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Nicotinic Acetylcholine Receptor-Mediated [^{3}H]-Dopamine Release from Hippocampus

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ABBREVIATIONS: ACh, acetylcholine; AChR, acetylcholine receptor; α-BTX, α-bungarotoxin; α-CtxMII, α-conotoxin MII; α-CtxImI, α-conotoxin ImI; DHβE, dihydro-β-erythroidine; DMPP, 1,1-dimethyl-4-phenyl-piperazinium iodide; DA, dopamine; EB, (±)-epibatidine; Hex, hexamethonium; 5-HT, serotonin; Mec, mecamylamine; MG 624, N,N,N-triethyl-2-[4-(2 phenylethenyl)phenoxy]-ethanaminium iodine; MLA, methyllycaconitine; nAChR, nicotinic cholinergic receptor; NE, norepinephrine; d-TC, d-tubocurare

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ABSTRACT

The mechanism of nicotinic acetylcholine receptor (nAChR)-induced hippocampal dopamine (DA) release was investigated using rat hippocampal slices. nAChRs involved in hippocampal DA and norepinephrine (NE) release were investigated using prototypical agonists and antagonists, as well as several relatively novel compounds, ABT-594, (±)-UB-165, and MG 624. (±)-Epibatidine (EB), (±)-UB-165, anatoxin-a, ABT-594, (-)-nicotine, 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP), and (-)-cytisine (in decreasing order of potency) evoked [^{3}H]-DA release in a mecamylamine-sensitive manner. Aside from (±)-UB-165, all agonists displayed full efficacy relative to 100 μM (-)-nicotine in [^{3}H]-DA release. In contrast, (±)-UB-165 was a partial agonist, evoking 58% of 100 μM (-)-nicotine response. Mecamylamine (Mec), MG 624, hexamethonium (Hex), d-Tubocurare (d-TC), and dihydro-β-erythroidine (DHβE) (in decreasing order of potency), but not α-conotoxin-MII (α-CtxMII), methyllycaconitine (MLA), α-conotoxin-ImI (α-CtxImI) or α-bungarotoxin (α-BTX), attenuated 100 μM (-)-nicotine -evoked [^{3}H]-DA release in a concentration-dependent manner. (±)-UB-165, ABT-594 and MG 624 exhibited different pharmacological profiles in the [^{3}H]-NE release assay when compared to their effect on [^{3}H]-DA release. ABT-594 was 4.5-fold more potent, and (±)-UB-165 was a full agonist, in contrast to its partial agonism in [^{3}H]-DA release. MG 624 potently and completely blocked NE release evoked by 100 μM (-)-nicotine, and 10 μM (±)-UB-165, whereas it only partially inhibited (-)-nicotine -evoked [^{3}H]-DA release. In conclusion, we provide evidence that [^{3}H]-DA can be evoked from the hippocampus, and that the pharmacological profile for nAChR-evoked hippocampal [^{3}H]-DA release suggests involvement of α3β4* and at least one other

nAChR subtype, thus distinguishing it from that of nAChR-evoked hippocampal [^{3}H]-NE release.

Introduction

The cholinergic system plays a major role in cognitive functions that involve attention, learning, and memory (for review see Levin and Simon, 1998). The cognitive-enhancing properties of nAChR agonists have been attributed, at least in part, to their ability to enhance transmission of key neurotransmitters, which are active in the cascade of events associated with memory. Although it is well established that presynaptic nAChRs facilitate the release of several neurotransmitters from various brain regions, including dopamine (DA), norepinephrine (NE), serotonin (5-HT), and acetylcholine (ACh) (for review see Wonnacott, 1997), the identity of all nAChR subtype(s) involved in these actions remains unknown. Functional nAChRs, existing as heteromers comprised of an α (α2-α6) with a β (β2-β4) subunit, or homomers comprised of α7, α8, or α9 subunits, have been identified throughout the central nervous system (for review, see Romanelli and Gualtieri, 2003), greatly increasing the potential for extensive diversity in the function of nAChRs to regulate neurotransmitter release.

A major contributor to cognitive function appears to be the neurotransmitter dopamine. Although precise contribution of dopamine remains unclear, it most likely plays a role in the regulation of several different aspects of cognitive brain function (for review see Nieoullon, 2002). Indeed, abnormal dopaminergic neurotransmission has been observed in several diseases/disorders that express cognitive dysfunctions, including Alzheimer's disease, Tourette's disease, Huntington's chorea, attention deficit hyperactivity disorder, and cognitive deficits associated with schizophrenia.

In the hippocampus, a critical area involved in attention and memory, the significance of nicotinic-dopaminergic interactions for cognitive function has been well documented

(for review see Levin and Simon, 1998), and includes evidence of a significant role for nicotinic receptors localized in the ventral hippocampus in working memory. Projections from the mesencephalic dopamine groups (ventral tegmental area-substantia nigra) to the hippocampus have been characterized, with the most innervated area being the ventral region of the hippocampal formation (for review see Jay, 2003). In addition, dopamine (for review see Jay, 2003), the dopamine transporter (Mennicken et al., 1992; Horn et al., 1990) and dopamine receptors (for review see Jay, 2003), have been detected in rat hippocampus, substantiating dopaminergic function. Moreover, dopamine influences the firing rate of hippocampal cells (Smialowski and Bijak, 1987) and plays a role in cognitive function in the hippocampus (for review see Jay, 2003), particularly with respect to working memory. *In vivo* microdialysis studies have demonstrated that behaviorally active doses of nicotine increase hippocampal DA release (Brazell et al., 1991). Moreover, dopaminergic neurons of the VTA-SN express mRNA for a number of nAChR subunits, including α3-α7, and β2-β4 (Azam et al., 2002; Klink et al., 2001; Charpantier et al., 1998). To date, the nAChR subtype(s) mediating hippocampal [^{3}H]-DA release have not been characterized. Therefore, using several different agonists and antagonists, the present study used our relatively novel method of measuring neurotransmitter release (Anderson et al., 2000; Puttfarcken et al., 2000) to investigate nAChR-evoked hippocampal [^{3}H]-DA release from the hippocampus. The potential involvement of α4β2 and α7 was investigated, since previous behavioral studies have demonstrated the involvement of these subtypes with working memory function in the hippocampus (for review see Levin and Simon, 1998). Furthermore, the role of α3β4 was examined by comparing results obtained for [^{3}H]-DA release to those obtained for

[^{3}H]-NE release from the hippocampus, a process reported to be primarily mediated by α3β4 (Clarke and Reuben, 1996; Wonnacott, 1997; Luo et al., 1998; Anderson et al., 2000).

