British Journal of Pharmacology (1996) 117, 595-606 © 1996 Stockton Press All rights reserved 0007-1188/96 $12.00

Release of [^{3}H]-noradrenaline from rat hippocampal synaptosomes by nicotine: mediation by different nicotinic receptor subtypes from striatal [^{3}H]-dopamine release

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1 The aim of the present experiment was to characterize nicotine-evoked [^{3}H]-noradrenaline ([^{3}H]-NA) release from rat superfused hippocampal synaptosomes, using striatal [^{3}H]-dopamine release for comparison.

2 (−)-Nicotine, cytisine, DMPP and acetylcholine (ACh) (with esterase inhibitor and muscarinic receptor blocker) increased NA release in a concentration-dependent manner (EC_{50} 6.5 μM, 8.2 μM, 9.3 μM, and 27 μM, respectively) with similar efficacy.

3 Nicotine released striatal dopamine more potently than hippocampal NA (EC_{50} 0.16 μM vs. 6.5 μM). (+)-Anatoxin-a also increased dopamine more potently than NA (EC_{50} 0.05 μM vs 0.39 μM), and maximal effects were similar to those of nicotine. Isoarecolone (10−320 μM) released dopamine more effectively than NA but a maximal effect was not reached. (−)-Lobeline (10−320 μM) evoked dopamine release, but the effect was large and delayed with respect to nicotine; NA release was not increased but rather depressed at high concentrations of lobeline. High K^+ (10 mM) released dopamine and NA to similar extents.

4 Addition of the 5-hydroxytryptamine (5-HT) reuptake blocker, citalopram (1 μM) to hippocampal synaptosomes affected neither basal NA release nor nicotine-evoked release.

5 The nicotinic antagonist, mecamylamine (10 μM), virtually abolished NA and dopamine release evoked by high concentrations of nicotine, ACh, cytisine, isoarecolone, and anatoxin-a. Although NA release evoked by DMPP (100 μM) was entirely mecamylamine-sensitive, DMPP-evoked dopamine release was only partially blocked. Dopamine release evoked by lobeline (320 μM) was completely mecamylamine-insensitive.

6 The nicotinic antagonists dihydro-β-erythroidine and methyllycaconitine inhibited nicotine-evoked dopamine release approximately 30 fold more potently than NA release. In contrast, the antagonist chlorisondamine, displayed a reverse sensitivity, whereas trimetaphan and mecamylamine did not preferentially block either response. None of these antagonists, given at a high concentration, significantly altered release evoked by high K^+.

7 Blockade of nicotine-evoked transmitter release by methyllycaconitine and dihydro-β-erythroidine was surmounted by a high concentration of nicotine (100 μM), but blockade by mecamylamine, chlorisondamine, and trimetaphan was insurmountable.

8 Nicotine-evoked NA release was unaffected by tetrodotoxin, whereas veratridine-evoked NA release was virtually abolished.

9 We conclude that presynaptic nicotinic receptors associated with striatal dopamine and hippocampal NA terminals differ pharmacologically. *In situ* hybridization studies suggest that nigrostriatal dopaminergic neurones express mainly $\alpha4$, $\alpha5$, and $\beta2$ nicotinic cholinoceptor subunits, whereas hippocampal-projecting noradrenaline (NA) neurones express $\alpha3$, $\beta2$ and $\beta4$ subunits. Pharmacological comparisons of recombinant receptors suggest that release of hippocampal NA may be modulated by receptors containing $\alpha3$ and $\beta4$ subunits.

Keywords: Nicotine; acetylcholine; dopamine; noradrenaline; nicotinic receptors; synaptosomes; striatum; hippocampus; chlorisondamine; dihydro-β-erythroidine

Introduction

Nicotine evokes the release of a number of transmitters in rat brain via a direct action on nerve terminals. Particular attention has been focused on the nigrostriatal dopamine system, and several groups have characterized the pharmacology of nicotine-evoked release in striatal synaptosomes or slices. Striatal dopamine release that is evoked by micromolar concentrations of nicotine is dependent upon external calcium (Grady *et al.*, 1992; El-Bizri & Clarke, 1994) and mediated by nicotinic cholinoceptors (AChRs). Thus, the response is stereoselective (Rapier *et al.*, 1988), susceptible to desensitization (Rapier *et al.*, 1988; Grady *et al.*, 1994; Rowell & Hillebrand, 1994), mimicked by a number of nicotinic agonists and inhibited by certain nicotinic AChR antagonists (Rapier *et al.*, 1988; Schulz & Zigmond, 1989; Rowell & Hillebrand, 1994; Rapier *et al.*, 1990; Grady *et al.*, 1992; 1994; El-Bizri & Clarke, 1994; Clarke *et al.*, 1994b).

