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Effect of novel α-conotoxins on nicotine-stimulated [^{3}H]dopamine release from rat striatal synaptosomes

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ABSTRACT

Nicotine's action on the midbrain dopaminergic neurons is mediated by nicotinic acetylcholine receptors (nAChRs) that are present on the cell bodies and the terminals of these neurons. Previously, it was suggested that one of the nAChR subtypes located on striatal dopaminergic terminals may be an α3β2 subtype, based on partial inhibition of nicotine-stimulated [^{3}H]dopamine release by α-conotoxin MII, a potent inhibitor of heterologously expressed α3β2 nAChRs. More recent studies indicated that α-conotoxin MII also potently blocks α6-containing nAChRs. In the present study, we have examined the nAChR subtype(s) modulating [^{3}H]dopamine release from striatal terminals by using novel α-conotoxins that have 37-78 fold higher selectivity for α6- vs. α3-containing nAChRs. All of the peptides partially (20-35%) inhibit nicotine-stimulated [^{3}H]dopamine release with IC_{50}s consistent with those obtained with heterologously expressed rat α6-containing nicotinic acetylcholine receptors. These results, together with previous studies by others, further support the idea that α6-containing nicotinic receptors modulate nicotine-stimulated dopamine release from rat striatal synaptosomes.

Nicotinic acetylcholine receptors (nAChRs) are members of the large family of ligand gated ion channels. Neuronal nAChRs have a pentameric structure composed of α and β subunits (Anand et al., 1991; Cooper et al., 1991). Through molecular cloning, six mammalian neuronal α (α2-α7) and 3 β (β2-β4) subunits have been identified in the brain (Sargent, 1993; McGehee and Role, 1995). Heteromeric receptors are composed of different combination of these subunits. Heterologous expression systems have revealed that α and β subunits both contribute to the diversity in biochemical and physiological properties of this class of receptors (Gross et al., 1991; Luetje and Patrick, 1991; Cachelin and Rust, 1995; McGehee and Role, 1995).

Nigrostriatal dopamine (DA) neurons, which originate from within the substantia nigra compacta (SNc) and send projections to the dorsal striatum, are involved in voluntary motor control. The selective loss of these neurons has been implicated in the pathology of the motor deficits associated with Parkinson's disease. Nicotine's actions on these neurons, and subsequent dopamine release within the striatum, may partly underlie this alkaloid's potential therapeutic effects in patients with Parkinson's. Midbrain (DA) neurons possess nicotine binding sites (Clarke and Pert, 1985) and nicotine directly activates these neurons (Pidoplichko et al., 1997; Yin and French, 2000). Moreover, nicotine stimulates release of DA within the striatal target region by direct action on nicotinic receptors on the DAergic terminals (Rapier et al., 1990; Grady et al., 1992; Clarke and Reuben, 1996; Wonnacott, 1997). *In situ* hybridization and immunohistochemical studies have revealed the presence of a variety of nAChR subunit mRNAs and protein within midbrain DAergic neurons, including α3-α7 and β2 and β3 (Charpantier et al., 1998; Elliott et al., 1998; Sorenson et al., 1998; Klink et al., 2001; Azam et al., 2002), that

could potentially result in numerous distinct subtypes of nAChRs. Therefore, due to lack of subtype specific ligands, it has been difficult to determine the exact subtypes of nAChRs mediating DA neurotransmission. Recently, it was shown that α-conotoxin MII (α-MII), a *Conus* toxin isolated from *C. magus* that potently inhibits the α3β2 nAChR subtype (Cartier et al., 1996), inhibited approximately 30-40% of nicotine stimulated [^{3}H]DA release from striatal synaptosomes (Kulak et al., 1997; Kaiser et al., 1998; Grady et al., 2001). However, more recent data indicate that α-MII also has high affinity for α6-containing receptors. Studies with mutant animals have shown that [^{125}I]α-MII binding is largely preserved in α3 knockout mice, but abolished in α6 knockout mice (Champtiaux et al., 2002; Whiteaker et al., 2002). These studies suggest that the primary target of α-MII in DA neurons may be an α6 rather than α3-containing receptor.

Until now no ligands that distinguish between α6*[1] and α3* nAChRs have been available. In the present study, we have characterized nicotine- modulated [^{3}H]DA release from rat striatal synaptosomes using the novel α-conotoxin PIA (α-PIA), isolated from *C. purpurascens* that has approximately 78-fold higher selectivity for α6- versus α3-containing receptors. In addition, three recently developed structural analogs of α-MII, with 37-, 54- and 75-fold selectivity for α6 vs. α3-containing receptors, were also used. The results implicate the involvement of α6* rather than α3* receptors in nicotine- evoked dopamine release from DAergic terminals.

