonist exposure. Since the present saturation studies clearly show that nicotine treatment results in a decrease in the maximal number of α-conotoxin MII sites, the decline in function is most likely due to a reduction in α6* nAChRs. Thus, the apparent dissociation between nAChR binding and functional activity in striatum may be related to opposing changes in multiple receptor subtypes.

The observation that α-conotoxin MII-sensitive receptors are decreased in response to chronic nicotine stimulation whereas other nAChRs, such as α4β2*, increase after the same treatment may suggest that different mechanisms regulate expression of the various nAChR subtypes. Marks and coworkers have suggested that upregulation of α4β2* subtypes by nicotine may be due to the establishment of an equilibrium between α4β2* receptor number and function to maintain an overall balance between these two parameters with chronic treatment (Marks et al., 2004). This contrasts with α7 nAChRs that appear relatively resistant to regulation by nicotine (Collins et al., 1994; Pauly et al., 1991; Pauly et al., 1996), as well as α3* nAChRs that are unchanged in both the central and peripheral nervous system (Davila-Garcia et al., 2003; Nguyen et al., 2003; Nguyen et al., 2004). α6* nAChRs may be regulated by yet another mechanism involving self-regulation of receptor levels by endogenous ligand similar to that for catecholamine receptors which are down-regulated after chronic agonist exposure (Creese and Sibley, 1981; Overstreet and Yamamura, 1979; Wonnacott, 1990).

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As an alternate approach to investigate changes in nAChR subtypes with nicotine treatment, studies were done using the anti-nAChR subunit mAbs 295 and 299. Since specificity of the mAbs used for immunoprecipitation is critical, we first showed that mAbs 295 (β2-directed) and mAb 299 (α4-directed) did not immunoabsorb residual nAChR binding sites in the corresponding knockout mice. The results show that there was an increase in sites that immunoabsorb to α4 and β2 directed-mAbs, consistent with the observed increase in ^{125}I-epibatidine and ^{125}I-A85380 binding.

In the present study we observed a decline in ^{125}I-α-conotoxin MII binding sites with chronic nicotine treatment, while other reports observed an increase or no change in α3* and/or α6* nAChRs with nicotine administration (Nguyen et al., 2003; Parker et al., 2004). This difference may relate to route of administration, although Salminen and coworkers also observed a decrease in ^{125}I-α-conotoxin MII binding in mice administered nicotine via chronic jugular infusion (Salminen et al., 2004b). Other important variables that have the potential to modulate drug-induced receptor expression include species (mice versus rats), method of determination of α-conotoxin binding sites and age. In fact, in the present study we show significant declines in α6* nAChRs only in older but not younger mice, although there was a trend for a decrease in the latter group. The age of the rats in the other studies appeared more similar to our young group of mice (Nguyen et al., 2003; Parker et al., 2004). This point is of relevance to studies that attempt to model human disease states since these generally occur in aged individuals. This includes neurodegenerative disorders such as Parkinson's and Alzheimer's disease that are characterized by deficits in the cholinergic system.

In summary, the present report shows that α6* nAChR sites in striatum are selectively decreased with nicotine administration in older mice. This treatment also resulted in a reduction

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in nAChR-mediated ^{3}H-dopamine release that appeared to be due primarily to a decline in function mediated through α-conotoxin MII-sensitive sites. These data suggest that the α6* nAChRs are important in the regulation of striatal dopamine release, particularly with chronic nicotine treatment which results in a reduction in both receptor sites and function. Knowledge of the changes in nAChRs after chronic nicotine treatment is critical for the development of subtype-selective therapies in Parkinson's disease and other neurological disorders with cholinergic deficits.