Nicotine also acts in the rat isolated hippocampus, resulting in the *in vitro* release of noradrenaline (NA) (Balfour, 1973; Arqueros *et al.*, 1978; Snell & Johnson, 1989). In the two earlier studies, effects of nicotine were obtained only at high concentrations (500 μM or greater) with properties suggestive of an intracellular, tyramine-like action. Subsequently, lower concentrations (10 and 100 μM) of nicotine were shown to release [^{3}H]-NA from superfused hippocampal slices (Snell & Johnson, 1989); this effect was inhibited by the nicotine AChR

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antagonist mecamylamine, but whether it derived from a direct action on NA terminals was not investigated. A recent report suggests that nicotine-evoked NA release from hippocampal slices may be largely indirect (Sacaan *et al.*, 1995).

The nicotinic AChR subtypes mediating nicotine-evoked release of striatal dopamine and hippocampal NA are not known, but evidence from *in situ* hybridization histochemistry suggests that they may differ. Thus, presumed nigrostriatal dopaminergic neurones contain abundant mRNA for α4, α5, and β2 subunits, with low levels of α3 signal (Wada *et al.*, 1989; 1990), whereas the locus coeruleus, which gives rise to the NA projection to hippocampus, contains high amounts of α3 message, and moderate β2 and β4 signals (Wada *et al.*, 1989; 1990; Dineley-Miller & Patrick, 1992). The present study therefore had two main goals: to characterize nicotine-evoked [^{3}H]-noradrenaline release from superfused rat hippocampal synaptosomes, and to identify pharmacological differences by comparison with striatal [^{3}H]-dopamine release.

Methods

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec), weighing 200–250 g, were maintained on a 12/12 h light-dark cycle. Rats were housed four per cage, and food and water were available *ad libitum*. Rats were allowed to accommodate to the housing conditions for 4 days after arrival, and were drug-naive prior to testing.

Dopamine and noradrenaline release from superfused synaptosomes

Methods for synaptosomal preparation and measurement of transmitter release were virtually identical to those described in detail elsewhere (Clarke *et al.*, 1994b). In each assay, crude synaptosomal (P2) fraction was prepared from dissected striata and/or hippocampus (approx. 140 and 150 mg per rat, respectively). Where the two tissues were compared in parallel, tissue was pooled from two rats per assay; where only one tissue was tested, tissue from four rats was pooled. The P2 fractions were resuspended (140–150 mg wet weight of original tissue ml^{-1}) in superfusion buffer (SB) composed of the following, in mM concentrations: NaCl 128, KCl 2.4, $CaCl_2$ 3.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 25, (+)-glucose 10, (+)-ascorbic acid 1 and pargyline 0.01 at pH 7.5. These synaptosomal preparations were incubated with 0.1–0.2 μM [^{3}H]-dopamine ([^{3}H]-DA; striatum) or 0.2 μM [^{3}H]-noradrenaline ([^{3}H]-NA; hippocampus) at 37°C for 10 min.

The superfusion apparatus comprised 32 identical channels, each comprising a small polypropylene retention chamber, through which superfusate was pumped at 0.4 ml min^{-1}. Each experiment comprised one or more assays. At the start of each assay, 100 μl of the synaptosomal suspension was introduced to each superfusion chamber. During the next 35 min, synaptosomes were superfused with superfusion buffer (SB) alone or with SB containing antagonist, as appropriate. Next, 12 samples per channel were collected in consecutive 1 min intervals: after a 5 min baseline collection period, a 1 min (0.4 ml) pulse of releasing drug or SB (prepared with or without antagonist as appropriate) was given. Finally, the filters holding the synaptosomes were removed in order to measure residual radioactivity (Wallac 1410 liquid scintillation counter, LKB, Sweden).

In each assay, data were collected simultaneously from all 32 channels. Each assay incorporated control (SB only) channels, and tissues and treatment conditions were counterbalanced across channels and assays. In striatal and hippocampal synaptosomes preloaded with [^{3}H]-dopamine or [^{3}H]-NA, tritium release largely corresponds to unmetabolized transmitter (Rapier *et al.*, 1988; Pittaluga & Raiteri, 1992). Therefore, in the present study, we refer to dopamine or NA release, as appropriate.