To our knowledge this is the first study to extensively characterize nAChR-mediated [^{3}H]-DA release from the hippocampus. We report on the effects of (R)-5-(2-azetidinylmethoxy)-2-chloropyridine (ABT-594), a compound with high affinity for the α4β2 subtype, which is also a potent agonist at α4β2 and (albeit to a somewhat lesser extent) α3β4 receptor in rat and human (Donnelly-Roberts et al., 1998). Furthermore, we report, for the first time, the pharmacological actions of two novel compounds, N,N,N-triethyl-2-[4-(2 phenylethenyl)phenoxy]-ethanaminium iodine (MG 624), and (2-chloro-5-pyridyl)-9-azabicyclo[4.2.1]non-2-ene ((±)-UB-165) in the hippocampus. Although very little is known regarding their selectivity for specific nAChRs in the rodent brain, MG 624 has been reported to display nanomolar affinity and antagonist activity against rat α7 receptors expressed in *Xenopus* oocytes (Di Angelantonio et al., 2000). (±)-UB-165, a hybrid of anatoxin-a and epibatidine, has been demonstrated to functionally activate recombinant rat α3β4 expressed in a mouse fibroblast cell (Sharples et al., 2002).

Methods

Materials. [^{3}H]-Dopamine ([^{3}H]-DA) (3,4-[ring-2,5,6-^{3}H]-dihydroxyphenylethylamine, 60 Ci/mmol) and [^{3}H]-norepinephrine ([^{3}H]-NE) (1-[ring-2,5,6-^{3}H]-norepinephrine) were purchased from PerkinElmer Life Science (Boston, MA). (-)-Nicotine bitartrate, (+)-anatoxin-a, 1,1 dimethyl-4-phenyl-pipazinium iodide (DMPP), (-)-cytisine, hexamethonium bromide (Hex), d-tubocurare (d-TC), mecamylamine (Mec), dihydro-β-erythroidine HBr (DHβE), methyllycaconitine (MLA), desipramine, pargyline and ascorbic acid were purchased from Sigma (St. Louis, MO). (±)-epibatidine (EB) and nomifensine was purchased from Sigma-RBI (Natick, MA). α-Conotoxin MII (α-CtxMII) was obtained from Tocris (Bristol, UK). (±)-UB-165 fumarate, and MG 624 were obtained from Tocris (Ellisville, MO). α-Conotoxin ImI (α-CtxImI) was purchased from either American Peptide Company, Inc. (Sunnyvale, CA) or Sigma (St. Louis, MO). ABT-594 was synthesized in house.

Animals. Male Sprague Dawley rats (250-300g) (Harlan, Madison, WI.) were housed four per cage, and food and water were available *ad libitum*. Rats were allowed to acclimate to housing conditions four days after arrival. Animals were treated in accordance with the IACUC guidelines.

Measurement of [^{3}H]-DA and [^{3}H]-NE release from tissue slices. Methods for tissue slice preparation and measurement of [^{3}H]-DA release were slightly modified from those described elsewhere (Puttfarcken et al., 2000). Briefly, rat hippocampal slices (250 μm) were prepared using a McIlwain tissue chopper. The tissue was washed three times with buffer (15 mM HEPES-NaOH; 137 mM NaCl; 4.7 mM KCl; 1.0 mM $MgSO_4$; 0.1 mM ascorbic acid; 2.5 mM $CaCl_2$; 1.25 mM NaH_2PO_4; 3.3 mM dextrose; 10 μM pargyline;

pH 7.4) and subsequently incubated with buffer containing uptake blockers for 5 minutes at 37°C under an atmosphere of 95% O_2 and 5% CO_2. Tissue was then loaded with either 100 nM [^{3}H]-DA or 50 nM [^{3}H]-NE for 30 minutes. The present study utilized a relatively low concentration of [^{3}H]-DA (100 nM) to ensure exclusive uptake by the dopamine transporter, since DA exhibits a weak affinity for both the norepinephrine and 5-HT transporter [K_D = 32 μM and > 100 μM, respectively, (Tatsumi et al., 1997)]. Desipramine, 100 nM, was included in the assay buffer during the loading of [^{3}H]-DA to prevent uptake into noradrenergic terminals. In experiments examining the actions of α-BTX, α-CtxMII and α-CtxImI, tissue was allowed to incubate at 37°C in buffer containing 0.1% BSA and 1 mM phenylmethylsulfonyl fluoride (PMSF). Following the incubation, the tissue suspension was allowed to settle under gravity and washed extensively to remove excess radioactivity. The slices were distributed in 50 μl/well to 96 well plates containing a 1.2 μm hydrophilic, low protein binding durapore™ membrane (Millipore MultiScreen™ BV clear plates, cat no. MABVN1210), and washed three times with 100 μl buffer/well using vacuum filtration (UniVac™ Polyfiltronics). Hippocampi from two animals were pooled to provide enough tissue for one 96-well plate. Following a 10 minute rest period at 37°C, the buffer was removed by vacuum filtration. For experiments involving α-BTX, slices were instead distributed to 96-well plates in the presence of the antagonist and allowed to incubate at 37°C for 60 minutes. Following incubation, the buffer was then removed by vacuum filtration. For the [^{3}H]-DA release assay, the subsequent buffer contained 100 nM nomifensine; for the [^{3}H]-NE release assay, 1 μM yohimbine and 30 nM nisoxetine were added. To collect the basal release, 100 μl of preoxygenated buffer was added to each well and allowed to

preincubate for 5 minutes at 37^{o}C. Preincubation was terminated by vacuum filtration into a 96 well plate to collect basal release. Following collection of the basal sample, 100 µl of buffer containing various concentrations of agonists (with or without antagonists) were added to each well, and allowed to incubate for an additional 5 minutes at 37^{o}C. Stimulated release was collected by rapid filtration into a new 96 well collecting plate. Following the collection of stimulated release, tissue samples were collected and counted for radioactivity as described above.

Data Analysis. Data are presented as fractional release for each well [stimulated release /(radioactivity in stimulated + tissue lysate) - basal release/(radioactivity in basal + stimulated + tissue lysate)] and expressed as means ± SEM. Relative efficacies were calculated using the release evoked by 100 µM (-)-nicotine as a standard. Both EC_{50} and IC_{50} values were determined by fitting the data to a sigmoidal logistic equation using the software Prism (Graph Pad, San Diego, CA).

