Chronic Nicotine Differentially Regulates α6- and β3-containing Nicotinic Cholinergic Receptors in Rat Brain

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Non-standard abbreviations:

A-85380: 5-iodo-3-(2(S)-azetidinylmethoxy)pyridine

α-CtxMII: α-conotoxin MII

nAChR: neuronal nicotinic acetylcholine receptor

EB: epibatidine

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ABSTRACT

We investigated the effects of chronic nicotine on α6- and β3-containing nAChRs in two rat brain regions using three methodological approaches: radioligand binding, immunoprecipitation, and nicotine-stimulated synaptosomal release of dopamine. Nicotine was administered by osmotic minipumps for two weeks. Quantitative autoradiography with [^{125}I]α-conotoxin MII, to selectively label α6* nAChRs, showed a 28% decrease in binding in the striatum, but no change in the superior colliculus. Immunoprecipitation of nAChRs labeled by [^{3}H]epibatidine in these two regions showed that chronic nicotine increased α4- and β2-containing nAChRs by 39-67%. In contrast, chronic nicotine caused a 39% decrease in α6-containing nAChRs in striatum, but no change in superior colliculus. No changes in β3-containing nAChRs were seen in either region after chronic nicotine. The decreased expression of α6-containing nAChRs persisted for at least three days, recovering to baseline by seven days after removal of the pumps. There was a small but significant decrease in total nicotine-stimulated dopamine release in striatal synaptosomes after nicotine exposure. However, the component of dopamine release that was resistant to α-conotoxin MII blockade was unaffected, whereas dopamine release that was sensitive to blockade by α-conotoxin MII was decreased by 56%. These findings indicate that the α6* nAChR is regulated differently from other nAChR subtypes, and they suggest that the inclusion of a β3 subunit with α6 may serve to inhibit nicotine-induced down-regulation of these receptors.

INTRODUCTION

Nicotine regulates expression of neuronal nicotinic acetylcholine receptors (nAChRs) both in vitro and in vivo. Chronic exposure to nicotine increases nAChR binding sites in post-mortem brains from rats (Schwartz and Kellar, 1983); mice (Marks et al., 1983), non-human primates (McCallum et al., 2006a) and human smokers (Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999). And recently, increased nAChRs were imaged in living human subjects who smoked (Staley et al., 2006). Studies using subunit-selective antibodies (Flores et al., 1992) as well as subtype-selective autoradiography (Nguyen et al., 2003; Marks et al., 2004) and knockout mice (McCallum et al., 2006b) demonstrated that most of this up-regulation is due to an α4β2* subtype. The effect of chronic nicotine exposure on other subtypes of nAChRs is less well-established. The lower affinity α7* nAChRs are up-regulated, although to a lesser degree and in fewer regions (Pauly et al., 1991; Rasmussen and Perry, 2006). The α3β4* subtype, which is prominent in certain midbrain and throughout the brainstem nuclei, as well as in autonomic ganglia, appears to be resistant to regulation by nicotine exposure (Dávila-García et al., 2003; Nguyen et al., 2003).

Much recent attention has focused on a class of nAChRs sensitive to the cone snail toxin α-conotoxin MII (α-CtxMII). Although originally described as selective for α3β2 nAChRs (Cartier et al., 1996), more recent evidence indicates that the selectivity extends to nAChRs containing α6* subunits (Champtiaux et al., 2002; Whiteaker et al., 2000; Zoli et al., 2002). This nAChR subtype is localized largely to catecholaminergic regions, visual structures, and the habenulo-peduncular pathway (Quik et al., 2000; Whiteaker et al., 2000; Champtiaux et al., 2002; Le Novere et al., 1996). Although not the major nAChR subtype in striatum, α6-containing nAChRs contribute disproportionately to nicotine-stimulated release of dopamine (Kulak et al., 1997; Salminen et al., 2004; Kaiser et al., 1998), and they are selectively affected in Parkinson's disease (Quik et al., 2004). Our original attempts to determine the effect of chronic nicotine on these α3/α6* receptors

used an indirect method to deduce subtype populations based on differential sensitivity of [^{125}I]epibatidine autoradiography to the competing ligands cytisine and A-85380; we found little evidence for change in most brain regions, including striatum and superior colliculus (Nguyen et al., 2003). Subsequent studies addressed this problem using more direct binding and functional methods. Parker et al., using an antibody directed at the α6 subunit and homogenate binding of [^{125}I]epibatidine and [^{125}I]α-CtxMII, reported that α6 receptors in rat brain were disproportionately increased by long-term self-administration of nicotine (Parker et al., 2004). However, more recent studies reported a *decrease* in striatal [^{125}I]α-CtxMII binding in nicotine-treated rats (Mugnaini et al., 2006) and in both nicotine-stimulated dopamine release and [^{125}I]α-CtxMII striatal binding in nicotine-treated mice (Lai et al., 2005).

In this study we used a paradigm of long-term nicotine exposure that we previously demonstrated up-regulates both α7* and α4β2* nAChR binding sites in a wide variety of rat brain regions, but has no effect on α3β4* receptors (Nguyen et al., 2003; Rasmussen and Perry, 2006). We used three different approaches to assess the effect of nicotine on α3/α6* nAChRs: binding of [^{125}I]α-CtxMII and [^{125}I]A-85380; immunoprecipitation with a battery of subunit-selective antibodies; and determination of α-CtxMII-sensitive and α-CtxMII-resistant striatal dopamine release. Our results show that chronic nicotine exposure results in down-regulation of α6* nAChR numbers and function in rat striatum, but not in superior colliculus. Surprisingly, nAChRs containing β3 subunits in these regions appear to be unaffected by nicotine exposure, suggesting a complex regulatory mechanism for α6-containing nAChRs.

METHODS

Materials

Striatum and superior colliculus used to determine the nAChR subunit profiles were dissected from brains of adult Sprague-Dawley rats purchased from Zivic Miller laboratories (Portersville, PA). The brain regions were dissected on ice, quickly refrozen on dry ice and stored at -80°C for later use. Rabbit antisera directed at bacterially expressed fusion proteins containing partial sequences of the cytoplasmic domains of nAChR α2, α4, α5, β3 and β4 subunits were kind gifts from Drs. Scott Rogers and Lorise Gahring (University of Utah, Salt Lake City). These antisera have been described previously (Rogers et al., 1992) (Flores et al., 1992). An antibody directed at the C-terminal peptide sequence of the rat nAChR α3 subunit was affinity purified from rabbit serum. This antibody has been described previously (Yeh et al., 2001). A monoclonal antibody, mAb 270, to the chick β2 subunit was made from hybridoma stocks (American Type Culture Collection, Manassas, VA). This mAb was originally developed and characterized by Whiting and Lindstrom (Whiting and Lindstrom, 1987). The specificity of most of the antibodies in the immunoprecipitation procedures was reported previously (Hernandez et al., 2004; Marritt et al., 2005; Turner and Kellar, 2005). For the α6 subunit, the following peptide was synthesized (ResGen, Inc., Carlsbad, CA) from the cytoplasmic loop region of the rat nAChR α6 subunit: GVKDPKTHTKRPAKVKFTHRKEPKLLKEC (Champtiaux et al. 2003**)**. Rabbits were immunized with this peptide (Lampire Biologicals, Pipersville, PA), and the antibody produced was then affinity purified from the rabbit serum. Evidence for the specificity of this antibody in immunoprecipitation assays is provided in the Results section. For simplicity, in this paper we use the term antibody to refer to unpurified antisera, as well as to affinity purified antisera and monoclonal antibody.