For each channel, the release occurring in each 1 min collection period was calculated as a percentage of basal release, determined from a 5 min baseline. For each individual channel, drug-evoked release was taken as the peak value occurring among the next 3 or 4 fractions. This measure of release is likely to be less affected than the time-averaged drug effect ('area under the curve') by receptor desensitization that occurs within the first minutes of drug administration (Rapier *et al.*, 1988; Grady *et al.*, 1994), although in practice, similar results have been obtained by both measures (data not shown).

Basal release of [^{3}H]-DA was 1900±100 d.p.m. min^{-1} (mean±s.e.mean across 12 experiments), i.e. approx. 3.4 fmol min^{-1} mg^{-1} original wet tissue, or 1% of residual radioactivity collected on the tissue filters. Basal release of [^{3}H]-NA was 1000±30 d.p.m. min^{-1}, i.e. approx. 0.47 fmol min^{-1} mg^{-1} original wet tissue, or 1.5% of residual radioactivity.

Drugs

Chemicals and suppliers were as follows: [^{3}H]-dopamine (dopamine, [8-^{3}H]-, specific activity 15.8 or 20.3 Ci mmol^{-1}) and [^{3}H]-noradrenaline (noradrenaline, [ring-2,5,6-^{3}H]-, specific activity 57.3 or 70.2 Ci mmol^{-1}) (New England Nuclear, Boston, MA, U.S.A.), (−)-nicotine hydrogen tartrate, cytisine, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), (−)-lobeline hydrochloride, and diisopropylfluorophosphate (DFP) (Sigma Chemical Corp., St. Louis, MO, U.S.A.), acetylcholine hydrochloride, (+)-anatoxin-a hydrochloride, pargyline hydrochloride, tetrodotoxin and veratridine (Research Biochemicals Inc., Natick, MA, U.S.A.). The following were generous gifts: chlorisondamine chloride (CHL) (Ciba-Geigy, Summit, NJ, U.S.A.), citalopram hydrobromide (H. Lundbeck A/S, Copenhagen, Denmark), dihydro-β-erythroidine (DHBE), mecamylamine hydrochloride (Mec) (Merck and Co, Rahway, NJ, U.S.A.), isoarecolone oxalate (Dr I.P. Stolerman), methyllycaconitine hydrochloride (MLA: Dr M.H. Benn), and trimetaphan camsylate (Hoffman-La Roche, Mississauga, Canada). Other chemicals and reagents were purchased from commerical sources. For superfusion, drugs were dissolved in buffer (SB).

Data analysis

Drug effects were examined by analysis of variance, using commercial software (Systat, Evanston, IL, U.S.A.). Multiple comparisons with a single control group were made with Dunnett's test (Dunnett, 1955); other multiple comparisons were made by Student's *t*-test with Bonferroni's correction (Glantz, 1992). Probability values are 2-tailed.

For each agonist, EC_{50} values were determined by non-linear regression using the Hill equation:

$$E = E_{max} \times C^{n_H}/(C^{n_H} + EC_{50}{}^{n_H})$$

where E is the peak release, expressed as the percentage increase above baseline, C is the concentration of agonist, and n_H is the Hill number. Linear regression yielded somewhat lower EC_{50} values (Clarke & Reuben, 1995a). IC_{50} values of antagonists were determined in an analogous manner, except that in certain assays, not all concentrations were tested in parallel; release data were therefore normalized within each assay with respect to the effect of agonist alone. Unless otherwise stated, dopamine and NA release were tested in parallel.

Results

Experiment 1: Effects of nicotine, acetylcholine, cytisine and DMPP on hippocampal [^{3}H]-NA release

In a set of four assays, nicotine, ACh, cytisine and DMPP were tested in parallel for their ability to induce hippocampal [^{3}H]-

NA release. All agonists except for ACh were tested at 1–100 μM. ACh was tested at 1–320 μM; for this agonist, the superfusion medium contained DFP 30 μM and atropine sulphate 1 μM, in order to inhibit hydrolysis of ACh and to prevent activation of muscarinic cholinoceptors, respectively. All four agonists increased [^{3}H]-NA release in a concentration-dependent manner; equivalent pulses of superfusion buffer (SB) alone were ineffective (Figure 1).