Results

The Effect of nAChR Agonists on Hippocampal [^{3}H]-DA Release

Activation of nAChRs evoked [^{3}H]-DA release in the rat hippocampus. The nAChR agonists, ABT-594, (±)-UB-165 (Fig 1A), EB, DMPP, anatoxin-a, and (-)-cytisine (Fig. 1B), evoked a concentration-dependent increase in [^{3}H]-DA release, with a rank order potency of EB > (±)-UB-165 > anatoxin-a > ABT-594 > (-)-nicotine > DMPP > (-)-cytisine (Table 1). With the exception of DMPP, pretreatment with Mec completely blocked release evoked by all agonists tested (Table 1). Mec only inhibited 48% of the release evoked by DMPP (Table 1). (-)-Cytisine was the weakest agonist examined, with an EC_{50} value approximately 3.4-fold less potent than that obtained for (-)-nicotine (Fig. 1B, Table 1). The maximal release produced by all agonists, aside from (±)-UB-165, was equivalent to that produced by (-)-nicotine (Table 1). In contrast, (±)-UB-165, the hybrid of anatoxin-a and epibatidine (Sharples et al., 2000), produced only 58% of the release evoked by (-)-nicotine (Fig. 1A, Table 1).

The Effect of (±)-UB-165 and ABT-594 on Hippocampal [^{3}H]-NE Release

The ability of ABT-594 and (±)-UB-165 to evoke [^{3}H]-NE release from hippocampus was also examined for further characterization of the nAChR subtypes involved in (-)-nicotine-evoked [^{3}H]-NE release (Fig. 1A and Table 1). Previous studies of ours (Anderson et al., 2000) have already examined the other agonists used in the present study. Both agonists evoked a concentration-dependent increase in [^{3}H]-NE release, with EC_{50} values of 0.47 μM for ABT-594 and 0.058 μM for (±)-UB-165. Pretreatment with Mec completely blocked release evoked by both agonists (Table 1). The maximal

release produced by ABT-594 and (±)-UB-165, was nearly equivalent to that produced by (-)-nicotine (Table 1).

The Effect of nAChR Antagonists on Hippocampal [^{3}H]-DA Release

Additional experiments examined the ability of various antagonists to affect hippocampal [^{3}H]-DA release evoked by (-)-nicotine (Fig. 2-4, and Table 2). A maximal concentration of (-)-nicotine, 100 μM, was used in these experiments. Most of the antagonists tested inhibited (-)-nicotine-evoked release, with a rank order of potency of Mec > MG 624 > Hex > d-TC > DHβE (Fig. 2, Table 2). The weakest antagonist tested, DHβE, only partially (by 67 ± 10%) inhibited the release (Fig. 2A, Table 2). In contrast to the antagonists mentioned above, three of the four reported α7 antagonists, MLA, α-CtxImI, and α-BTX, examined, had no effect on 100μM (-)-nicotine-evoked [^{3}H]-DA release (Fig. 3A-C). Interestingly, MG 624, the fourth α7 antagonist, originally characterized in chick (Gotti et al., 1998), blocked about 60% of (-)-nicotine-evoked [^{3}H]-DA release (Fig. 2B, Table 2), and was one of the most potent compounds examined (IC_{50} = 0.54 μM). In contrast, α-CtxMII, the α3β2β3/α6β2β3 antagonist, had no activity against [^{3}H]-DA release from the hippocampus (Fig. 3D).

In order to obtain an idea of the mechanism underlying antagonism, (-)-nicotine concentration response curves were carried out in the absence and presence of each antagonist (Fig. 4). Antagonist concentrations were selected based on IC_{50} values obtained in earlier experiments (see Table 2). The inhibition produced by each antagonist was found to be non-surmountable, suggesting a non-competitive mechanism.

The Effect of MG 624 on Hippocampal [^{3}H]-NE Release

Since the effect of MG 624, a reported α7 receptor antagonist, on nicotine-induced [^{3}H]-DA release was different from that of other α7 receptor antagonists, further experiments examined its ability to affect other nAChR-mediated neurotransmitter release processes. A maximal concentration of (-)-nicotine, 100 μM (Fig.2B, Table2), and (±)-UB-165, 10 μM (Table 2), were used to evoke hippocampal [^{3}H]-NE release. In contrast to hippocampal [^{3}H]-DA release, MG 624 completely inhibited [^{3}H]-NE release evoked by (-)-nicotine and (±)-UB-165 with IC_{50} values of 0.34 μM and 0.42 μM, respectively.

Discussion

To characterize nAChR(s) subtypes involved in (-)-nicotine-evoked [^{3}H]-DA release, we examined the ability of nAChR agonists to evoke [^{3}H]-DA release in rat hippocampus. Each agonist elicited a concentration-dependent release of hippocampal [^{3}H]-DA with a rank order potency somewhat similar to that for hippocampal [^{3}H]-NE release. This suggests that at least one of the same nAChR subtypes is involved in mediating release of both neurotransmitters from the hippocampus. Since previous studies have proposed that $\alpha3\beta4$ is a major nAChR subtype involved in hippocampal [^{3}H]-NE release (Clarke and Reuben, 1996; Wonnacott, 1997; Luo et al., 1998; Anderson et al., 2000), the current data suggest that $\alpha3\beta4$ may also be involved in hippocampal [^{3}H]-DA release. In support, (-)-cytisine displayed full agonism suggesting involvement of $\beta4$ rather than $\beta2$ nAChRs (Leutje and Patrick, 1991; Papke and Heinemann, 1994). Additionally, the rank order potency for (-)-cytisine was similar for hippocampal [^{3}H]-DA release to that measured using $\alpha3\beta4$ human recombinant nAChRs (Stauderman et al., 1998). Although DMPP appeared much more efficacious in evoking [^{3}H]-NE release (178%; Anderson et al., 2000), Mec did not fully block release for either neurotransmitter. Indeed, a non-nicotinic, Ca^{2+}-independent component of DMPP-mediated [^{3}H]-NE release has been previously reported (Kiss et al., 1997; Anderson et al., 2000). Since the concentration of nomifensine present in the assay is not expected to fully inhibit rat dopamine transporters (Richelson and Pfenning, 1984), DMPP could influence [^{3}H]-DA uptake, resulting in a carrier-mediated increase of extracellular DA. Finally, maximal DMPP responses equal to or greater than (-)-nicotine have been shown only with $\alpha3$ subtypes (Luetje and Patrick, 1991).