Protein-G Sepharose beads were purchased from Amersham Biosciences Corporation (Piscataway, NJ). Normal rabbit serum (NRS) was purchased from Calbiochem (San Diego, CA). All other chemicals unless otherwise noted were obtained from Sigma Aldrich (St. Louis, MO). [^{3}H]Epibatidine ([^{3}H]EB; 55 Ci/mmol) and [^{125}I]A-85380 (2200 Ci/mmol) were obtained from Perkin Elmer Life & Analytical Sciences (Boston, MA). [7,8-^{3}H]Dopamine (40-60 Ci/mmol) was obtained from GE Healthcare (Piscataway, NJ). [^{125}I]α-CtxMII was synthesized by a reported method (Whiteaker et al., 2000). Tyr^{0}-α-CtxMII (25 nmol) was dissolved in 25 μl dH_2O; to this was added NH_4acetate (40 μl of 0.3 M, pH 5.3) and 10 μCi Na^{125}I (22 μl; Perkin Elmer Life and Analytical Sciences, Boston, MA). The reaction was initiated by addition of chloramine-T (40 μl of 0.4 mM), followed by incubation for 10 min at room temperature. The reaction was terminated by addition of ascorbic acid (65 μl, 0.5 M) followed by trifluoracetic acid (0.8 ml, 0.1%). The reaction mixture was then purified by HPLC using an analytical Vydac C18 reversed phase column. A 50 min linear gradient was run from 75% solvent A (0.1% trifluoracetic acid) to 75% solvent B (0.09% trifluoracetic acid, 60% acetonitrile) at 1 ml/min; absorbance was monitored 215 nm. Fractions were collected in polypropylene tubes containing 10 μl of 20 mg/ml lysozyme to decrease adsorption; fractions were then concentrated to dryness by vacuum centrifugation and resuspended in 40% methanol, and stored at -40°C until use. This protocol readily separates unreacted Tyr^{0}-α-CtxMII from the mono-iodo and di-iodo forms (Whiteaker et al., 2000); only the mono-iodo form was utilized, and based on the purification was assumed to be maximally iodinated (approx. 2200 Ci/mmol).

Animal Treatment

Osmotic minipumps (Alzet model 2002; Durect Corporation, Cupertino, CA) were filled with sterile saline or with nicotine hydrogen tartrate in saline, at concentrations calculated to achieve a dose of 6 mg/kg/day, calculated as nicotine free base (37 μmole/kg). We have previously found that this dose produced blood levels of 0.56 μM for nicotine and 3.5 μM for cotinine (Nguyen et al., 2004), which is comparable to levels achieved in humans who are moderate to heavy smokers (Rose et al., 1999). Others have reported that this same protocol using half the dose (i.e. 3 mg/kg/day free base) achieved brain levels of nicotine of approximately 1.5 μM (Mugnaini et al., 2006); nicotine has been shown to accumulate in the brain over time with continuous dosing (Ghosheh et al., 2001).

Male Sprague-Dawley rats (225-275g; Hilltop Lab Animals, Scottdale, PA) were anesthetized with isoflurane and the minipumps inserted into a subcutaneous pocket via a small incision made over the shoulders. While under anesthesia, animals were administered buprenorphine (0.1 mg/kg, s.c.) for post-operative pain. The wound was closed with clips and the area swabbed with antiseptic. After recovery from anesthetic (10-30 min), animals were returned to individual cages. Fourteen days after minipump implantation, animals were lightly anesthetized with isoflurane and either sacrificed by decapitation, or, to measure the reversibility of effects of nicotine on α6-containing receptors, the minipumps were removed and the rats were then sacrificed 1, 3, 7 or 30 days later. In those recovery experiments, the saline control rats were sacrificed as a group on day 14, so the results do not take into account any developmental changes that might take place over the following 30 days. However, since these rats were young adults, it is unlikely that such changes contributed significantly to the results. Animal use and procedures were approved by the George Washington University Medical Center Institutional Animal Care and Use Committee.

Autoradiography

Following decapitation, brains were rapidly removed and frozen on dry ice. Frozen coronal brain sections (16 μm) were cut and mounted onto Superfrost Plus slides (Fisher Scientific, Newark, DE) and stored at -80°C until use. Autoradiographic methods were adapted from the work of Whiteaker et al. (Whiteaker et al., 2000). Sections were preincubated for 15 min in buffer 1 (20 mM HEPES, pH 7.5, 144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM PMSF, 0.1 % BSA) at room temperature. This was followed by incubation for 60 min at room temperature in buffer 2 (= buffer 1 plus 5 mM EGTA, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml peptidase A, 10 μg/ml leupeptin) containing 0.8 nM [^{125}I]α-CtxMII. Adjacent sections were incubated in the same buffer with 100 μM nicotine added to determine non-specific binding. Slides were then rinsed for 5 min at room temperature in buffer 1, followed by 10 min in buffer 1 on ice, then sequential dips in ice-cold 5 mM HEPES and H_2O followed by rapid air-drying. For [^{125}I]A-85380, slides were first pre-incubated in buffer 1 for 15 min at room temperature, followed by incubation for 60 min at room temperature in buffer 1 with 0.6 nM [^{125}I]A-85380 plus 100 nM unlabeled α-CtxMII, followed by two five minute rinses in buffer 1, a water dip and rapid air-drying. Adjacent sections were incubated with the addition of 100 μM nicotine to determine non-specific binding. After overnight desiccation, the sections were apposed to film (Kodak BioMax MR) for 3-5 days along with ^{125}I standards (Amersham, Arlington Heights, IL); film was developed in an automatic developer. Film images were digitized and quantitative densitometric analysis of binding was done using the Loats Inquiry digital densitometry system (Loats Associates, Winchester, MD). Quantification of binding was done by comparison with standard curves constructed from ^{125}I standards; regions were identified by comparison with the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1998). Non-specific binding in adjacent sections was subtracted from the

total binding in the paired section to calculate specific binding. Values for specific binding of nicotine- and saline-treated animals were compared using student's t-test with a Bonferroni correction. Statistically significant differences between means were accepted at $p<0.05$.

Immunoprecipitation assays.

Brain tissues were homogenized in 50 mM Tris HCl buffer (pH 7.4 at 24°C), centrifuged twice at 35,000 x g for 10 minutes and the membrane pellets were resuspended in fresh buffer. The receptors were solubilized by incubating the homogenates in 2% Triton X-100 with gentle rotation for three hours at 4°C. After centrifuging the mixture at 35,000 x g for 10 minutes, aliquots of the clear supernatant from striatum (equivalent to 6 mg tissue) and superior colliculus (equivalent to 4 mg tissue) were added to sample tubes containing ~1.5 nM [^{3}H]EB. One of the subunit-specific antibodies at an optimal concentration, which had been determined previously, or an equivalent volume of normal rabbit serum (NRS), was added to each sample tube. The final volume of the assay was 150 µl. The samples were then rotated gently overnight at 4°C. After the addition of 50 µl of a 50% slurry of Protein-G Sepharose beads, rotation at 4°C was continued for another hour. The samples were then centrifuged at 12,000 x g for 1 minute and the supernatants were removed and filtered over GF/B filters that had been pre-wet with 0.5% polyethyleneimine. Radioactivity on the filters was then measured by liquid scintillation counting. The remaining pellets were washed by re-suspension in 1.2 ml 50 mM cold Tris HCl buffer (pH 7.0), followed by centrifugation at 12,000 x g for 1 minute. The pellets were then dissolved in 200 µl of 0.1N NaOH and the radioactivity was quantified by liquid scintillation.

The counts precipitated in tubes containing NRS, which was used as control for nonspecific precipitation, were subtracted from those in the pellets immunoprecipitated with antibodies. For determination of subunit profile, the calculated number of radiolabeled nAChRs

immunoprecipitated by each antibody was compared to the total number of [^{3}H]EB-labeled receptors, as measured in both the supernatants and the pellets after immunoprecipitation, and the data are presented as the percent of the total nAChRs immunoprecipitated. For comparison of the number of nAChRs immunoprecipitated by specific antibodies in tissues from saline- and nicotine-treated rats, data are presented as the calculated number of [^{3}H]EB-labeled nAChRs per mg tissue, which was carefully weighed before homogenization while still frozen.

A one-sample *t*-test was used to determine if residual values in immunoprecipitation assays were different from 0. Statistical analyses of the differences between group means were assessed using Student's *t*-test. The recovery of α6-containing receptors to control levels after exposure to nicotine was evaluated by regression analysis to determine the reversibility of the nicotine effects and to estimate its time course.

[^{3}H]Dopamine Release

The methods for measurements of [^{3}H]dopamine release were adapted from the work of Grady et al. (Grady et al., 1997). Immediately following decapitation, striatal tissue was removed and placed into ice-cold 0.32 M sucrose buffered with 5 mM HEPES at pH 7.5. Tissue was homogenized with 10-20 strokes of a Teflon homogenizer, then centrifuged 20 min at 12,000 x G. Pellets were resuspended in 1.6 ml 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, 10 μM pargyline) to create the synaptosomal mixture. This mixture was then incubated for 10 min at 37°C before addition of 100 nM [^{3}H]dopamine, followed by an additional 5 minute incubation at 37°C. All subsequent steps were done at room temperature.