Within the concentration-range tested, responses to nicotine and cytisine tended to plateau at high concentrations, whereas responses to ACh and DMPP did not (Figure 1). To test whether higher concentrations of ACh and DMPP produced a similar maximal effect to nicotine, ACh (1000 μM, with DFP 30 μM and atropine 10 μM present) and DMPP (320 μM) were tested in parallel against a maximally effective concentration of nicotine (100 μM) ($n = 8-10$ channels per condition). The drug effects were similar in magnitude: mean ± s.e.mean peak release was 186.3±5.2 and 197.6±8.5% vs. 185.2±8.8% of basal release, respectively (main effect of agonist: $F = 0.85$, d.f. 2.25, $P > 0.4$).

The concentration-response data (Figure 1) were therefore analyzed by non-linear regression analysis, assuming equal efficacy and constraining the maximal response to be 210% of basal (a value based on data inspection). Estimated EC_{50} values (±s.e.) were: nicotine 6.5±1.1 μM, ACh 27±8 μM, cytisine 8.2±1.8 μM, DMPP 9.3±2.6 μM. The time course of release was similar for each agonist (Figure 2).

Experiment 2: Effects of nicotine, anatoxin-a, isoarecolone and lobeline on hippocampal [^{3}H]-NA vs. striatal [^{3}H]-dopamine release

Each agonist was tested individually in a set of two or more assays. Within each assay, hippocampal [^{3}H]-NA release and striatal [^{3}H]-dopamine release were tested in parallel. Nicotine was considerably more potent in releasing [^{3}H]-dopamine (EC_{50}±s.e. 0.16±0.04 μM) than [^{3}H]-NA (6.5±2.1 μM). Although the peak effects were similar (Figure 3a), release of [^{3}H]-dopamine was somewhat more prolonged (Figure 4). This difference was subsequently observed in four out of eight groups of assays where the two transmitters were again tested in parallel.

Anatoxin-a increased [^{3}H]-dopamine-release more potently than [^{3}H]-NA release (EC_{50}±s.e. 0.051±0.023 μM vs. 0.39±0.18 μM). The maximal effects of anatoxin-a were similar in the two tissues, and comparable to those of nicotine (Figure 3b). Unlike nicotine, anatoxin-a did not appear to evoke a more prolonged release of [^{3}H]-dopamine than of [^{3}H]-NA (Figure 4). Isoarecolone (1–320 μM) enhanced the release of both transmitters, but the concentration-response curves did not plateau; at a given concentration, isoarecolone had a greater effect on [^{3}H]-dopamine release (Figure 3c).

Lobeline differed in several respects from the other agonists tested. Firstly, although it increased [^{3}H]-dopamine release (Figure 3d), this effect was delayed by 2 min compared with that seen in channels tested in parallel with nicotine 100 μM (Figure 5). Secondly, [^{3}H]-dopamine release evoked by a high concentration of lobeline did not appear to plateau, and exceeded the maximal effect of nicotine (Figure 3d). Thirdly, lobeline failed to increase [^{3}H]-NA release at any concentration (Figure 3d); at high concentrations, a depressant effect was seen which peaked 2 min after the stimulatory nicotine peak (Figure 5).

In order to determine whether striatal [^{3}H]-dopamine release was more sensitive than hippocampal [^{3}H]-NA release to a non-nicotinic secretagogue, high K^+ was tested. A concentration of K^+ (10 mM) was selected that gave a submaximal response. This stimulus evoked similar amounts of [^{3}H]-dopamine and [^{3}H]-NA release (% basal release 131±2 vs. 149±6). Responses to nicotine, tested in parallel, were 173±8 vs. 196±13.

Experiment 3: Effect of the 5-HT transporter blocker, citalopram

In a recent study, hippocampal synaptosomes were pre-loaded with [^{3}H]-NA in the presence of a selective 5-HT reuptake blocker, as a precaution against possible uptake of [^{3}H]-NA into 5-hydroxytryptaminergic terminals (Pittaluga & Raiteri, 1992). This eventually was tested as follows. Pooled hippocampal synaptosomes from 4 rats were divided into 26 samples of 140 μl, each equivalent to 21 mg original wet tissue. Equal numbers of randomly assigned samples were preincubated for 10 min at RT with added buffer or citalopram, at a concentration (1 μM) reported to block the 5-HT transporter without significantly inhibiting the NA transporter (Hyttel, 1994). [^{3}H]-NA was then added according to the standard