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Figure Legends

Fig. 1. Differential changes in nAChRs with nicotine treatment in older mice. Nicotine (300 μg/ml) was administered in the drinking water to mice (>8 month) for 1 to 6 wk. ^{125}I-α-conotoxin MII (CtxMII) binding in the striatum (A) was significantly decreased after 1 wk of chronic nicotine administration. In contrast, in cortex and striatum, ^{125}I-epibatidine and ^{125}I-A85380 binding (B, D) were both elevated with nicotine treatment, while ^{125}I-α-bungarotoxin (α-Bgt) binding (C, E) remained unchanged. Control binding for striatum was: 3.18 $\pm$ 0.18 fmol/mg (^{125}I-epibatidine), 7.89 $\pm$ 0.71 fmol/mg (^{125}I-A85380), 1.02 $\pm$ 0.04 fmol/mg (^{125}I-α-Conotoxin MII), and 6.46 $\pm$ 0.19 fmol/mg (^{125}I-α-bungarotoxin); and for cortex was 2.66 $\pm$ 0.18 fmol/mg (^{125}I-epibatidine), 4.33 $\pm$ 0.32 fmol/mg (^{125}I-A85380), and 6.72 $\pm$ 0.20 fmol/mg (^{125}I-α-bungarotoxin). Four or more animals were used for each time point, that is, at 1, 2, 4, 5 and 6 wk. This time course was then repeated with an additional 5 or more animals per group. The data represent the combined results of both these experiments, with each point representing the mean $\pm$ S.E.M. of 9-22 animals per group. Significance of difference from control, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 2. Saturation analyses and Scatchard plot (inset) of ^{125}I-α-conotoxin MII (^{125}I-α-CtxMII) binding to striatum from control and nicotine-treated mice. Mice (>8 month) were given saccharin or nicotine in the drinking water (300 μg/ml) for 2 weeks. Each value represents the mean $\pm$ SEM of 8-9 determinations at each concentration of ^{125}I-α-conotoxin MII. Saturation analyses yielded a B_{max} of 6.04 and 3.25 fmol/mg tissue, and K_d of 1.45 and 1.51 nM, for control and nicotine-treated mice, respectively.

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Fig. 3. Specificity of mAb 295 for β2* and mAb 299 for α4* nAChRs in immunoabsorption assays. Panels A and B show the effects of immunoabsorption of ^{125}I-epibatidine binding to solubilized nAChRs by mAbs 295 and 299, respectively, in wild-type mice. Both mAbs removed the majority of ^{125}I-epibatidine binding sites from each region tested, with the exception of IPN, MH and OB. Panels C and D show that mAbs 295 and 299 do not immunoabsorb the residual ^{125}I-epibatidine binding sites expressed in regions from $\beta2^{-/-}$ and $\alpha4^{-/-}$ null mutant mice, indicating that the mAbs do not interact with nAChR subunits other than β2 and α4, respectively. CX, cortex; ST, striatum; CB, cerebellum; HB, hindbrain; HP, hippocampus; HT, hypothalamus; IC, inferior colliculus; IPN, interpeduncular nucleus; MB, midbrain; MH, medial habenula; OB, olfactory bulb; OT, olfactory tubercle; SC, superior colliculus; TH, thalamus. Each bar represents the mean ± S.E.M. of 3 animals.

Fig. 4. Nicotine-evoked ^{3}H-dopamine release from striatal synaptosomes with chronic nicotine administration. Mice received nicotine (300 μg/ml) in the drinking water for 2 wk. Striatal synaptosomes were prepared as described in Methods. A dose response curve for total ^{3}H-dopamine release (A) showed there was a significant decrease in the V_{max} values in nicotine-treated mice, with no change in the EC_{50} values (1.68 ± 0.48 μM and 1.55 ± 0.35 μM for control and nicotine animals, respectively). Nicotine treatment did not significantly affect potassium-evoked or baseline ^{3}H-dopamine release (B). Analyses of α-conotoxin MII (50 nM)-resistant and α-conotoxin MII-sensitive nicotine-stimulated ^{3}H-dopamine release (C, D) showed that the decline in nicotine-evoked ^{3}H-dopamine release was due to a loss of the α-conotoxin MII nAChR response. Significance of difference from controls, * $p < 0.05$, *** $p < 0.001$.

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TABLE 1

Plasma cotinine levels in nicotine-treated mice

Mice (>8 month) were given 300 μg/ml nicotine in the drinking water. Blood was collected via the orbital sinus 1 wk after treatment with 300 μg/ml nicotine or from the trunk during sacrifice. Plasma levels of cotinine, the primary metabolite of nicotine, were significantly increased in mice treated with nicotine. Plasma cotinine levels were not detectable in mice when treatment was stopped 18 h prior to blood collection. Values expressed as mean ± S.E.M. for the indicated number of animals.