Synaptosomes (80 μl aliquots) were applied to glass fiber filters and perfused with perfusion buffer (uptake buffer containing 0.1% BSA and 10 μM nomifensine) at 1 ml/min for 10

min before beginning collection of fractions. For some samples, the second 5 min of this perfusion included 50 nM α-CtxMII; this concentration and time were previously shown by Grady et al. (Salminen et al., 2004) to yield optimal inhibition of the α6-receptor component of release. Fractions were then collected every 18 sec for 4 min; nicotine-stimulated [^{3}H]dopamine release was obtained by perfusing with different concentrations of nicotine in perfusion buffer for a total of 1 min during the collection period. The radioactivity of the fractions as well as that remaining on the filter after perfusion was measured by scintillation counting.

Radioactivity in fractions immediately before and after the stimulated peak was used to calculate basal release as a single exponential decay (SigmaPlot 2001, SPSS, Inc., Chicago, IL). This basal release was then subtracted from the values in the peak (fractions that exceeded the baseline by 10% or more). Peak values were then summed and expressed as a percent of total counts (sum of all fractions collected plus filter radioactivity). Nicotine-stimulated release data were fit to a sigmoidal dose-response curve using Prism 4.0 (GraphPad Software, San Diego, CA), and E_{max} and EC_{50} values calculated. Comparison of treatment groups (i.e. +/- nicotine) was accomplished by an F test of the parameters generated from the fitted curves (differences accepted at $p<0.05$).

RESULTS

Receptor autoradiography

We used quantitative autoradiography to measure the effects of nicotine treatment on nAChR binding sites in the striatum and the superficial gray layer of the superior colliculus, two regions previously demonstrated to have relatively high fractions of α6/α3* receptor subtypes (Whiteaker et al., 2000; Salminen et al., 2004; Zoli et al., 2002; Gotti et al., 2005a; Gotti et al., 2005b). Adjacent sections from striatum and superior colliculus were labeled with either [^{125}I]α-CtxMII, or [^{125}I]A-85380 in the presence of 100 nM α-CtxMII. Although [^{125}I]α-CtxMII can bind to both α6* and α3β2* nAChRs (Cartier et al., 1996; Whiteaker et al., 2000; Whiteaker et al., 2002; Whiteaker et al., 2002;), in both of these brain regions [^{125}I]α-CtxMII binding appears to represent α6-containing receptors predominantly (Champtiaux et al., 2002; Whiteaker et al., 2002). [^{125}I]A-85380 binds to all β2-containing receptors (Mukhin et al., 2000; Xiao and Kellar, 2004); but by including α-CtxMII to mask binding to α6β2* and α3β2* nAChRs, it labels predominantly α4β2* nAChRs in these brain regions.

Representative autoradiographs of these two radioligands from these two brain regions in saline- and nicotine-treated rats are shown in Figures 1 and 2, and the results from quantitative analyses of the autoradiographic study are shown in Table 1. In the striatum from nicotine-treated rats, [^{125}I]α-CtxMII binding was reduced by 28% ($p<0.01$). In the superior colliculus, in contrast, no significant change in [^{125}I]α-CtxMII binding was found. Binding of [^{125}I]A-85380 in the presence of 100 nM of unlabeled α-CtxMII, which represents primarily α4β2* nAChRs, was significantly increased in the striatum from nicotine-treated rats; in the superior colliculus, there was a trend toward an increase that did not quite reach statistical significance.

Immunoprecipitation

The specificity of the α6 antibody for immunoprecipitation of nAChRs is shown in Table 2. It immunoprecipitated 23% and 28% of the nAChRs in the striatum and superior colliculus, respectively; while in the thalamus, less than 4% of the receptors were immunoprecipitated. No nAChRs were immunoprecipitated with the α6 antibody in the hippocampus, cerebral cortex or cerebellum, where no gene transcript or α6 subunit protein has been reported, but which do express α2, α3, α4, α5, β2 and β4 subunits of nAChRs. These results indicate that that this α6 antibody is highly selective for the α6 subunit of nAChRs.

We first determined the nAChR subunit profiles in rat striatum and superior colliculus by immunoprecipitation with antibodies selective for different nAChR subunits. As shown in Fig. 3, α4 and β2 subunits are the predominant subunits in striatum as well as superior colliculus. But both regions also have a significant fraction of heteromeric nAChRs containing the α6 subunit, ~23% and ~28% of the total nAChRs in striatum and superior colliculus, respectively. This is consistent with earlier studies using similar immunoprecipitation procedures (Zoli et al., 2002; Gotti et al., 2005b), as well as with the relatively high level of [^{125}I]α-CtxMII binding in these two regions, as shown in Figures 1 and 2 and also found previously (Champtiaux et al., 2002). A smaller but significant percentage of the nAChRs also contain the β3 subunit, which is associated primarily with the α6β2* subtype(s) in these brain regions (Cui et al., 2003; Champtiaux et al., 2003; Gotti et al., 2005b). Additionally, significant levels of receptors containing α3 and α5 subunits were detected in striatum and superior colliculus. No receptors containing α2 or β4 subunits were detected in striatum or superior colliculus. The absence of the β4 subunit in these brain areas indicates that both regions require β2 subunits to form agonist-binding sites for all their heteromeric nAChRs.

Results from immunoprecipitation assays in striatum from saline- and nicotine-treated rats are shown in Fig. 4. Because nAChRs containing α4 and β2 subunits are expressed at a much higher level than the nAChRs containing other subunits in striatum, the data for the receptors containing these predominant subunits and the less prevalent α6 and β3 subunits are presented in separate graphs with appropriate scales for easier visualization (Figs. 4A and 4B). Chronic exposure to nicotine increased the number of striatal nAChRs immunoprecipitated by the antibodies to α4 and the β2 subunits by ~40% each (Fig. 4A), consistent with the quantitative autoradiographic data for [^{125}I]A-85380 binding shown in Table 1. In contrast, immunoprecipitation with the antibody directed at the α6 subunit demonstrated a 40% decrease in the striatal nAChRs containing α6 subunits in nicotine-treated rats (Fig. 4B); this too is consistent with the quantitative autoradiographic data for [^{125}I]α-CtxMII binding in striatum (Table 1). Interestingly, no change was detected in the number of nAChRs containing β3 subunits in the nicotine-treated rats (Fig. 4B), indicating that chronic exposure to nicotine did not affect the β3-containing nAChRs in the striatum.

Results from immunoprecipitation assays in superior colliculus from saline- and nicotine-treated rats are shown in Figure 5. Again, for better visualization of the data, the results for the nAChRs containing the predominant α4 and β2 subunits and the less abundant α6 and β3 subunits are presented in separate graphs with different scales (Figs. 5A and 5B). The nicotine treatment increased the number of nAChRs immunoprecipitated by the α4 and the β2 antibodies by >50% (Fig. 5A). Interestingly, no significant change in the nAChRs containing α6 subunits was detected in the superior colliculus (Fig. 5B), which is in marked contrast to the striatum, but consistent with the [^{125}I]α-CtxMII autoradiographic data (Table 1). Again, as in striatum, the number of nAChRs immunoprecipitated with the β3 antibody in the superior colliculus was not changed by the nicotine treatment.

To assess the persistence of nicotine-induced down-regulation of α6-containing nAChRs in the striatum, rats were treated with saline or nicotine for 14 days and then either sacrificed immediately, or the pumps were removed and the rats were sacrificed 1 to 30 days later. The α6-containing nAChRs were then measured by immunoprecipitation assays. The α6-containing nAChRs were significantly decreased up to 3 days following the end of nicotine treatment but recovered to near control levels by 7 days and completely by 30 days after removal of the pumps (Fig. 6).

[^{3}H]Dopamine release

To determine whether chronic exposure to nicotine affected the function of nicotinic receptor subtypes, nicotine-stimulated release of [^{3}H]dopamine was measured in striatal synaptosomes from saline- and nicotine-treated rats, in the presence and absence of 50 nM α-CtxMII. In control (saline-treated) animals, 38% of total nicotine-stimulated release of [^{3}H]dopamine was inhibited by 50 nM α-CtxMII (compare values for saline treated groups in Figs.7A and 7B), which is consistent with the percentage of α-CtxMII-sensitive release reported by others in rat (Kulak et al., 1997; Kaiser et al., 1998; Cao et al., 2005) and mouse (Salminen et al., 2004) striatum. As shown in Fig. 7C, which is derived by subtracting the values in 7b from those in 7A, maximal α-CtxMII-sensitive [^{3}H]dopamine release was decreased by ~54% in nicotine-treated compared to saline-treated rats. (Although the EC_{50} value for α-CtxMII-sensitive release in saline controls appeared to be significantly higher than in nicotine-treated animals, because of the lower values and shallow slopes, the log scale might lead to exaggerated differences that are more apparent than real.)