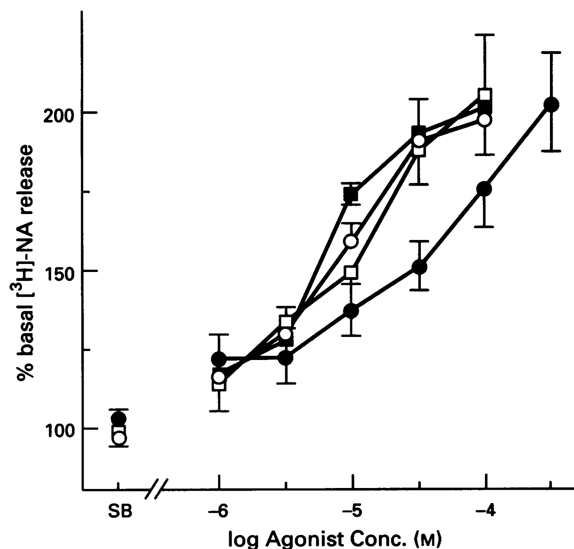

Figure 1 Effects of nicotinic agonists on [^{3}H]-noradrenaline release from hippocampal synaptosomes. Synaptosomes were superfused with superfusion buffer (SB) for 35 min prior to administration of a 1 min pulse of the agonist or SB (controls). Agonists were tested in parallel: (−)-nicotine (■), acetylcholine (●), 1,1-dimethyl-4-phenylpiperazinium (DMPP: □), and cytisine (○). The vertical axis represents the mean (±s.e.mean) peak release, calculated as a percentage of basal release ($n = 5-7$ channels).

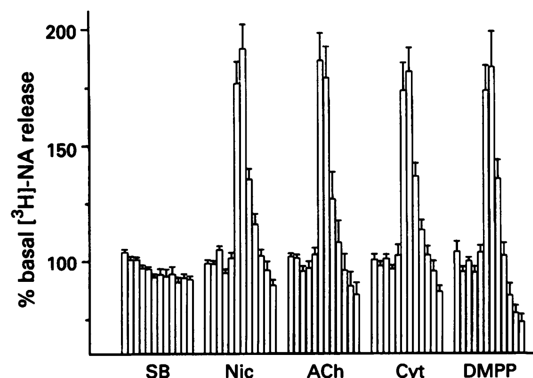

Figure 2 Time course of [^{3}H]-NA release from hippocampal synaptosomes evoked by nicotinic agonists. Serial 1 min samples are shown. After a 5 min basal collection period, a 1 min pulse was given, either of superfusion buffer (SB), nicotine (Nic), acetylcholine (ACh), cytisine (Cyt), or 1,1-dimethyl-4-phenylpiperazinium (DMPP). For each agonist, data were pooled from the two highest concentrations shown in Figure 1. The vertical axis represents the mean (±s.e.mean) release, calculated as a percentage of basal release ($n = 10$ channels for each agonist, $n = 20$ for SB).

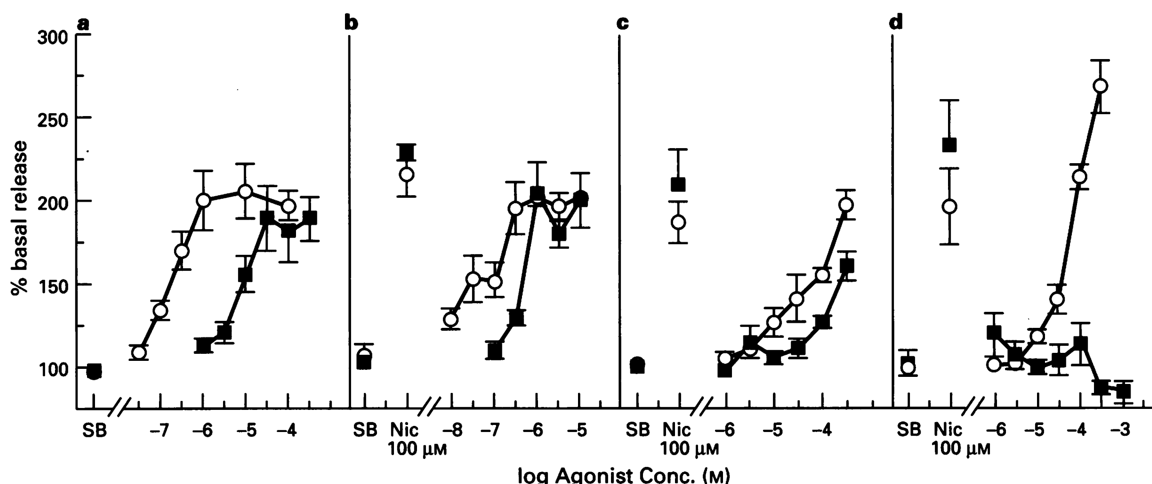

Figure 3 Comparison of agonist-evoked release of striatal [^{3}H]-dopamine (○) and hippocampal [^{3}H]-NA (■). Within each panel, tests were conducted in parallel. (a) (−)-nicotine; (b) (+)-anatoxin-a; (c) isoarecolone; (d) lobeline. In each assay, additional channels were challenged with superfusion buffer (SB) or a high concentration of nicotine (100 μM). The vertical axis represents the mean (±s.e.mean) peak release, calculated as a percentage of basal release (n=5–7 channels).