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Interestingly, despite similar agonist rank order potency for both neurotransmitters, the response to (±)-UB-165 (Sharples et al., 2000) and ABT-594 differed, suggesting distinct profiles of nAChRs involved in modulating release of each neurotransmitter. Although ABT-594 was equally efficacious in both assays, it was approximately 4.5-fold more potent in the [^{3}H]-NE release assay. Furthermore, whereas (±)-UB-165 only partially evoked hippocampal [^{3}H]-DA release it showed full efficacy in [^{3}H]-NE release, in agreement with efficacy reported for human (Sharples et al., 2000) and rat (Sharples et al., 2002) recombinant α3β4* nAChRs. If the pharmacological profile of (±)-UB-165 at rat recombinant and native nAChRs is similar, the partial efficacy of (±)-UB-165 suggests that other subtypes, such as α4β2, α2β2 and/or an nAChR(s) composed of multiple α and β subunits, that are not significantly activated by (±)-UB-165 in human, may also function in nAChR-mediated rat hippocampal [^{3}H]-DA release. Although the expression of the α2 nAChR subunit is weak to moderate in hippocampus (Wada et al., 1989), the weak antagonism by DHβE does not support a role for α2β2 (IC_{50} at human α2β2= 850 nM; Chavez-Noriega et al, 1997). Furthermore, α4β2* is most likely not involved, since the IC_{50} value of DHβE in the present this study is reminiscent of the weak antagonist activity displayed by DHβE in two cell lines expressing α3, but not α4-containing nAChRs (Decker et al., 1995; Puttfarcken et al., 1997). Although we have previously described DHβE as a competitive antagonist for nAChR-mediated striatal [^{3}H]-DA release using our 96-well format (Anderson et al., 2000), and others have reported competitive properties for nAChR-mediated [^{3}H]-NE release from hippocampal synaptosomes (Clarke and Reuben, 1996) and [^{3}H]-DA release from rat striatal synaptosomes (Rapier et al., 1990; el-Bizri and Clarke, 1994), in hippocampus DHβE

behaved as a noncompetitive antagonist of [^{3}H]-DA release, as also reported for [^{3}H]-NE release (Anderson et al. 2000). Although the mechanism remains unclear, the apparent noncompetitive inhibition of hippocampal [^{3}H]-NE release (Anderson et al., 2000) by DHβE was not attributable to differences in tissue preparation (slice versus synaptosomes), method of measuring release (superfusion versus 96-well format), or preincubation times (Anderson et al., 2000). Competitive antagonists can be classified into two different subtypes, depending upon the interaction between antagonist and receptor (Waud D.R., 1968). The strength and/or mode of interaction between DHβE and receptor may vary depending on the subunit composition, and DHβE may behave as a nonequilibrium, or irreversible, competitive antagonist of specific nAChRs, rather than as an equilibrium competitive antagonist. These types of antagonists show insurmountable competition, as is also typical of non-competitive antagonists. The exact composition of nAChRs involved in hippocampal [^{3}H]-DA release is not yet fully defined, and the presence of a modulatory subunit, such as α5 (demonstrated by RT-PCR to be present in SN and VTA dopaminergic neurons (Charpantier et al., 1998)), may thus influence the properties of DHβE. In support, α5 has been shown to alter nAChR sensitivity to different antagonists (Yu and Role, 1998).

The inability of three α7 antagonists, α-CtxImI, MLA, and α-BTX, to reverse (-)-nicotine-evoked [^{3}H]-DA release, suggests that α7 is most likely not involved. However, the response of MG 624, a reported competitive antagonist at chick α7 receptors (Gotti et al., 1998; Maggi et al, 1999), differed between the two neurotransmitters. Although MG 624 was nearly equipotent, it only blocked 60% of nicotine-evoked [^{3}H]-DA release, whereas it fully antagonized nicotine- and UB-165-evoked [^{3}H]-NE release.

Furthermore, in contrast to findings reported for the chick α7 receptor (Maggi et al., 1999), MG 624-mediated inhibition in the rat [^{3}H]-DA release assay appeared to be through a non-competitive mechanism. This difference in apparent mode of interaction may be due to different receptor subtype(s) involved and/or to species-specific differences in the pharmacological profile of oxystilbene derivatives (Di Angelantonio et al, 2000). Since α3β4 is thought to be a predominant nAChR involved in hippocampal [^{3}H]-NE release (Clarke and Reuben, 1996; Wonnacott, 1997; Luo et al., 1998; Anderson et al., 2000), these data suggest that MG 624 is an antagonist at α3β4* nAChRs. Indeed, previous binding studies have shown that although oxystilbene derivatives, such as MG 624, retain their potency toward α7-containing receptors in mammals, they are also active at non-α7-containing receptors (Gotti et al., 2000). In support, F3, a structurally related 4-oxystilbene derivative, competitively inhibits nicotine-evoked currents, in a preparation that prevalently contains rat α3β4* nAChRs (Di Angelantonio et al., 2000). The partial blockade of [^{3}H]-DA release by MG 624 suggests that other subtypes, aside from α3β4, are also involved in the release process. Results obtained with (±)-UB-165 further support the role of multiple nAChRs in the hippocampal [^{3}H]-DA release process.

The use of d-TC and Hex, which show selectivity for α3-containing nAChRs, provides further evidence for the involvement of α3-containing nAChRs in hippocampal [^{3}H]-DA release. Although not potent, d-TC inhibited hippocampal DA release with the same range of potency as reported for human recombinant α3-containing nAChRs (Stauderman et al., 1998; Chavez-Noriega et al., 1997). Furthermore, as observed in the present study, DHβE was reported to be less potent than d-TC at human recombinant

α3β4 nAChRs (Chavez-Noriega et al., 1997). Finally, the lack of effect with α-CtxMII eliminates a role for α3β2β3 and/or α6β2β3*.

In summary, results obtained from rank order studies support a role for α3β4* nAChRs in hippocampal [^{3}H]-DA release. However, differences in pharmacology suggest that there are some differences in nAChR subtypes mediating release of each neurotransmitter. While (±)-UB-165 elicited a full response, relative to nicotine, in [^{3}H]-NE release, it behaved as a partial agonist in [^{3}H]-DA release. Since the pharmacological profile for (±)-UB-165 has not been fully characterized in rodent brain, it is currently difficult to determine other nAChRs involved in rat hippocampal [^{3}H]-DA release. Furthermore, the magnitude of blockade produced by MG 624 for each neurotransmitter suggests differences, and supports the involvement of more than one receptor in hippocampal [^{3}H]-DA release. Since our studies do not reveal the likely involvement of other well-established nAChR subtypes, the possibility exists that more complex heteromers, such as those containing α5 and/or β3, may also contribute to the hippocampal [^{3}H]-DA release we observe. Presently, the exact localization of nAChRs involved in dopamine release in the hippocampus is not clearly defined. DA release may be mediated directly by receptors localized on terminals of dopaminergic afferents projecting from SN and/or VTA, predominantly to the ventral hippocampus, or it could be evoked indirectly by non-dopaminergic neurons that then enhance DA release from other neurons located in the hippocampus.