DISCUSSION

nAChR up-regulation during chronic nicotine exposure has long been viewed as somewhat of a paradox; and we now know sensitivity to up-regulation in vivo varies by receptor subtype, with α4β2* receptors being highly sensitive, α7* receptors less sensitive, and α3β4* receptors virtually unaffected (Flores et al., 1997; Dávila-García et al., 2003; Nguyen et al. 2003). The development of α-CtxMII greatly facilitated the characterization of α6-containing nAChRs and aided studies of their regulation. In the striatum, these receptors assemble with β2 subunits (Salminen et al., 2005); thus, they might be expected to share sensitivity to up-regulation with the α4β2* subtype. In fact, The initial report of regulation of α6* nAChRs by chronic nicotine found an increase in these receptors in rat brain following 18 days of nicotine self-administration (Parker et al., 2004). However, subsequent studies in mice (Lai et al., 2005) and rats (Mugnaini et al., 2006) found a *decrease* in [^{125}I]α-CtxMII binding in brain following chronic nicotine exposure. To further address this question, we administered nicotine to rats chronically and measured α6 nAChRs three ways: by autoradiography with [^{125}I]α-CtxMII, by quantitative immunoprecipitation, and by nicotine-stimulated dopamine release from striatal synaptosomes. All three measurements yielded similar results, which lead to the conclusion that chronic nicotine exposure in this model causes either a decrease or no change in α6* nAChRs, depending on brain region. Furthermore, although β3 subunits are frequently associated with α6-containing nAChRs (Cui et al., 2003; Gotti et al., 2005a), immunoprecipitation studies indicate that the receptor subtypes containing β3 subunits are not decreased by nicotine treatment.

The decrease in binding we detected by [^{125}I]α-CtxMII autoradiography in rat striatum is consistent with that reported in mice after 1-6 weeks of nicotine given in drinking water (Lai et al., 2005) and in rats after 2 weeks of nicotine infused at 3 mg/kg/day via minipump (Mugnaini et al., 2006). In contrast to the decrease in the striatum, binding of [^{125}I]α-CtxMII in the superior

colliculus was unaffected by chronic nicotine exposure. The difference in the responsiveness to nicotine of putative α6-containing receptors in the striatum and superior colliculus confirms studies reported previously (Mugnaini et al., 2006). This difference is unlikely to be due to a difference in cellular location of the α6-containing receptors, since in both regions these receptors appear to be located exclusively on incoming axon terminals (Zoli et al., 2002; Gotti et al., 2005b; Cox et al., 2006). In contrast to its effect to decrease striatal α6-containing receptors, nicotine treatment increased striatal binding of [^{125}I]A-85380, which in the presence of excess α-CtxMII labels predominantly α4β2* nAChRs.

To further delineate the α6-containing nAChR subtype(s) affected by nicotine treatment, we immunoprecipitated [^{3}H]EB-labeled nAChRs with subunit-selective antibodies. We detected α6-containing receptors in significant numbers in both striatum and superior colliculus but few or none in the other regions surveyed, which is consistent with other studies (Whiteaker et al., 2000; Champtiaux et al., 2002). The effects of nicotine on α6-containing nAChRs measured by immunoprecipitation are entirely consistent with the autoradiography results—that is, down-regulation in striatum, but no change in superior colliculus. In contrast, the α4β2 nAChRs were markedly increased in both brain regions. The immunoprecipitation studies showed also that the down-regulation of α6 nAChRs in the striatum persists for at least three days after stopping nicotine administration. Interestingly, Mugnaini et al. (2006), using autoradiographic measurements, reported that nicotine-induced down-regulation of α6 nAChRs lasted longer in dopamine terminal fields than in cell body regions. This difference could reflect the time required to transport newly-synthesized receptors down the axons.

An important new finding revealed by our immunoprecipitation studies is that nicotine treatment does not change the density of the nAChRs containing β3 subunits. A majority of the β3 subunits is associated with α6 and β2 subunits in both the rat striatum and superior colliculus

(Champtiaux et al., 2003; Gotti et al., 2005a; Gotti et al., 2005b). Thus, nicotine's differential regulation of nAChRs containing α6 subunits versus those containing β3 subunits suggests that the population of α6β2* receptors labeled by [^{125}I]α -CtxMII is heterogeneous. Consistent with this, we found more nAChRs containing α6 subunits than β3 subunits in both brain regions. This agrees with results from an earlier study in the rat striatum (Zoli et al., 2002), but differs from a study in superior colliculus (Gotti et al., 2005b). Moreover, although knocking out the β3 subunit in mice decreased a majority of the [^{125}I]α-CtxMII binding sites in the striatum and a smaller fraction in the midbrain (Cui et al., 2003), a substantial fraction of these sites remained. Together, these data suggest that [^{125}I]α-CtxMII labels at least two populations of α6β2* nAChRs in the brain— one with the β3 subunit incorporated and the other without. Our immunoprecipitation studies distinguish between these β3-containing receptors in the striatum, and suggest that the α6β2β3* subtype does not down-regulate in response to nicotine in vivo.

The mechanisms underlying the decrease in α6-containing nAChRs are not yet known. But Lindstrom and colleagues (Tumkosit et al., 2006) have suggested that the decrease in α6β2* receptors and the increase in α4β2 receptors, as seen in the striatum, could be related; thus, if the availability of the β2 subunit is limiting and nicotine increases assembly of α4β2 nAChRs (Kuryatov et al., 2005; Sallette et al., 2005) the density of α6β2* nAChRs might be decreased because of depletion of the pool of β2 subunits (Tumkosit et al., 2006). In heterologous cell models, the presence of the β3 subunit increases the expression of α6β2β3 receptors, suggesting that the β3 subunit stabilizes and/or allows more efficient assembly of this receptor subtype (Tumkosit et al., 2006). This might then explain why β3-containing nAChRs in the striatum are not decreased by nicotine.

The superior colliculus also appears to contain a larger fraction of nAChRs with α6 subunits than β3 subunits, again indicating that some α6 receptors contain β3 subunits and some do

not. Yet there was no measurable change in either [^{125}I]α-CtxMII binding sites or nAChRs immunoprecipitated by the α6 or β3 antibodies, suggesting that even in the absence of the β3 subunit, some α6-containing receptors are resistant to nicotine-induced down-regulation. Most of the nAChRs in the superior colliculus are on retinal ganglion cell axons that innervate the superficial layers of the colliculus (Gotti et al., 2005b; Cox et al., 2006). Therefore, the absence of a nicotine-induced decrease in α6-containing receptors in the superior colliculus even while α4β2 receptors are increased suggests that these receptors are expressed by different retina ganglion cells.

Because stimulation of nAChRs in dopamine terminal regions enhances dopamine release, we assessed this activity in striatal synaptosomes prepared from rats treated with nicotine or saline. Our finding that 38% of the nicotine-stimulated dopamine release in saline-treated animals is blocked by α-CtxMII is consistent with reports of from 30-70% block in rodents and monkeys (Kaiser et al., 1998; McCallum et al., 2005; Champtiaux et al., 2003; Salminen et al., 2004). Neurotoxin studies demonstrate that α6-containing nAChRs in this region are localized to dopamine terminals, whereas non-α6 nAChRs are found on multiple cell types (Zoli et al., 2002; Champtiaux et al., 2003). This probably explains why the proportion of α-CtxMII-sensitive dopamine release is higher than would be expected by the overall proportion of α6-containing nAChRs. Our finding of a 54% decrease in the E_{max} for nicotine-stimulated dopamine release confirms that the decrease in α6* receptor number is matched by a change in receptor function in striatum. Similarly, in mice treated chronically with oral nicotine, α-CtxMII-sensitive striatal dopamine release declined 65% (Lai et al., 2005).