METHODS

Materials. The chemicals were obtained from the following sources: (-)Nicotine hydrogen tartarate, pargyline HCl, bovine serum albumin, ascorbic acid (Sigma, St. Louis, MO), [^{3}H]dopamine (dihydroxyphenylethylamine 3,4 [7-^{3}H], 28-30 Ci/mmol) (Dupont NEN, Boston, MA), Ecolume scintillation cocktail (ICN, Costa Mesa, CA). α-Conotoxins were synthesized as previously described (Cartier et al., 1996; McIntosh et al., 2004).

Tissue Preparation. Adult male albino rats (Simonsen Laboratories, Gilroy, CA) were kept two per cage on a 12:12 hr light/dark cycle, with food and water available *ad libitum.* For each experiment, four adult male rats between 60 and 90 days old, were used. The rats were decapitated and brains quickly removed. This procedure was approved by the Institutional Animal Care and Use Committee and is consistent with Federal guidelines. Synaptosomes were prepared essentially as described by Kulak et al. (1997). Briefly, the striata was quickly dissected on ice and placed in ice-cold 0.32 M sucrose buffer, pH 7.4-7.5. The dissected striata were homogenized by 14 gentle up and down strokes, followed by centrifugation at 1000*g* for 10 min at 4^{o}C. The supernatant was centrifuged at 12,000*g* for 20 min at 4^{o}C. The resulting P2 pellet was resuspended in 2 ml of Krebs-HEPES buffer (superfusion buffer) with composition (in mM): NaCl 128, KCl 2.4, KH_2PO_4 1.2, $MgSO_4$ 0.6, $CaCl_2$ 3.2, HEPES 25, Glucose 10 and supplemented with 1mM ascorbic acid and 0.1mM pargyline. The synaptosomes were incubated for 10 min at 37^{o}C to equilibrate with the superfusion buffer, followed by another 10 min incubation with 0.12 μM [^{3}H]dopamine (specific activity 28-30 Ci/mmol) at 37^{o}C. The synaptosomes were centrifuged at 3500 rpm for 5 min in order to get rid of excess radiolabeled dopamine. The pellet was re-suspended in 4 ml of superfusion buffer and 1 ml was transferred into each of four conical tubes containing 3 ml of superfusion buffer and subsequently loaded

into the superfusion chambers containing 13 mm diameter A/E glass fiber filters (Gelman Sciences, Ann Arbor, MI). One tube (4 ml total volume) contained enough synaptosomes for six chambers of the superfusion apparatus.

Superfusion. The superfusion system had 12 identical channels and was set up as described in Kulak et al. (1997). Once synaptosomes were loaded into the superfusion apparatus, they were washed for 20 min with either superfusion buffer alone or buffer plus varying concentrations of the toxins, at a rate of 0.5 ml/min. α-conotoxin MII[E11A], due to its slow on-rate, was flowed on for 40 min. Following the wash period, 2-min fractions were collected in 5 ml polypropylene vials containing 4 ml of Ecolume scintillation cocktail. At the end of the third 2-min fraction, a 1-min pulse of nicotine or nicotine plus toxin was applied, followed by five 2-min washes with superfusion buffer alone. For studies where α- MII and α-PIA or α-MII and α-MII[E11A] were co-applied, synaptosomes were perfused for 20 or 40 min, respectively, with buffer containing 100nM α-MII and 100nM α-PIA or 100nM α-MII and 10nM α-MII[E11A] and pulsed with nicotine as described above. At the end of the superfusion, filters containing the synaptosomes were taken out and placed directly in vials containing 4 ml Ecolume to determine total [^{3}H]DA uptake. Radioactivity collected in each fraction was quantitated by liquid scintillation spectroscopy, with Beckman 5801 and 9800 liquid scintillation counters, tritium efficiency approximately 50%.

Data Analysis. Throughout this paper, tritium release is presumed to correspond directly to amounts of radiolabeled transmitter release, as it has been shown previously that tritium

released by nAChR agonists is proportional to total radiolabeled transmitter released (Rapier et al., 1988).

Baseline release was determined as average of two fractions before and two fractions after the peak release. Average baseline was subtracted from the evoked release and the resulting values divided by the baseline to yield the evoked release as a percent over baseline. For all data, except the release profiles in figure 1, % release over baseline was normalized to average release with nicotine alone. IC_{50}s were determined by non-linear regression analysis using Prism (Graphpad, San Diego, CA). All statistical analysis was performed with Prism. Toxin effects were analyzed by one-way ANOVA, followed by Dunnett's *post-hoc* for comparisons with nicotine control or Neumann-Keuls or Bonferroni's (where indicated) for multiple pairwise comparisons.