Interestingly, although dopamine functions in the regulation of different aspects of cognitive brain functions (for review see Nieoullon, 2002), and α4β2 and α7 are the nAChRs identified thus far to be involved in working memory function in the

hippocampus, these receptors do not appear to play a role in either hippocampal [^{3}H]-DA or [^{3}H]-NE release (Clarke and Reuben, 1996; Wonnacott, 1997; Luo et al., 1998; Anderson et al., 2000). In support of the novel findings of a significant role for α3β4* nAChRs in DA release from hippocampus and the possible relevance to cognition that we describe, SIB1553A, a β4-preferring nAChR agonist (Rao et al., 2003), has recently been reported to exhibit cognitive enhancing properties, particularly in working memory, in several models (Bontempi *et al.*, 2003). As more selective nAChR ligands are developed, it will be worth further investigating the involvement of α3β4* in cognition, as well as in other brain functions which may respond to increased DA release in hippocampus.

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Figure Legends

Fig. 1. Concentration-response curves of agonist-induced [^{3}H]-DA and [^{3}H]-NE release from rat hippocampal slices. Rat hippocampal slices were preloaded with either [^{3}H]-DA (A and B) or [^{3}H]-NE (A) and exposed to different agonists, as described in Methods. Results were normalized and expressed as a percent of 100 μM (-)-nicotine-induced release. Data points represent the mean ± SEM of 4-5 separate experiments performed in quadruplets.

Fig. 2. Concentration-inhibition curves of (-)-nicotine-induced [^{3}H]-DA (A and B) and [^{3}H]-NE (B) release from rat hippocampus by antagonists. Preloaded rat hippocampal slices were preincubated for 5 minutes with the indicated concentrations of antagonist prior to the addition of 100 μM (-)-nicotine. Treatment with antagonists alone had no effect on basal levels. Data were normalized to release evoked by 100 μM (-)-nicotine in the absence of antagonist. Data points represent the mean ± SEM of 5-6 separate experiments performed in quadruplets.

Fig. 3. Concentration-inhibition curves of (-)-nicotine-induced [^{3}H]-DA release from rat hippocampus by methylycaconitine (MLA) (A), α–conotoxin IMI (α-CtxIMI) (B), α–bungarotoxin(α-BTX)(C), and α–conotoxin MII (α-CtxMII) (D). Basal release was collected after a 5 minute preincubation with the indicated concentrations of antagonist prior to the addition of 100 μM (-)-nicotine. In α-BTX experiments, slices were incubated for 60 minutes prior to collection of basal samples. Treatment with antagonists alone had no effect on basal dopamine levels. Data were normalized to release evoked by

100 μM (-)-nicotine in the absence of antagonist (indicated by dotted line). Data points represent the mean ± SEM of five separate experiments performed in quadruplets.

Fig. 4. Effect of mecamylamine (Mec) (A), hexamethonium (Hex) (B), dihydro-β-erythroidine (DHβE) (C), and MG 624 (D) on (-)-nicotine-induced [^{3}H]-DA release. Basal release was collected after a 5 minute preincubation with the indicated concentrations of antagonist prior to the exposure of different concentrations of (-)-nicotine. Data were normalized to release evoked by 100 μM (-)-nicotine in the absence of antagonist. Data points represent the mean ± SEM of 3-4 separate experiments performed in quadruplets.

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Table 1 Summary of concentration response assays of nicotinic agonist-evoked [^{3}H]-DA and [^{3}H]-NE release from slices of hippocampus

Maximal responses are normalized to that evoked by 100 μM (-)-nicotine. (CI, confidence interval; Con., concentration tested).

Drug	EC_{50}[a] (μM) (95 % CI)	Maximal response (Mean ± SEM)	Maximum inhibition by Mec	
			Con. (μM)	% Inhibition (Mean ± SEM)
A. [^{3}H]-DA Release				
EB	0.0065 (0.0010, 0.047)	106 ± 12	10	112 ± 18
(±)-UB-165	0.12 (0.058, 0.24)	58 ± 2	10	90 ± 9
Anatoxin-a	0.32 (0.075, 1.40)	104 ± 22	10	118 ± 7
ABT-594	2.14 (1.19, 3.84)	112 ± 7	100	78 ± 4
(-)-Nicotine	4.79 (1.74, 13.2)	113 ± 9	100	97 ± 7
DMPP	7.41 (2.66, 20.7)	109 ± 4	100	48 ± 5
(-)-Cytisine	16.2 (3.75, 70.2)	157 ± 27	100	98 ± 7
B. [^{3}H]-NE Release				
(±)-UB-165	0.058 (0.036, 0.092)	86 ± 6	10	103 ± 7
ABT-594	0.47 (0.16, 1.39)	120 ± 14	10	103 ± 1

[a] Calculated from pEC_{50}: $EC_{50} = 10^{-pEC_{50}}$.

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Table 2 Summary of concentration inhibition assays of nicotinic antagonist blockade of hippocampal [^{3}H]-DA and [^{3}H]-NE release

[^{3}H]-DA or [^{3}H]-NE release was evoked by 100 μM (-)-nicotine or 10 μM (±)-UB-165. (N.D., not able to determine; CI, confidence interval; Con., concentration tested.).

Drug	**IC_{50}[a] (μM) (95 % CI)**	**Maximum inhibition**	
		Con. (μM)	**% Inhibition (Mean ± SEM)**
A. 100 μM (-)-nicotine - [^{3}H]-DA			
Mec	0.20 (0.07, 0.57)	100	86 ± 10
MG 624	0.54 (0.18, 1.59)	100	60 ± 11
Hex	3.16 (0.53, 18.9)	1000	87 ± 6
d-TC	6.31 (1.87, 21.3)	1000	79 ± 8
DHβE	61 (21, 183)	1000	67 ± 10
MLA	N.D.	0.010	-4 ± 6
α-CtxImI	N.D.	1.0	-4 ± 11
α-BTX	N.D.	0.4	-1.0 ± 0.9
α-CtxMII	N.D.	0.1	12 ± 14
B. 100 μM (-)-nicotine - [^{3}H]-NE			
MG 624	0.34 (0.17, 0.69)	10	111 ± 5
C. 10μM (±)-UB-165 - [^{3}H]-NE			
MG 624	0.42 (0.22, 0.80)	10	100 ± 5

[a] Calculated from pIC_{50}: $IC_{50} = 10^{-pIC_{50}}$.