Chronic nicotine exposure had essentially no effect on α-CtxMII-resistant dopamine release. Similar results were reported in mice exposed to chronic oral nicotine (Lai et al., 2005). This may seem surprising in light of the increase in α4β2* nAChRs; however, a large fraction of

these receptors are not located on dopamine terminals (Champtiaux et al., 2003; Zoli et al., 2002) and thus do not directly mediate dopamine release. Furthermore, a significant fraction of striatal α4β2* receptors co-express the α5 subunit, and virtually all of these α4β2α5 receptors are located on dopamine terminals (Zoli et al., 2002; Champtiaux et al., 2003). Interestingly, we recently found that α4β2 nAChRs that contain the α5 subunit are not up-regulated by nicotine treatment in vivo (Perry et al., 2005). Thus, despite the overall increase in striatal α4β2* nAChRs induced by chronic nicotine, it seems likely that the α4β2* nAChRs on dopamine terminals are not up-regulated by chronic nicotine. In fact, the nicotine-induced release of dopamine *in vivo* appears to be mediated by α4β2 nAChRs in the cell body areas (Corrigall et al., 1994; Nisell et al., 1997; Champtiaux et al., 2003).

In conclusion, we have used both receptor autoradiography and subunit-selective immunoprecipitation to demonstrate that chronic exposure of rats to nicotine leads to a decrease in α6-containing nAChRs in the striatum, but not in the superior colliculus. The presence of the β3 subunit and perhaps other subunits in the α6-containing receptors appears to modulate the regulatory effects of nicotine on these receptors. The decrease in striatal α6* nAChRs persisted for at least three days following termination of nicotine, and was accompanied by a decline in α-CtxMII-sensitive dopamine release in striatal synaptosomes, demonstrating the functional significance of the decrease in receptor number. The shift in the nicotinic receptor profile following such exposure may be relevant to nicotine dependence and its treatment.

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LEGENDS FOR FIGURES

Figure 1. Representative autoradiographic images from coronal rat brain sections through striatum.

A-C: [^{125}I]α-CtxMII binding; D-E: [^{125}I]A-85,380 binding in presence of 100 nM α-CtxMII. A, D: Saline-treated animal, total binding. B, E: Nicotine-treated animal, total binding. C, F: non-specific binding.

Figure 2. Representative autoradiographic images from coronal rat brain sections through superior colliculus.

A-C: [^{125}I]α-CtxMII binding; D-E: [^{125}I]A-85,380 binding in the presence of 100 nM α-CtxMII. A, D: Saline-treated animal, total binding. B, E: Nicotine-treated animal, total binding. C, F: non-specific binding. SuG: superficial gray layer, superior colliculus.

Figure 3. Subunit profiles of heteromeric nAChRs in striatum and superior colliculus.

nAChRs from A) striatum and B) superior colliculus were solubilized, labeled with [^{3}H]EB, and immunoprecipitated with each of the subunit-specific antibodies shown. Non-specific immunoprecipitation was measured with normal rabbit serum (NRS) and has been subtracted. Data are mean ± SEM from 3 to 6 experiments.

Figure 4. Effects of 14 days chronic nicotine exposure on nAChRs in rat striatum labeled by [^{3}H]EB and immunoprecipitated by various antibodies.

A. nAChRs immunoprecipitated by α4 and by β2 antibodies (n=5 for each). B. nAChRs immunoprecipitated by α6 and β3 antibodies (n=18 for each). Note different y-axis scale in A and B. Different from saline controls, **p<0.01; ***p<0.001.

Figure 5. Effects of 14 days chronic nicotine exposure on nAChRs in rat superior colliculus labeled by [^{3}H]EB and immunoprecipitated by various antibodies.

A. nAChRs immunoprecipitated by α4 and by β2 antibodies (n=5 for each). B. nAChRs immunoprecipitated by α6 and β3 antibodies (n=18 for each). Note different y-axis scale in A and B. Different from saline controls, ***p<0.001.

Figure 6. Recovery from effects of chronic nicotine exposure on nAChRs in rat striatum labeled by [^{3}H]EB and immunoprecipitated by α6 antibodies.

After 14 days treatment by osmotic minipumps, animals (n=5-6) were either sacrificed (saline; Nic+0) or pumps removed, followed by sacrifice at indicated days (+1, +3, +7, +30). Different from saline controls, *p<0.05.

Figure 7. Effects of chronic nicotine exposure on release of [^{3}H]dopamine from rat striatal synaptosomes.

Data points represent percent of total tissue [^{3}H]dopamine per fraction, +/- S.E.M.; n=9 for each treatment group. Lines represent best fit of data to a sigmoidal dose-response curve. **A.** Total release; for saline-treated: E_{max} 3.85 +/- 0.13; EC_{50} 0.13 μM; for nicotine-treated, *E_{max} 3.33+/- 0.13; EC_{50} 0.14 μM. **B**. α-CtxMII-resistant release (in presence of 50 nM α-CtxMII); for saline-treated: E_{max} 2.39+/-0.10; EC_{50} 0.12 μM; for nicotine-treated, E_{max} 2.53+/-0.11; EC_{50} 0.18 μM. **C.** α-CtxMII-sensitive release (calculated by A-B for each nicotine concentration); for saline-treated: E_{max} 1.95+/-0.14; EC_{50} 5.9 μM; for nicotine-treated, *E_{max} 0.90+/- 0.14; *EC_{50} 0.91 μM. *Different from saline controls, p<0.05.

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Table 1. Autoradiographic binding in striatum and superior colliculus in rats treated chronically with saline or nicotine.

	Striatum			Superior Colliculus		
	Saline	Nicotine	%change	Saline	Nicotine	%change
[^{125}I]αCtxMII	0.40 ± 0.03	0.29 ± 0.03*	-28%	4.1 ± 0.23	4.0 ± 0.11	-2%
[^{125}I]A-85,380	1.3 ± 0.14	2.0 ± 0.19**	+54%	2.3 ± 0.20	2.8 ± 0.09	+22%

Values represent means +/- S.E.M. of specific binding (fmol/mg) of [^{125}I]-α-CtxMII, or [^{125}I]A-85,380 in the presence of 100nM unlabeled α-CtxMII, measured in the striatum and the superficial gray layer of the superior colliculus (N=7-8). Significantly different from saline-treated animals, *$p<0.05$; **$p<0.01$.

Table 2. Specificity of the α6 antibody.

Region	% of total heteromeric nAChRs immunoprecipitated
Striatum	23.0* ± 0.4
Superior Colliculus	28.5* ± 1.2
Thalamus	3.5* ± 0.7
Hippocampus	0 ± 0
Cerebral Cortex	0.3 ± 0.3
Cerebellum	2.6 ± 3.2