RESULTS

Inhibition of nicotine-stimulated [^{3}H]DA release by α-conotoxin PIA and MII

A one-min pulse of 3 μM nicotine (a submaximal concentration) resulted in [^{3}H]DA release approximately 80-100% above baseline (Fig. 1). It has previously been shown that this nicotine-evoked release is completely Ca^{2+} dependent and is blocked by the non-selective nAChR antagonist mecamylamine at a concentration of 100 μM (Kulak et al., 1997). Both α-PIA and α-MII dose-dependently inhibited nicotine-stimulated [^{3}H]DA release with IC_{50}s of 1.48 nM and 1.04 nM, respectively (Fig. 2, Table 1). However, this inhibition only reached significance at concentrations ≥1 nM for α-MII and ≥10 nM for α-PIA (Fig. 2). Maximum inhibition for both toxins was reached at 10 nM, as application of higher concentrations of either toxin did not produce significantly more inhibition than that seen with 10 nM. At the same toxin concentrations, block of nicotine-stimulated [^{3}H]DA release was not significantly different between α-MII and α-PIA.

In order to determine if the similar inhibition of nicotine-stimulated [^{3}H]DA release seen with α-MII and α-PIA is due to their action on the same site, the toxins were simultaneously applied in the presence of 3 μM nicotine. Co-application of both toxins at a maximally effective concentration (100 nM) did not produce a greater inhibition than either one applied alone (Fig. 3), thus suggesting that both toxins exert their effects by action on the same nAChR site(s).

Inhibition of nicotine-stimulated [^{3}H]DA release by α-MII and α-PIA in presence of varying concentrations of nicotine

A number of studies have suggested that [^{3}H]DA release from striatal terminals is modulated by at least two different nAChR subtypes (Kulak et al., 1997; Sharples et al., 2000; Grady et al., 2002), which have different affinities for nicotine (Kuryatov et al., 2000;

Champtiaux et al., 2003). To determine whether different proportion of α-MII and α-PIA sensitive nAChRs are activated at different nicotine concentrations, 10 nM α-MII and α-PIA were tested with nicotine concentrations in the range 300 nM-10 μM. Both toxins were used at a concentration of 10 nM because electrophysiological studies in our laboratory had indicated that for α-PIA, this concentration would selectively block a larger proportion of α6 rather than α3-containing receptors (Dowell et al., 2003) (also see Table 1). At 10 nM, α-PIA significantly inhibited nicotine-stimulated release at all concentrations of nicotine tested (approximately 20%, $p<0.001$), and this inhibition was similar across all nicotine concentrations (Fig. 4A). Similarly, α-MII significantly inhibited nicotine-stimulated [^{3}H]DA release at all concentrations of nicotine ($p<0.001$, Fig. 4B). However, α-MII displayed a trend towards greater inhibition of nicotine-stimulated [^{3}H]DA release at 300 nM and 1 μM nicotine compared to 3 μM and 10 μM nicotine, although this difference did not reach significance ($p=0.165$, Fig. 4B). Between α-MII and α-PIA, there was significantly more inhibition of nicotine-stimulated [^{3}H]DA release with α-MII at 300 nM and 1 μM nicotine compared to α-PIA at the same nicotine concentrations (Fig. 4).

Effects of α-conotoxin MII analogs, α-MII[H9A], α-MII[L15A] and α-MII[E11A], on nicotine-stimulated [^{3}H]DA release

Although α-conotoxin MII does not distinguish well between α3 and α6-containing receptors (Kuryatov et al., 2000; Dowell et al., 2003; McIntosh et al., 2004), substitution of Ala for His9 shifts the selectivity of α-MII towards α6-containing receptors, with an approximately 75-fold higher preference for α6 vs. α3-containing nAChRs (Table 1). Similarly, replacement of Leu15 with Ala and Glu11 with Ala increases the selectivity of α-MII for α6-containing nAChRs by approximately 37 and 54-fold, respectively. Double Ala substituted α-MII analogs

further increase selectivity (McIntosh et al., 2004). However, rapid off-rate kinetics combined with an apparent binding preference for the resting state of the nAChR make these analogs unsuitable for experiments with prolonged agonist application (unpublished observations). The three singly substituted α-MII analogs were examined for their effects on nicotine-stimulated [^{3}H]DA release in order to further assess the role of α6-containing receptors in modulation of this release. All analogs displayed dose-dependent inhibition of nicotine-stimulated [^{3}H]DA release, with IC_{50}s of 1.25 nM for α-MII[H9A], 0.84 nM for α-MII[L15A] and 0.025nM for α-MII[E11A] (Fig. 5, Table 1). α-MII [H9A] and α-MII[L15A] significantly inhibited release at concentrations ≥10 nM ($p<0.01$, Fig. 5), whereas α-MII[E11A] was the most potent of the analogs, significantly inhibiting [3H]DA release at concentrations ≥0.1nM ($p<0.001$, Fig. 5). For α-MII[H9A] and α-MII[L15A], application of 100 nM and 1 μM did not produce any greater inhibition than seen with 10 nM, whereas for α-MII[E11A], inhibition at 0.1 nM was not statistically different from that at higher concentrations (Fig. 5). α-MII[E11A] displayed a trend toward larger maximal inhibition than the other toxins; however this difference did not reach statistical significance. α-MII[E11A] blocks α4β2 nAChRs with an IC_{50} > 10,000 nM and therefore any larger block by α-MII[E11A] is unlikely to be due to blockade of this receptor subtype. Additionally, co-application of α-MII with α-MII[E11A] did not produce a statistically greater inhibition that either one alone (32 ± 7.9% inhibition with 100nM α-MII, 31.4 ± 11.8% with 10nM α-MII[E11A], 42 ± 13% inhibition with 100nM α-MII plus 10nM α-MII[E11A]), therefore suggesting action on the same site.