Treatment	**# Mice**	**Weight (g)**	**Time off nicotine (h)**	**Cotinine levels (ng/ml)**
Saccharin	12	35.7 ± 0.6	-	<10 .
Saccharin+Nicotine	11	32.6 ± 0.5	0	254 ± 29*
Saccharin+Nicotine	18	35.6 ± 0.3	18	<10 .

Significance of difference from saccharin, * $p < 0.001$.

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TABLE 2

Effect of nicotine administration on nAChRs in striatum and cortex of young mice

Mice (2-4 month) received nicotine (300 μg/ml) in 2% saccharin drinking water for 1-6 wk. Increases were observed in ^{125}I-A85380 and ^{125}I-epibatidine binding in cortex and striatum, with no change in ^{125}I-α-bungarotoxin (α-Bgt) binding. ^{125}I-α-conotoxin MII (α-CtxMII) binding sites in striatum were not significantly different from control. Control binding in striatum was 1.34 ± 0.38 fmol/mg (^{125}I-epibatidine), 4.13 ± 0.94 fmol/mg (^{125}I-A85380), 0.72 ± 0.05 fmol/mg (^{125}I-α-CtxMII), and 6.37 ± 0.14 fmol/mg (^{125}I-α-bungarotoxin), and for the cortex, 1.22 ± 0.33 fmol/mg (^{125}I-epibatidine), 3.01 ± 0.69 fmol/mg (^{125}I-A85380), and 7.29 ± 0.11 fmol/mg (^{125}I-α-bungarotoxin). ND, not detected. Values expressed as mean ± S.E.M. of the indicated number of animals.

Brain region	**Nicotine treatment (wk)**	**# mice**	**Specific binding (% control)**			
			^{125}I-α-CtxMII	**^{125}I-Epibatidine**	**^{125}I-A85380**	**^{125}I-α-Bgt**
Striatum	0	27-34	100.0 ± 2.3	100.0 ± 1.4	100.0 ± 1.3	100.0 ± 1.5
	1	14	86.2 ± 3.8	108.9 ± 1.8**	125.0 ± 3.9***	95.5 ± 2.7
	2	14	84.6 ± 7.1	94.2 ± 2.0	113.8 ± 1.1***	101.1 ± 1.8
	4	15	93.2 ± 9.2	107.0 ± 2.9**	113.9 ± 2.0***	103.7 ± 2.3
	6	15	89.5 ± 6.1	99.8 ± 1.3	113.6 ± 2.0***	98.5 ± 2.1
Cortex	0	20-27	ND	100.0 ± 1.5	100.0 ± 1.2	100 ± 0.9
	1	8-14	ND	121.4 ± 1.3***	137.7 ± 2.6***	91.8 ± 3.4
	2	8-14	ND	109.6 ± 1.4***	136.3 ± 2.1***	105.4 ± 1.2
	4	7-15	ND	121.5 ± 3.2***	130.3 ± 2.2***	106.3 ± 1.1
	6	9-15	ND	117.9 ± 2.2***	128.7 ± 2.8***	104.5 ± 1.6

Significance of difference from control, ** $p < 0.01$, *** $p < 0.001$.

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TABLE 3

Effect of nicotine administration on subunit-selective ^{125}I-epibatidine immunoprecipitation in striatal extracts from mice

Mice (>8 month) received nicotine (300 μg/ml) in 2% saccharin drinking water for 2 wk. MAbs specific to nAChR subunits were used to measure changes in receptor subtypes. There was an increase in both α4* and β2* nAChRs with nicotine treatment. Values expressed as mean ± S.E.M. of the indicated number of samples, each of which represents pooled tissue from 2 mice.

mAb	Subunit-selectivity	Treatment	N	^{125}I-Epibatidine binding	
				fmol/mg prot	% control
295	β2	Control	10	0.0176 ± 0.003	100 ± 8
		Nicotine	10	0.0219 ± 0.003	130 ± 7*
299	α4	Control	10	0.0079 ± 0.001	100 ± 12
		Nicotine	10	0.0147 ± 0.002	168 ± 16**

Significance of difference from control, * $p < 0.05$, ** $p < 0.01$.