*significantly different from zero, $p<0.05$; $N\geq3$

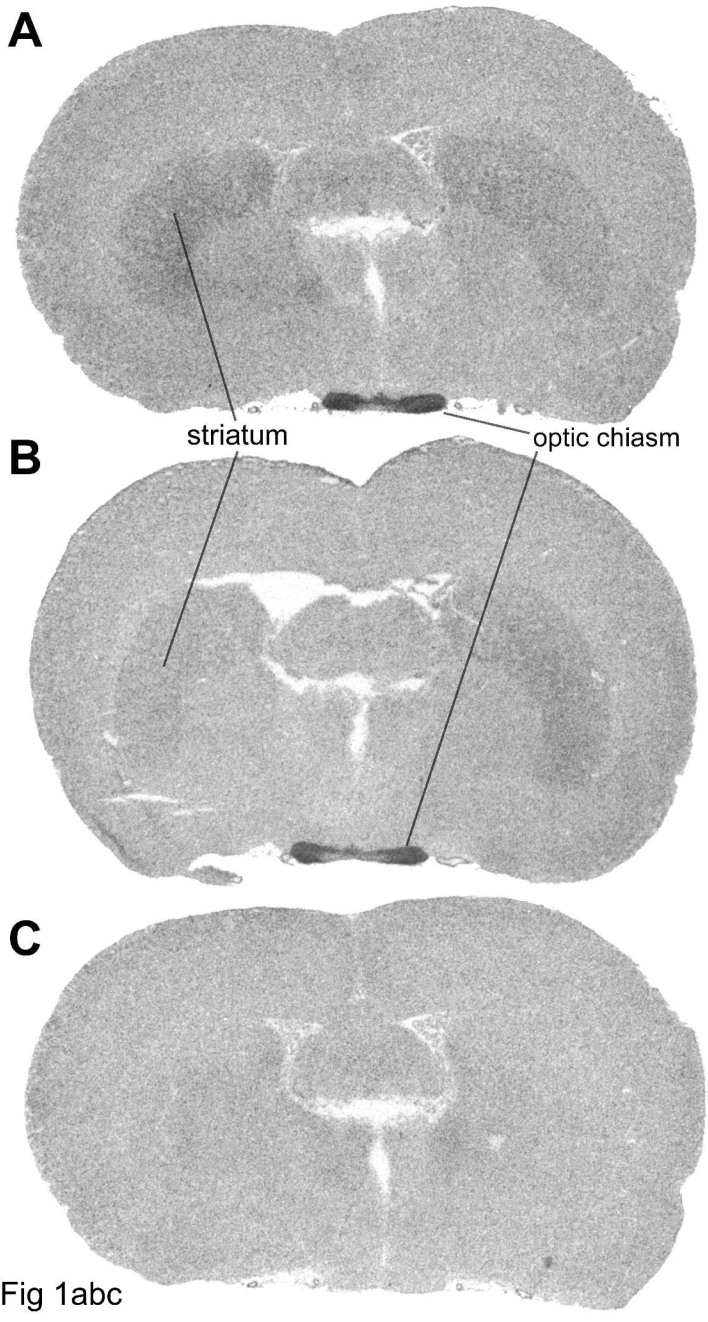


Fig 1abc

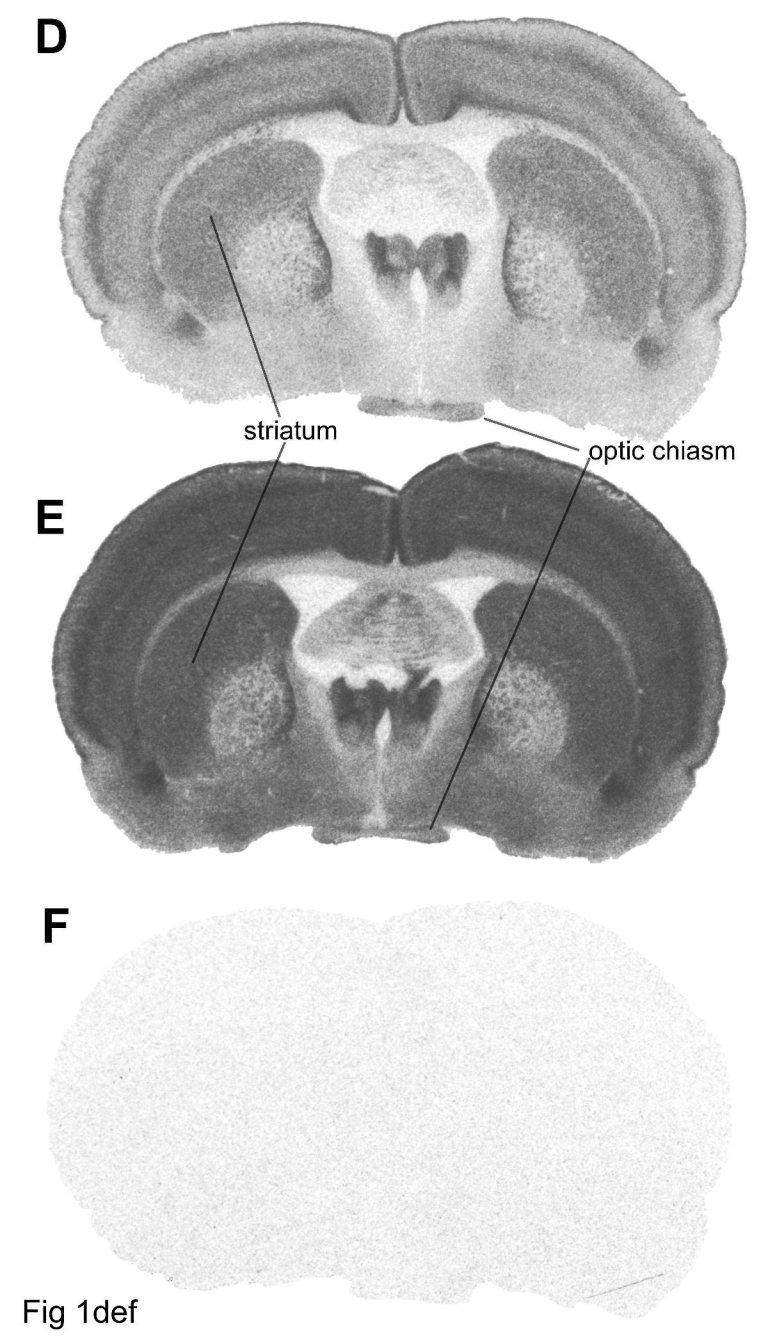
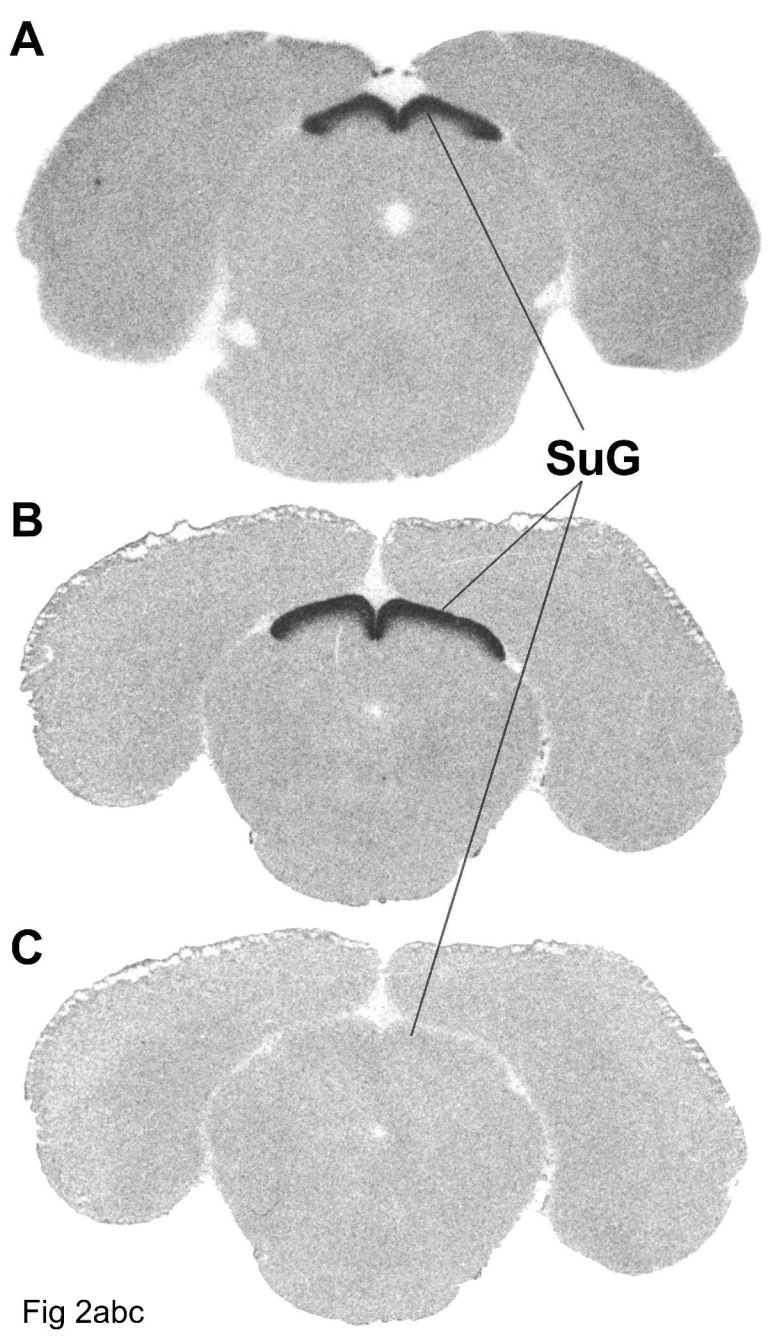