Discussion

In the present study, we have used novel α-conotoxins to assess the subtypes of nAChRs involved in nicotine-stimulated DA release in rat striatum. All of the toxins partially inhibit nicotine-stimulated [^{3}H]DA release, consistent with there being at least two population of nAChRs that modulate DA release (Sharples et al., 2000; Grady et al., 2002). The IC_{50}s of the toxins are similar to IC_{50}s of α6-containing nAChRs heterologously expressed in oocytes, suggesting that the nAChRs located on striatal DAergic terminals most likely contain an α6 rather than an α3 subunit (Table 1). The selectivity of these conotoxin peptides have allowed us to perform the first functional characterization of α6* vs. α3* nAChRs in rat striatal synaptosomes.

Previously, it had been suggested that [^{3}H]DA release from striatal terminals was modulated in part by an α3β2 nAChR subtype (Kulak et al., 1997; Kaiser et al., 1998; Sharples et al., 2000). This conclusion was based, in part, on inhibition of nicotine-stimulated [^{3}H]DA release by α-conotoxin MII. α-conotoxin MII was previously shown to block the α3β2 subunit combination with two to four orders-of-magnitude lower IC_{50} than other subunit combinations, including α2β2, α2β4, α3β4, α4β2, and α4β4 (Cartier et al., 1996). However, α-MII was subsequently shown to also have low nanomolar affinity for α6-containing receptors (Vailati et al., 1999; Kuryatov et al., 2000; Vailati et al., 2000; Barabino et al., 2001). Recent studies with mutant mice suggest that α-conotoxin MII binds with high affinity to α6* nAChRs in the central nervous system (Champtiaux et al., 2002; Whiteaker et al., 2002).

The findings in the present study strongly suggest that α-MII sensitive sites on rat striatal DAergic terminals contain an α6 rather than an α3 subunit. Although α-MII displays similar potency in blocking rat α3 and α6-containing receptors in oocytes (Dowell et al., 2003;

McIntosh et al., 2004), the results with the other toxins that have relatively lower potency for receptors containing the α3 subunit indicate the presence of an α6* nAChR subtype on striatal DAergic terminals. The high affinity of α-MII for both α3 and α6 subunits may be explained by the high homology (~80%) in the extracellular region (ligand binding site) between α3 and α6 subunits, as well as conservation of residues between α3 and α6 that confer selectivity to α-MII (Harvey et al., 1997). The residues within the toxins conferring the high selectivity for α6* and lower selectivity for α3* nAChRs are not currently known, although amino acids within the second disulfide loop are strong candidates. α-MII and α-PIA have identical amino acid sequence in the first loop, but their amino acid sequences are highly divergent in the second loop (Dowell et al., 2003). In addition, the three α-MII analogs that display high selectivity for α6* nAChRs have the alanine substitutions in their second loops (McIntosh et al., 2004).

Consistent with the current findings, recent immunoprecipitation studies have shown the presence of the α6 subunit in approximately 20% of [^{3}H]epibatidine-labeled receptors in the rat striatum, with the α3 subunit present in only 3% of the receptors. Moreover, there was an almost complete disappearance of nAChRs containing the α6 subunit in 6-OHDA lesioned striata, with no change in the α3 subunit (Zoli et al., 2002), indicating that the α6-containing sites are located primarily or exclusively on the DAergic terminals. The percentage of immunoprecipitated α6-containing [^{3}H]epibatidine sites closely resembles the maximum inhibition of nicotine-stimulated [^{3}H]DA release by the toxins in the present study. In addition to both β2 and β3, approximately 40% of α6-containing striatal nAChRs also contain the α4 subunit (Zoli et al., 2002, Champtiaux et al., 2003). However, none of the toxins in our present study may be able to distinguish between α6β2* and α6α4β2* nAChR subtypes. Toxin block of α6α4β2* nAChRs would likely

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be dependent on the position of the α6 subunit. If the α6 subunit is present at a ligand binding interface, the α-conotoxins used in this study would be expected to block the receptor, due to their high affinity for the α6/β2 subunit interface (Dowell et al., 2003; McIntosh et al., 2004). If the α6 subunit is located at an α6β2 interface in both subunit combinations, then the binding of the toxin to that interface would be sufficient for complete block, based on the assumption that two molecules of nicotine are required to activate the nAChR (Lester and Dani, 1995). The binding of the α-conotoxins to the α4β2 interface is unlikely, as all of the toxins, with the exception of α-MII, do not block heterologously expressed α4β2 nAChRs at concentrations used in the present study (Cartier, 2003; McIntosh et al., 2004). Results from other studies suggest that the large α-conotoxin-insensitive component of nicotine-evoked [^{3}H]DA release is most likely mediated by α4β2* nAChRs (Sharples et al., 2000; Zoli et al., 2002; Champtiaux et al., 2003).