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Fig. 1

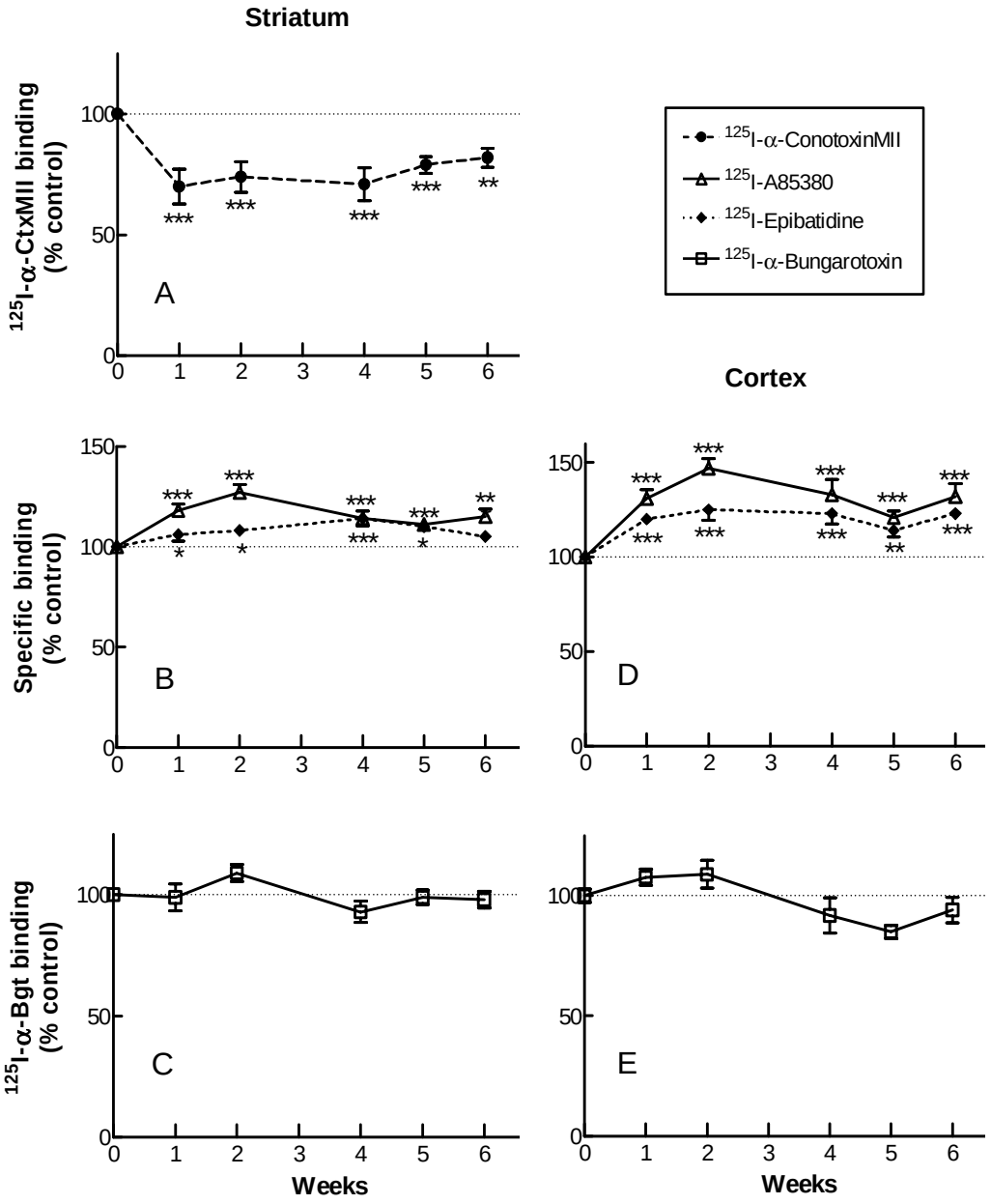

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Fig. 2