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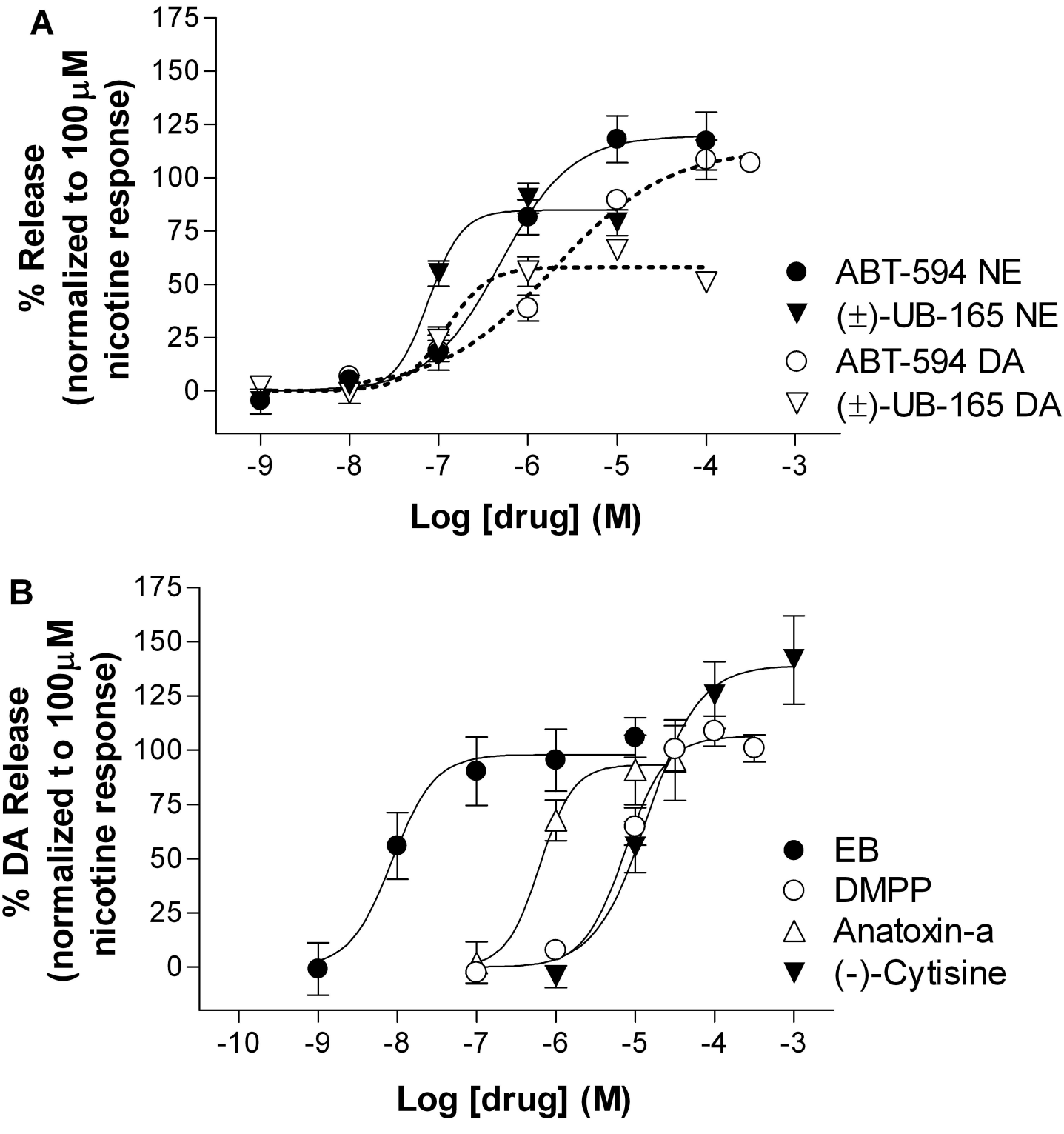

Fig. 1

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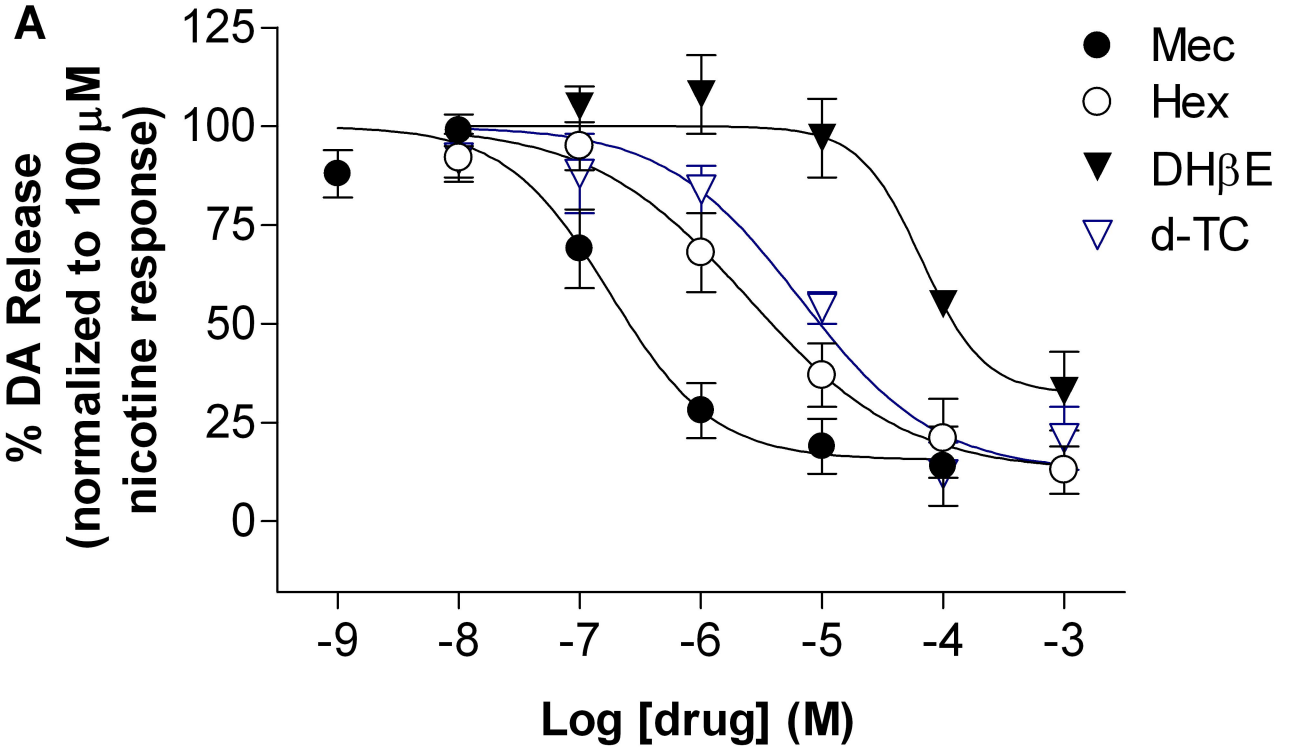