α-MII and α-PIA both showed similar inhibition of nicotine-stimulated [^{3}H]DA release across different concentrations of nicotine. While this may be indicative of non-competitive antagonism, it is more likely attributable to the slow off-rate kinetics of these toxins. Since the receptors have been pre-incubated with the toxins for at least 20 min, almost all the toxin molecules would be expected to be bound to the receptor when the nicotine is applied. However, at 300 nM and 1 μM nicotine, α-MII displayed significantly greater inhibition of nicotine-stimulated [^{3}H]DA release than PIA. This suggests possible presence of a high affinity nAChR subtype that is blocked by α-MII, but not α-PIA. Possible candidates for this nAChR are α4β2 and/or α6α4β2 subtypes, which have EC_{50}s of 340 nM and 1.3 μM for nicotine, respectively (Kuryatov et al., 2000). Both subtypes appear to be present on striatal DAergic terminals (Sharples et al., 2000; Zoli et al., 2002; Champtiaux et al., 2003). Although native α4β2*

nAChRs display very low affinity to α-MII, as shown by immunoprecipitation and competition binding studies of native receptors (Zoli et al., 2002), it is possible that native α4β2* receptors are more sensitive to functional blockade by this toxin. α-MII has been shown to block heterologously expressed α4β2 nAChRs with an IC_{50} of 470 nM (Cartier, 2003), whereas α-PIA does not show any block of this receptor subtype even at micromolar concentrations (Dowell et al., 2003). Additionally, the presence of the α5 subunit in a significant proportion of α4β2* nAChRs on DAergic terminals (Zoli et al., 2002), could affect the sensitivity of the receptor for α-MII. Immunoprecipitation studies indicate that some α6β2* nAChRs on DAergic terminals also contain the putative structural β3 subunit (Zoli et al., 2002). In addition, knock-out studies in mice suggest that most α-MII sensitive sites in the striatum (presumably located exclusively on DAergic terminals) contain the β3 subunit (Cui et al., 2003). Until recently, we have had difficulty expressing functional receptors in *Xenopus* oocytes with only the chimeric α6/α3 and β2 subunits and not the β3 subunit. However, utilizing the β2 subunit in the high expressing vector pGEMHE (Parker et al., 1998) with only the α6/α3 subunit routinely yielded functional receptors, albeit at low levels. Both PIA and MII[E11A] block α6/α3β2 vs. α6/α3β2β3 nAChRs with approximately equal IC_{50}s (Dowell et al., 2003; McIntosh et al., 2004), suggesting that the presence of the β3 subunit has little influence on the potency of these toxins. Finally, each of the toxins tested in this study also has high affinity for α6β4 nAChRs (Barabino et al., 2001; Dowell et al., 2003; McIntosh et al., 2004). However, it is unlikely that the β4 subunit is involved in nicotine-stimulated dopamine release in the striatum for several reasons. Both *in situ* hybridization (Winzer-Serhan and Leslie, 1997; Azam et al., 2002) and single-cell RT-PCR studies (Klink et al., 2001) have indicated an absence of the β4 subunit mRNA in SNc DAergic

neurons. In addition, immunoprecipitation studies indicate a lack of the β4 subunit protein on striatal DAergic terminals (Zoli et al., 2002). Finally, the IC_{50}s of block of nicotine-stimulated [^{3}H]DA release by α-conotoxins are consistent with α6β2* rather than α6β4* nAChRs (Dowell et al., 2003; McIntosh et al., 2004).

One caveat of comparing results obtained from heterologously expressed nAChRs to properties of nAChRs in the native system is that heterologously expressed receptors may not have the same post-translational modifications as the native mammalian nAChRs and therefore may not display the same affinity for a given ligand. However, the toxins used in the present study have been tested in competition binding studies with [^{125}I]α-MII using mouse striatal homogenates and they exhibited K_i values similar to IC_{50}s obtained for heterologously expressed α6/α3β2β3 nAChRs (Table 1).

In conclusion, data from the present study implicate α6β2* nAChRs as one of the modulators of nicotine stimulated striatal DA release. Subpopulations of α6β2* nAChRs containing α4 and/or β3 also appear to be present (Zoli et al., 2002; Champtiaux et al., 2003; Cui et al., 2003). However, at present, there are no ligands to further functionally discriminate among these subunit combinations.