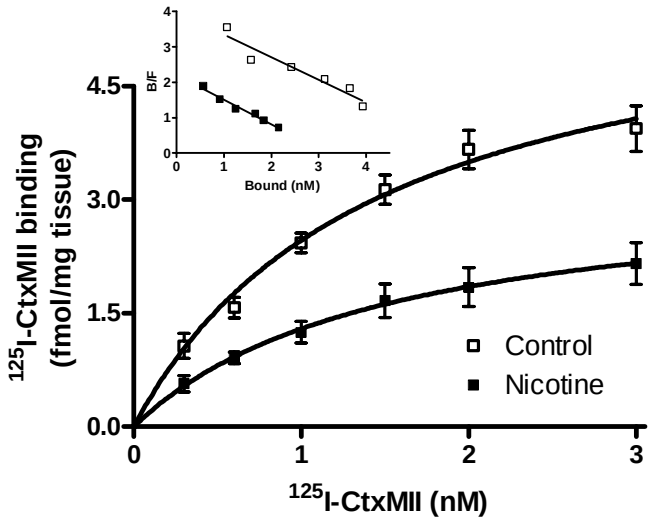

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Fig. 3

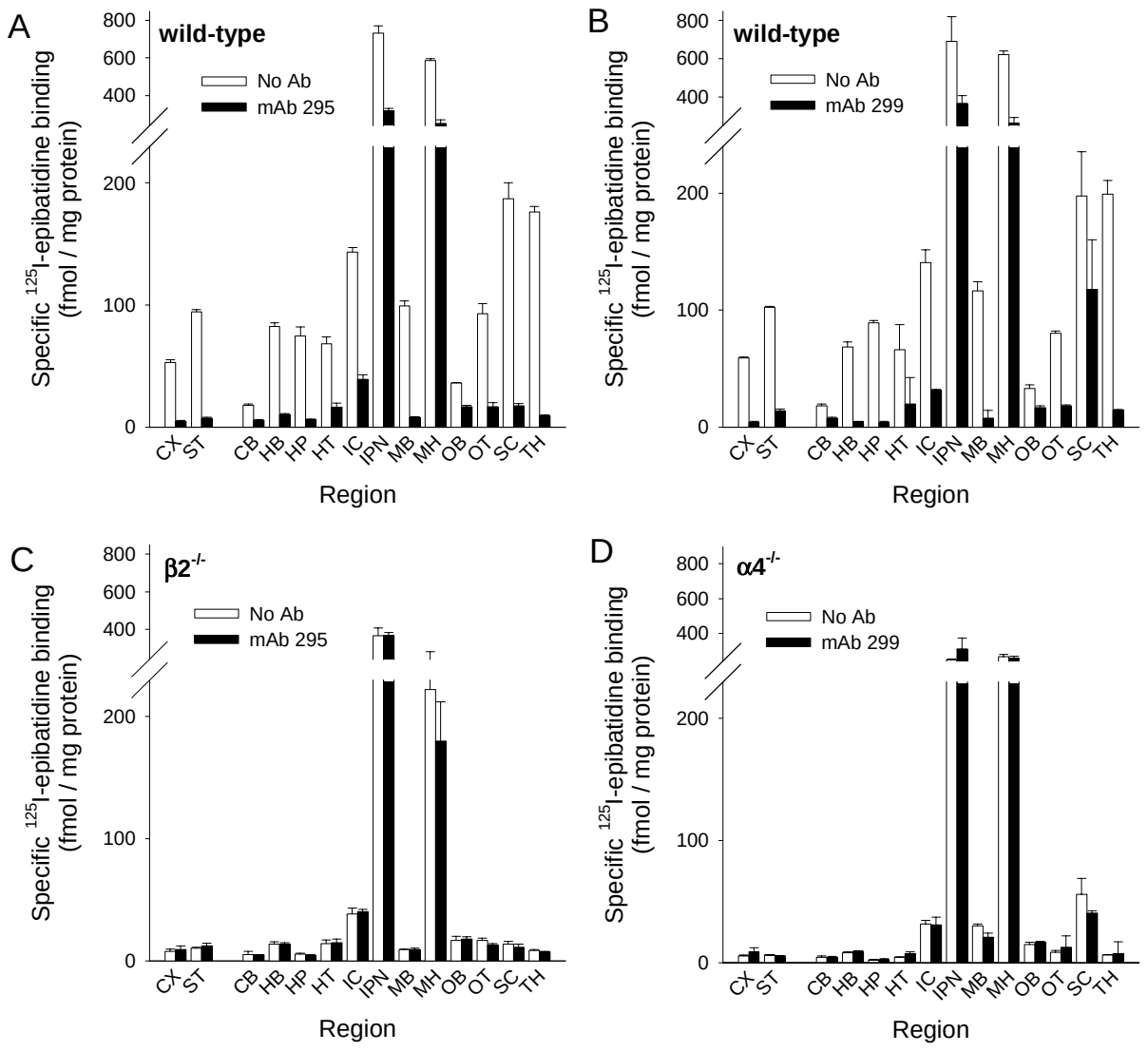