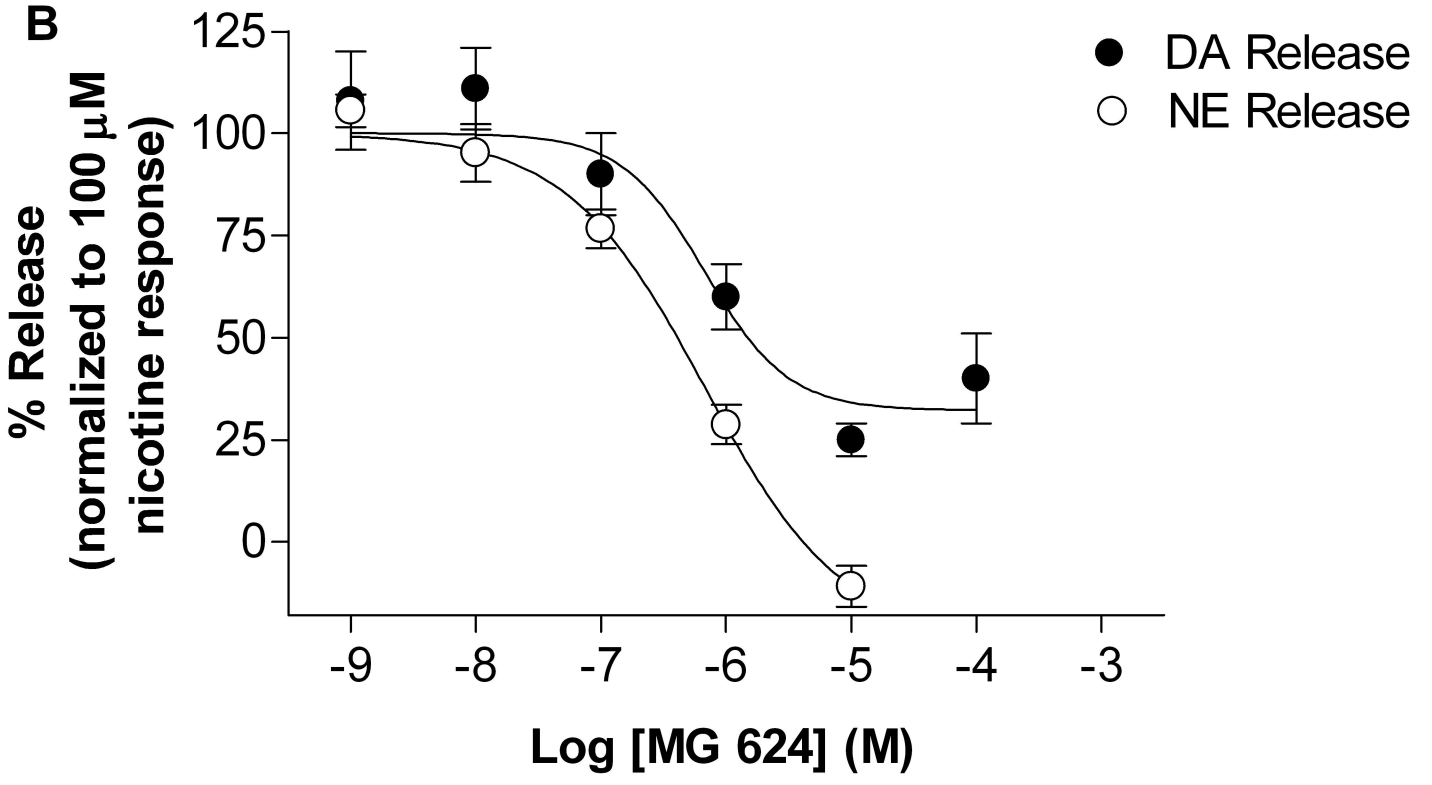


Fig. 2

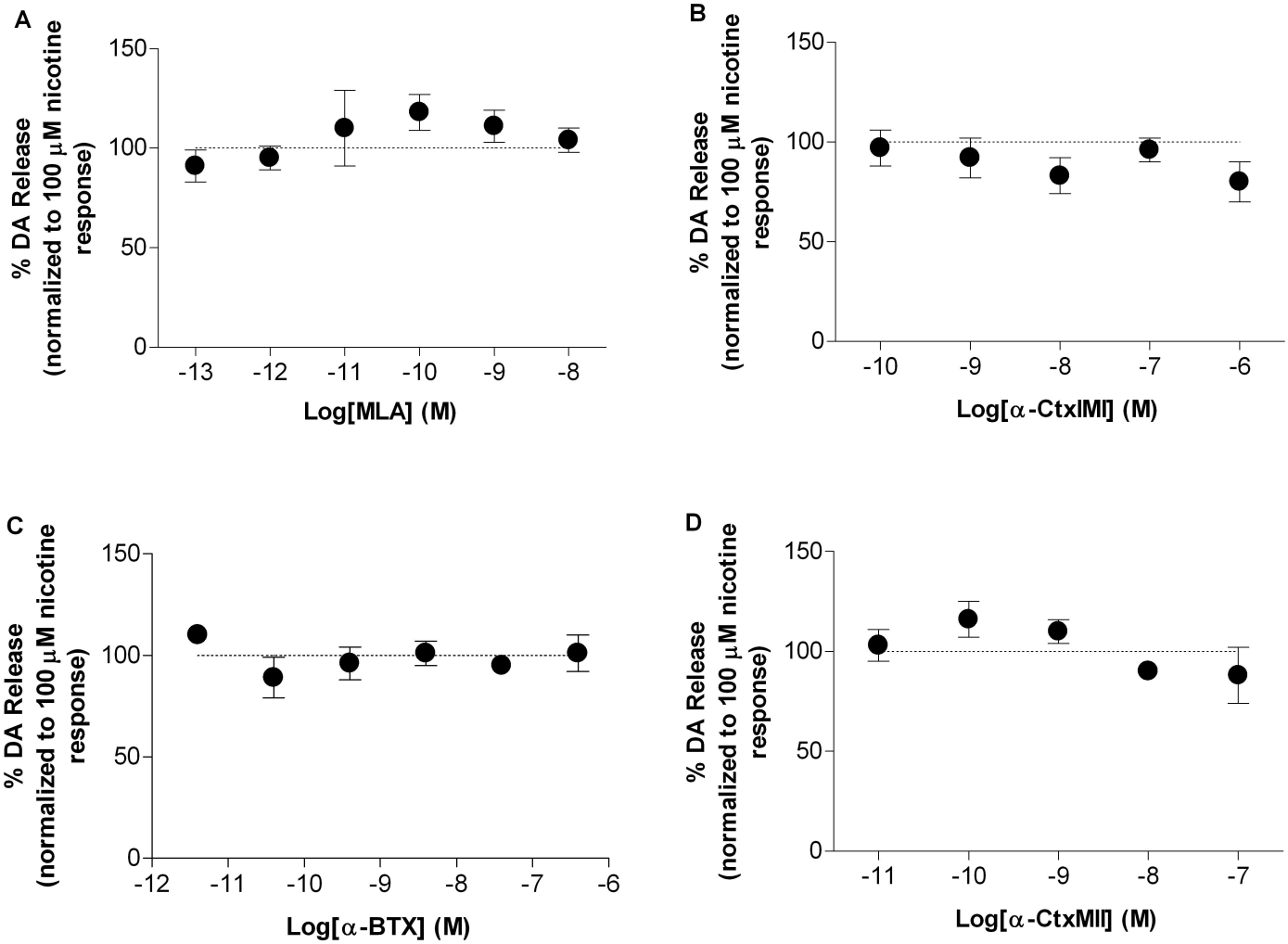
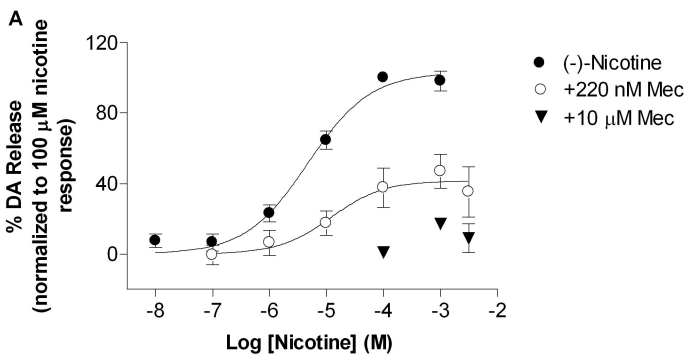
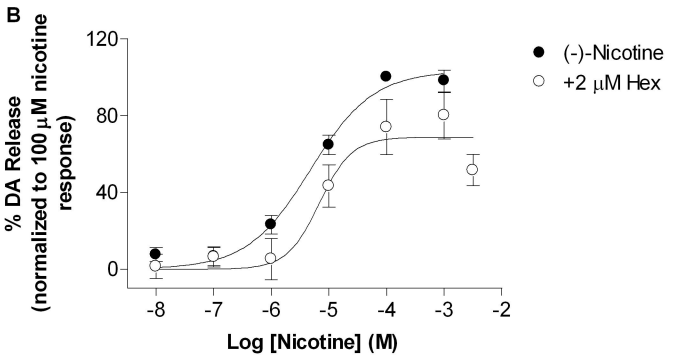
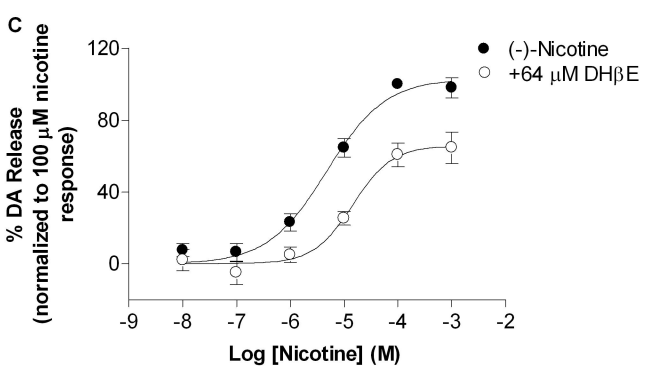


Fig. 3


A
% DA Release
(normalized to 100 μM nicotine response)
120
80
40
0
-8
-7
-6
-5
-4
-3
-2
Log [Nicotine] (M)
(-)-Nicotine
+220 nM Mec
+10 μM Mec


B
% DA Release
(normalized to 100 μM nicotine response)