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FOOTNOTES

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[1]Asterisk indicates the possible presence of other subunits

Figure Legends

Figure 1: Representative profiles of nicotine-stimulated [^{3}H]DA release from striatal synaptosomes. Striatal synaptosomes preloaded with [^{3}H]DA were stimulated with a 1-min pulse of 3 μM nicotine (indicated by arrow). This concentration of nicotine stimulated a peak release of 80-100% above baseline (solid lines denoted as "control"). Simultaneous application of 1 μM α-conotoxins (a saturating concentration) inhibited the peak release by approximately 20-30% (broken lines). The α-conotoxins that were tested are α-conotoxin MII (A), α-conotoxin PIA (B), α-conotoxin MII[H9A] (C), α-conotoxin MII[L15A] (D) and α-conotoxin MII[E11A] (E).

Figure 2: α-MII and α-PIA inhibition of nicotine-stimulated [^{3}H]DA release from striatal synaptosomes. The values are normalized to release obtained with 3 μM nicotine alone (100% release above baseline). Both toxins dose-dependently inhibited nicotine-evoked [^{3}H]DA release with IC_{50}s in the low nanomolar range. For α-MII, inhibition was significantly different from nicotine control at concentrations of 1 nM and greater (***$p<0.001$, Dunnett's post-hoc), whereas for α-PIA, inhibition became significant at concentrations of 10 nM and greater (###$p<0.001$, Dunnett's post-hoc). Values are mean ± SEM from 4-9 separate experiments.

Figure 3: Inhibition of nicotine-evoked [^{3}H]DA release from striatal synaptosomes in the presence of both α-MII and α-PIA. Co-application of maximally effective concentrations of α-MII and α-PIA (100 nM) did not produce a greater inhibition than that seen with 100 nM of each toxin alone. Percent [^{3}H]DA release above baseline is normalized to release obtained with 3 μM

nicotine alone (100% release above baseline). Values are mean ± SEM from 4 separate experiments. ***p<0.001, compared to nicotine control, Student's t-test.

Figure 4: α-MII and α-PIA inhibition of nicotine-stimulated [^{3}H]DA release in presence of varying concentration of nicotine. Percent [^{3}H]DA release above baseline is normalized to release in presence of the corresponding nicotine concentration alone (control). (A) α-PIA (10 nM) significantly inhibited nicotine-stimulated [^{3}H]DA at all nicotine concentrations as compared to corresponding nicotine control (***p<0.001, Dunnett's post-hoc) and this inhibition was similar across all nicotine concentrations. (B) α-MII (10 nM) significantly inhibited nicotine-stimulated [^{3}H]DA release at all nicotine concentrations as compared to corresponding nicotine control (***p<0.001, Dunnett's post-hoc), although this inhibition was slightly, although insignificantly, greater at 300 nM and 1 μM nicotine. However, at 300 nM and 1 μM nicotine, α-MII significantly inhibited more [^{3}H]DA release than α-PIA at the same nicotine concentrations. Absolute values for nicotine control (percent above baseline): panel A, 300nM: 61.7 ± 8.2, 1μM: 78.1 ± 8.4, 3μM: 101.3 ± 7.6, 10μM: 118.9 ± 12.3; panel B, 300nM: 70.9 ± 11.7, 1μM: 94.1 ± 7.1, 3μM: 101.7 ± 11.9, 10μM: 122.9 ± 17.2 (#p<0.05, ##p<0.01, Bonferroni's selected pairs of columns post-hoc). Values are mean ± SEM from 3-4 separate experiments.

Figure 5: α-MII[H9A], α-MII[L15A] and α-MII[E11A] inhibition of nicotine-stimulated [^{3}H]DA release from striatal synaptosomes. Percent [^{3}H]DA release above baseline is normalized to release obtained with 3 μM nicotine alone (100% release above baseline). All three analogs dose-dependently inhibited nicotine stimulated [^{3}H]DA release with IC_{50}s in the

picomolar to low nanomolar range. For α-MII[H9A] and α-MII[L15A], inhibition was significant at concentrations of 10 nM and greater (**p<0.01, Dunnett's post-hoc) whereas α-MII[E11A] showed the highest potency, with significant inhibition obtained at 0.1nM and greater(###p<0.001, Dunnett's post-hoc). Values are mean ± SEM from 3-4 separate experiments.

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Table1: IC_{50} and maximum inhibition of nicotine-stimulated [^{3}H]DA release for toxins used in this study. All subunits used to form receptors in oocytes were from rat clones. α6/α3β2β3 subtype is composed of an α6/α3 chimera, which contains the extracellular region of α6 and the remaining portion of the α3 subunit. . α-MII is the only toxin that does not distinguish between α3* and α6* nAChRs. For each toxin, the IC_{50} for [^{3}H]DA release closely resembles that for inhibition of heterlogously expressed α6* nAChRs. The substituted residues in the α-MII analogs are underlined. CI: Confidence Interval.