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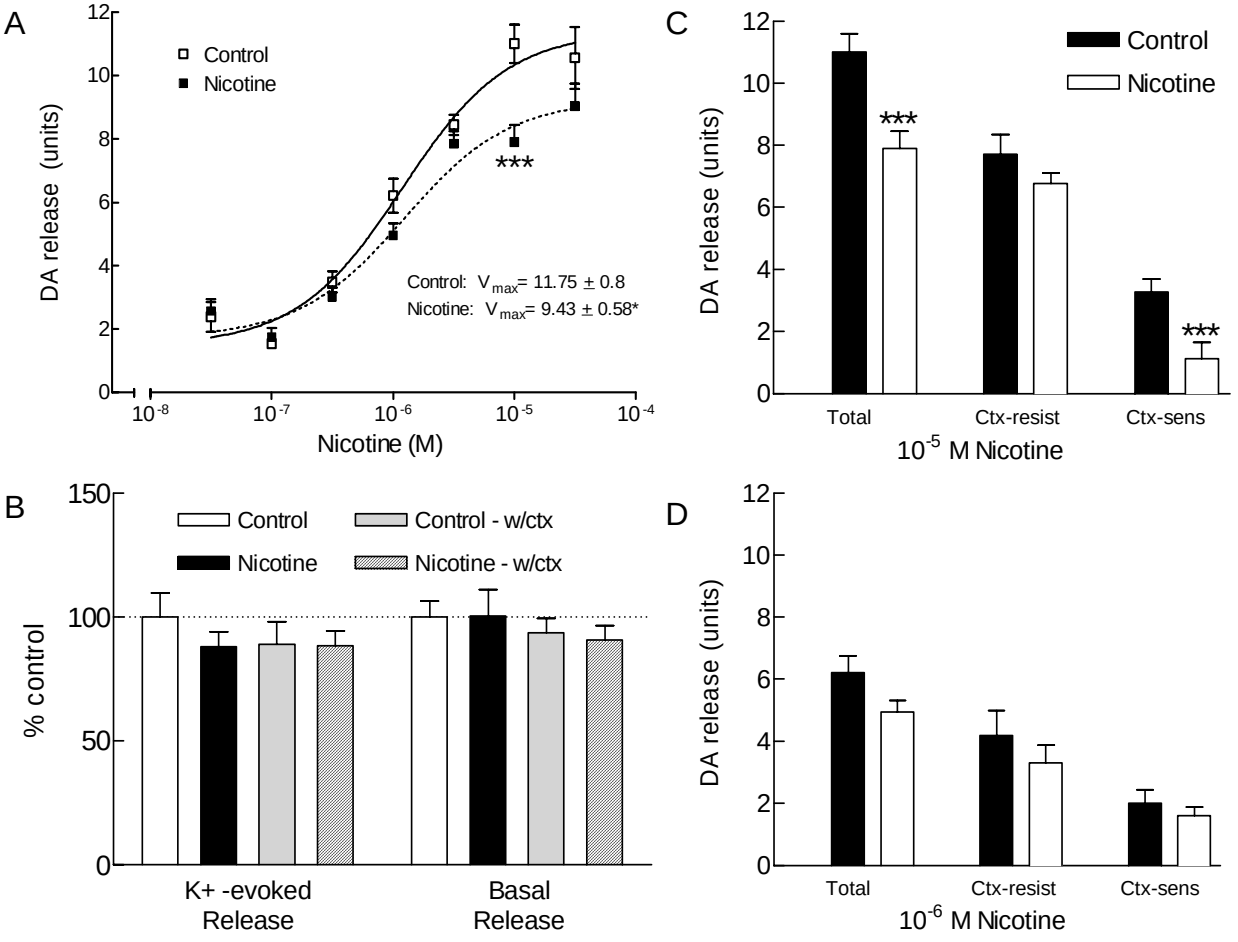
Fig. 4



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