Fig 1def



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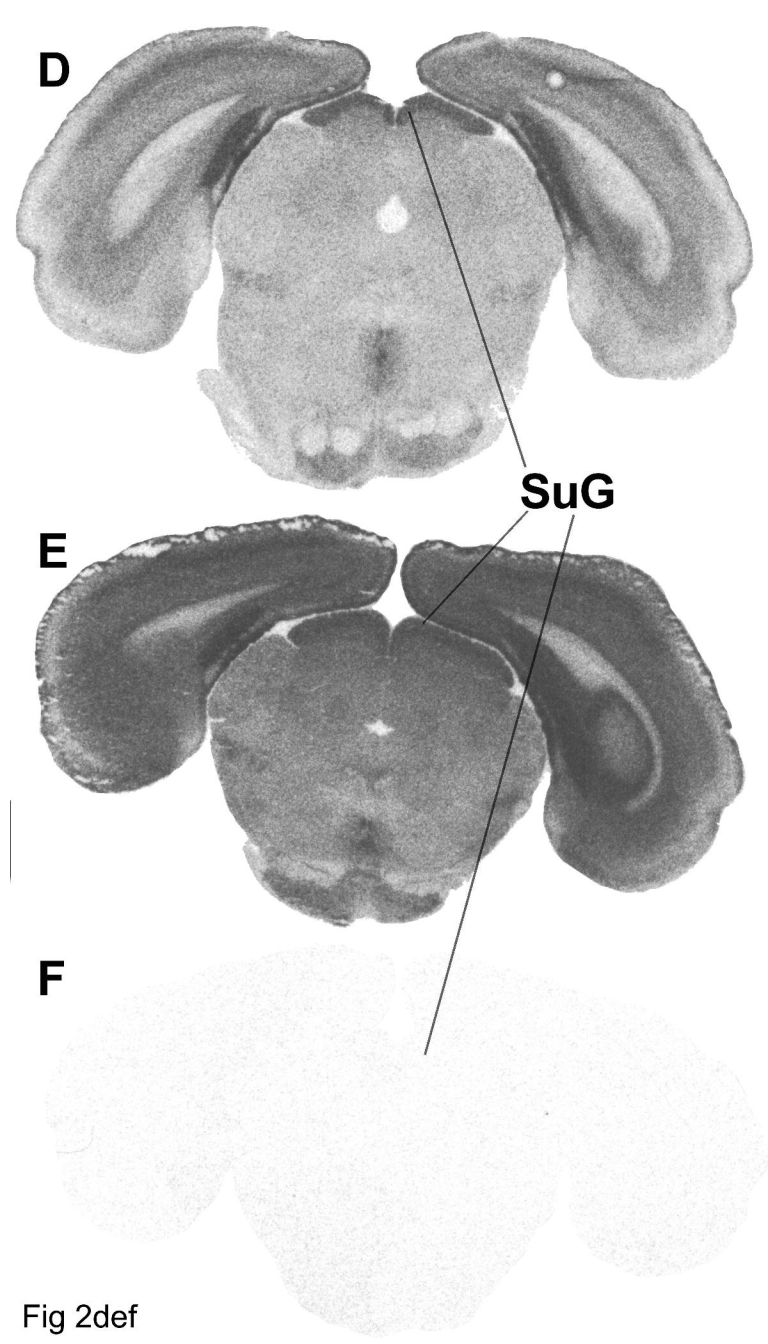

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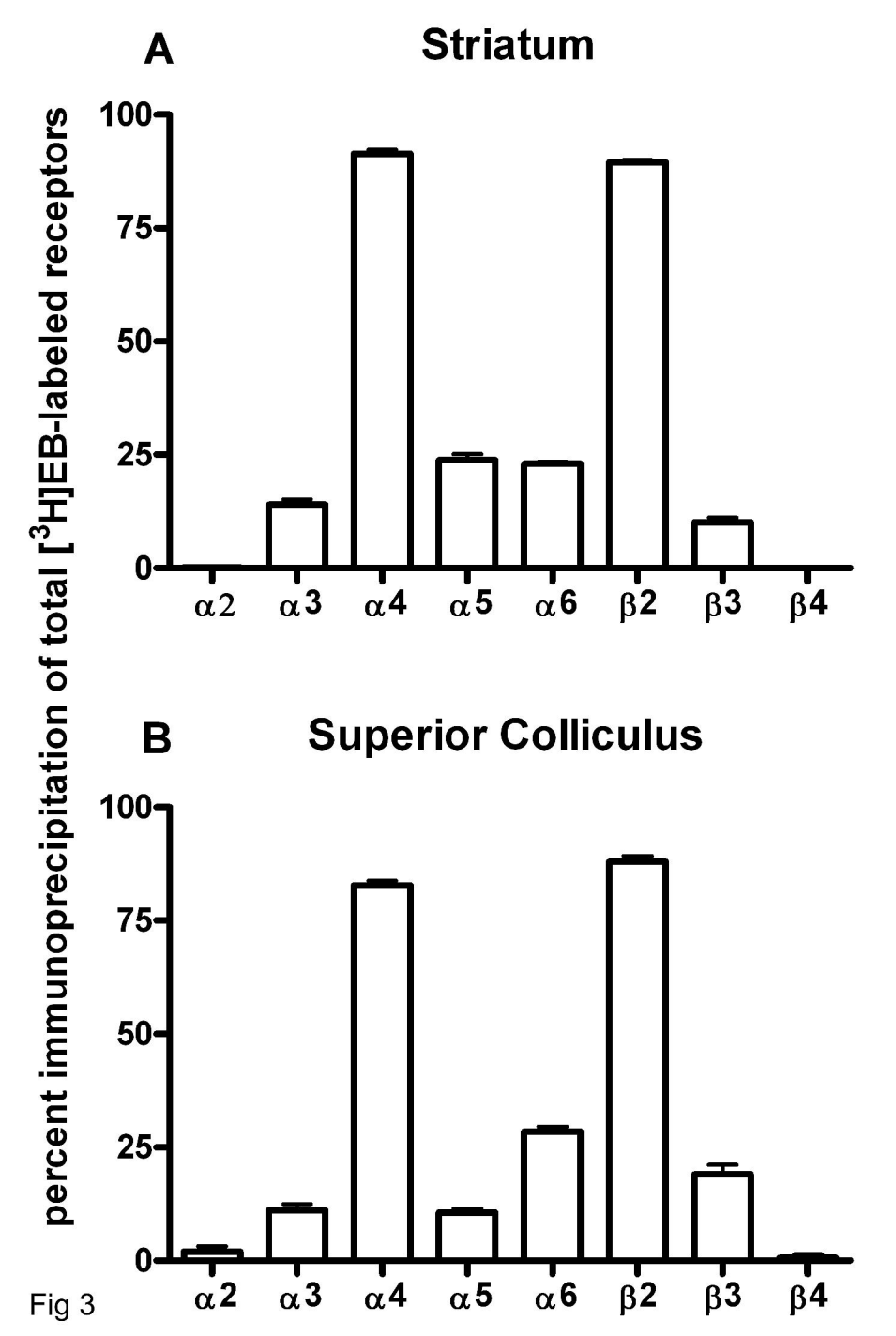
A
Striatum
100
75
50
25
0
percent immunoprecipitation of total [3H]EB-labeled receptors
α2
α3
α4
α5
α6
β2
β3
β4
B
Superior Colliculus
100
75
50
25
0
α2
α3
α4
α5
α6
β2
β3
β4