120
80
40
0
-8
-7
-6
-5
-4
-3
-2
Log [Nicotine] (M)
(-)-Nicotine
+2 μM Hex


C
% DA Release
(normalized to 100 μM nicotine response)
120
80
40
0
-9
-8
-7
-6
-5
-4
-3
-2
Log [Nicotine] (M)
(-)-Nicotine
+64 μM DHβE

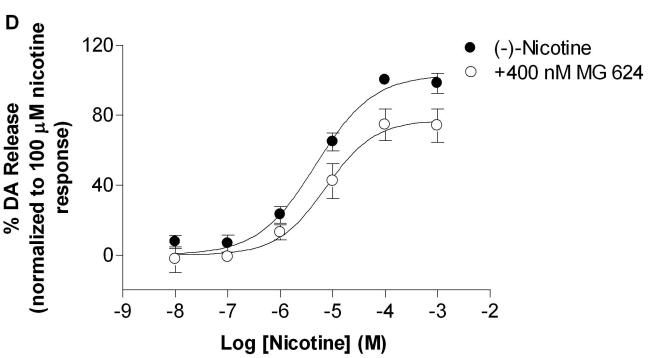

D
% DA Release
(normalized to 100 μM nicotine response)
120
80
40
0
-9
-8
-7
-6
-5
-4
-3
-2
Log [Nicotine] (M)
(-)-Nicotine
+400 nM MG 624

Fig. 4