Toxin	Sequence	IC_{50} Oocytes (nM) α3β2	IC_{50} Oocytes (nM) α6/α3β2β3	K_i (nM) α-MII sites	IC_{50} [^{3}H]DA release (95% CI) (nM)	% maximum inhibition
α-MII	GCCSNPVCHLEHSNLC	2.18[a] (1.2-3.8)	0.39[a] (0.28-0.55)	0.22[a] (0.20-0.25)	1.04 (0.47-2.3)	25 ± 1.8%
α-MII[L15A]	GCCSNPVCHLEHSN**A**C	34.1[a] (19-60)	0.92[a] (0.65-1.3)	0.30[a] (0.21-0.45)	0.84 (0.26-2.8)	22 ± 2.4%
α-MII[H9A]	GCCSNPVC**A**LEHSNLC	59[a] (44-79)	0.79[a] (0.56-1.1)	1.1[a] (0.84-1.6)	1.25 (0.32-4.9)	21 ± 1.8%
α-MII[E11A]	GCCSNPVCHL**A**HSNLC	8.7[a] (6.8-11.1)	0.16[a] (0.13-0.19)	0.27[a] (0.19-0.37)	0.025 (0.010-0.061)	32 ± 3.5%
α-PIA	RDPCCSNPVCTVHNPQIC	74.2[b] (49-110)	0.95[b] (0.71-1.3)	not tested	1.48 (0.25-8.7)	26 ± 3.3%