Fig 3

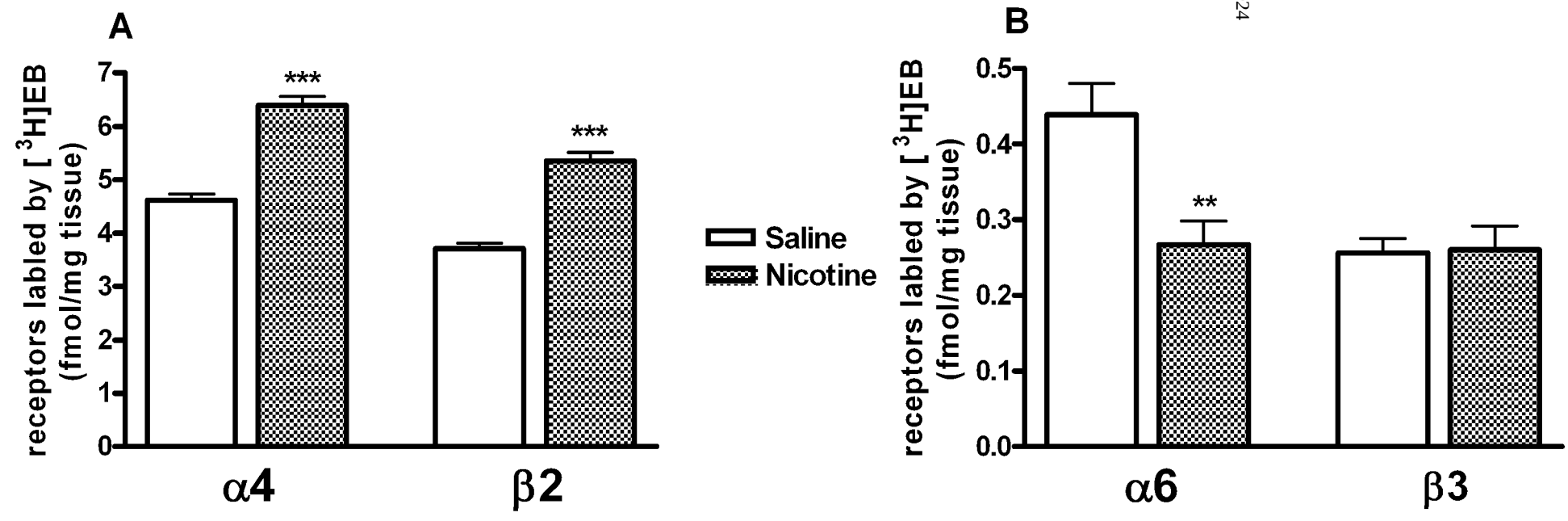

Fig 4

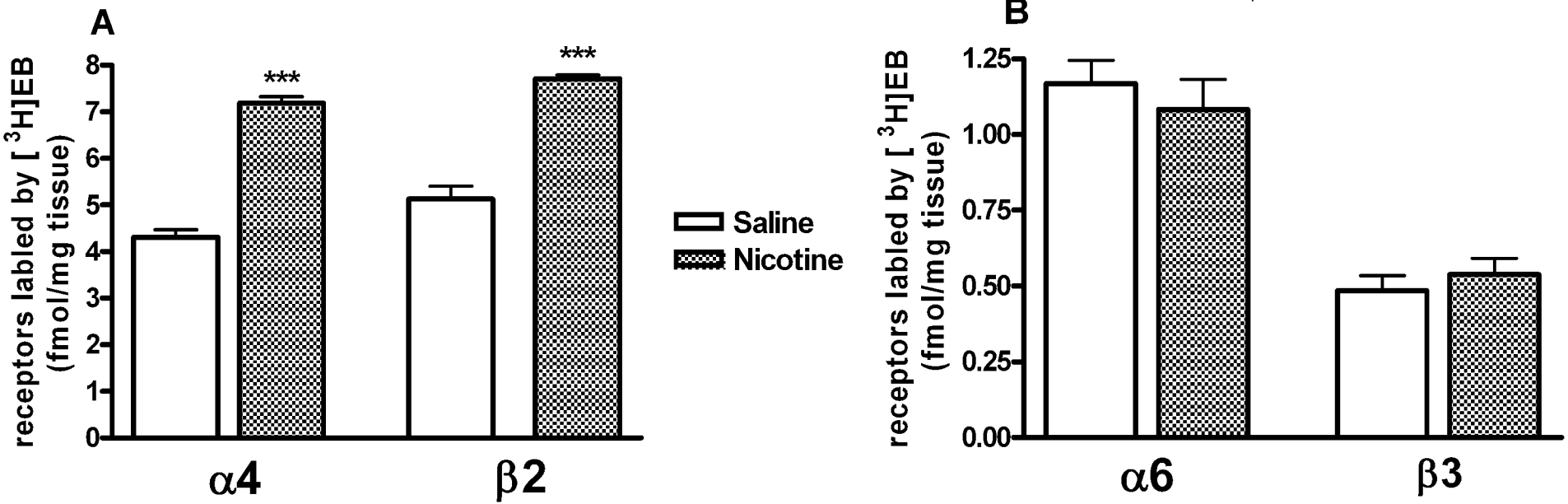
A
receptors labled by [3H]EB
(fmol/mg tissue)
0
1
2
3
4
5
6
7
8
***
***
α4
β2
Saline
Nicotine
B
receptors labled by [3H]EB
(fmol/mg tissue)
0.00
0.25
0.50
0.75
1.00
1.25
α6
β3

Fig 5

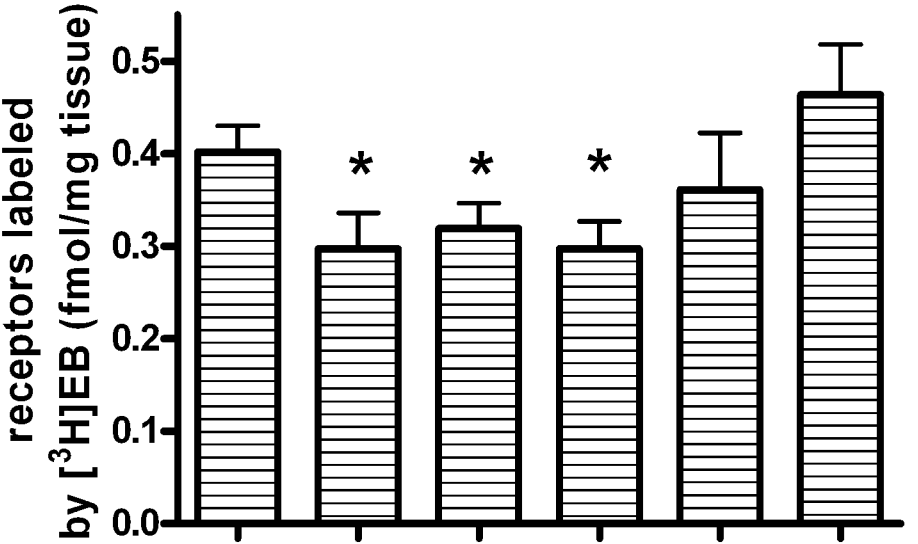
receptors labeled
by [3H]EB (fmol/mg tissue)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
saline
NIC +0
+1
+3
+7
+30

Fig 6

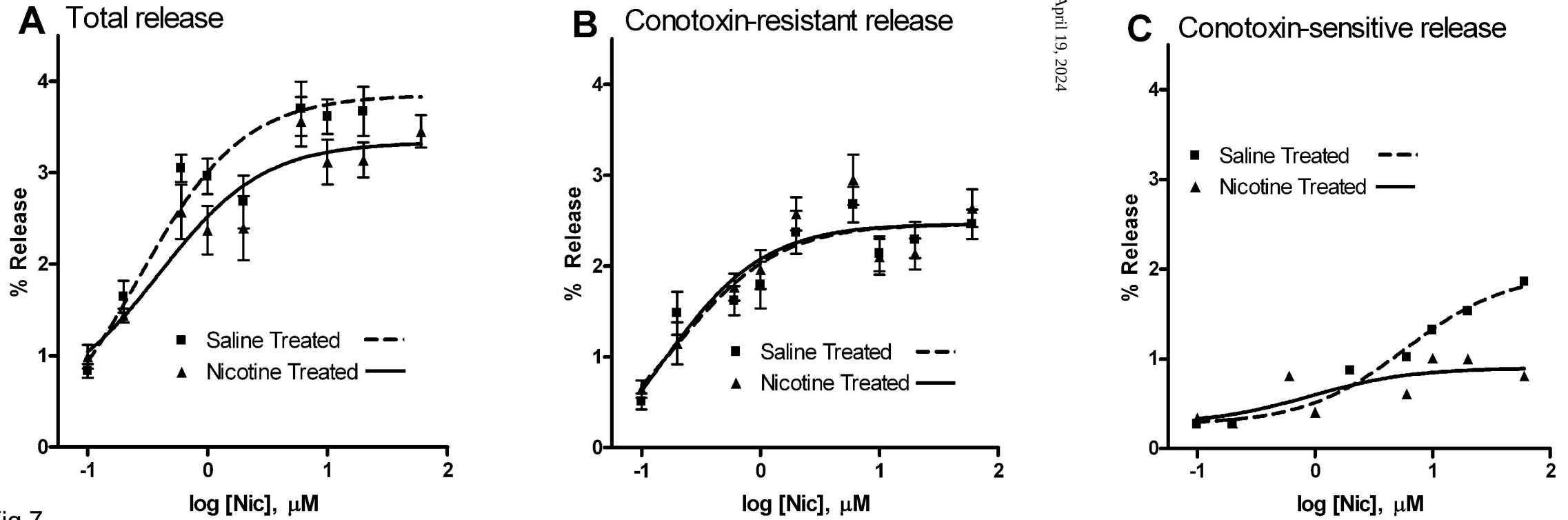

Fig 7