[a]Taken from McIntosh et al., 2004
[b]Taken from Dowell et al., 2003

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Figure 1

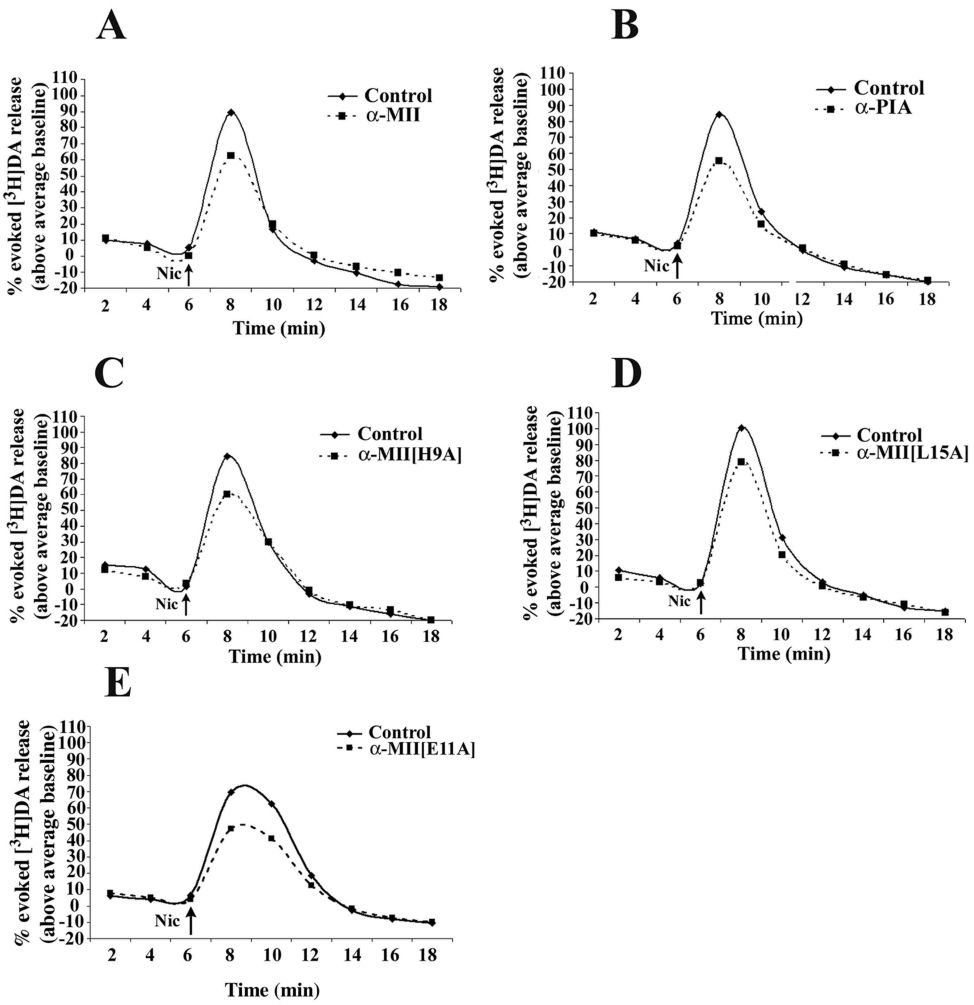

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Figure 2

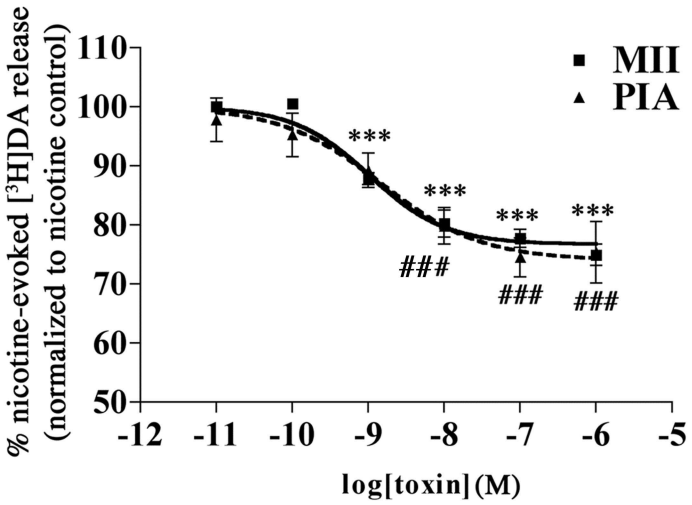

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Figure 3

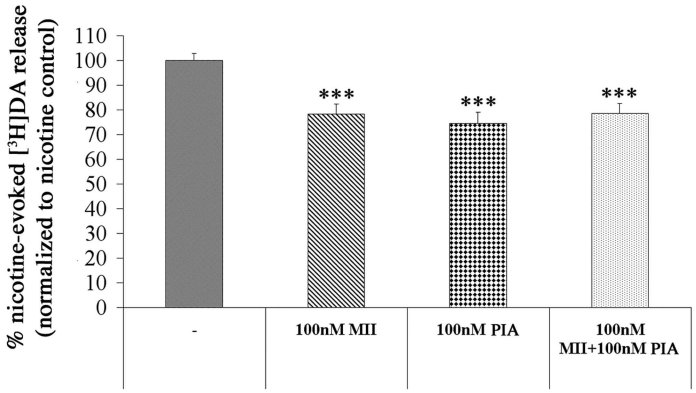

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Figure 4

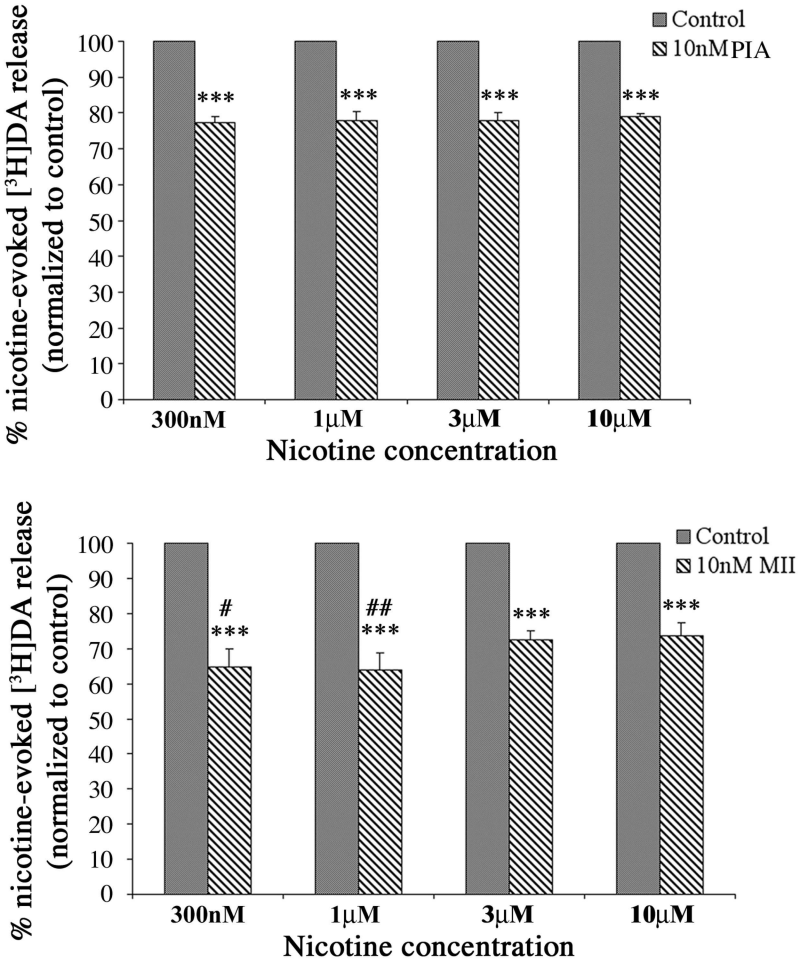

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Figure 5