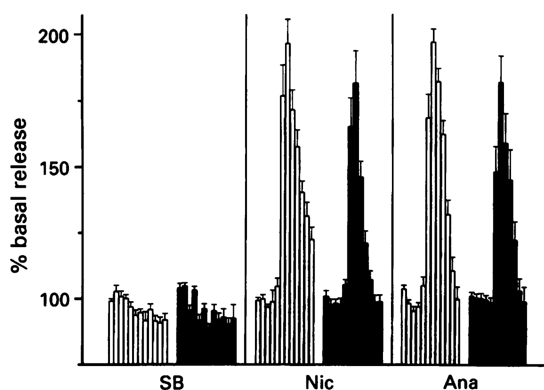

Figure 4 Time course of nicotine- and (+)-anatoxin-a-evoked release of striatal [^{3}H]-dopamine (open columns) and hippocampal [^{3}H]-NA (solid columns). After a 5 min basal collection period, a 1 min pulse was given, either of superfusion buffer (SB), nicotine (Nic), or anatoxin-a (Ana). For each agonist, data were pooled from the two (nicotine) or three (anatoxin-a) highest concentrations shown in Figure 3. The vertical axis represents the mean (±s.e.mean) release, calculated as a percentage of basal release (n=12–15 channels for each agonist, n=7 for SB).

protocol. Each sample was tested in a different superfusion channel, either with nicotine 100 μM or with buffer, in a counterbalanced design.

Synaptosomes that had been incubated with or without citalopram did not differ significantly on any measure. Respective mean values were as follows: basal release 1310±100 vs. 1100±30 d.p.m. min^{-1} (t=1.93, d.f. 24,P>0.06), nicotine-induced release (% basal: 166±9 vs. 157±10), and residual radioactivity (59,700±6,700 vs. 53,300±2,500 d.p.m.).

Experiment 4: Mecamylamine-sensitivity of agonist-induced [^{3}H]-NA and [^{3}H]-dopamine release

The aim was to test whether the stimulatory effects of high agonist concentrations were sensitive to the nicotinic AChR blocker, mecamylamine. Antagonism of nicotine-evoked [^{3}H]-dopamine and [^{3}H]-NA release by mecamylamine is insurmountable (El-Bizri & Clarke, 1994; Experiment 8). Mecamylamine was applied in a high concentration (10 μM) in the superfusion buffer, and was present for 35 min before as well as during agonist challenge.

Mecamylamine virtually abolished hippocampal [^{3}H]-NA release evoked by all agonists tested: nicotine 100 μM, DMPP 100 μM, anatoxin-a 10 μM, isoarecolone 320 μM, ACh 100 μM, and cytisine 100 μM (Figure 6). Mecamylamine also abolished striatal [^{3}H]-dopamine release evoked by nicotine 100 μM, anatoxin-a 10 μM, isoarecolone 320 μM, ACh 100 μM, and cytisine 10 μM (Figure 6). In contrast, [^{3}H]-dopamine release induced by lobeline 320 μM was unaffected, and that evoked by DMPP 100 μM was only somewhat reduced (Figure 6).

Mecamylamine did not significantly alter basal release in any group of assays. Mean (±s.e.mean) values of basal release of [^{3}H]-NA with vs. without mecamylamine were, respectively: 930±30 vs. 980±50 d.p.m. min^{-1} (anatoxin-a, isoarecolone), 1110±20 vs. 1180±30 d.p.m. min^{-1} (ACh, cytisine), and 1040±20 vs. 990±30 d.p.m. min^{-1} (nicotine, DMPP). Equivalent values for [^{3}H]-dopamine release were: 1930±80 vs. 1860±70 d.p.m. min^{-1} (lobeline, anatoxin-a, isoarecolone), and 1620±40 vs. 1670±60 d.p.m. min^{-1} (ACh, cytisine).

Experiment 5: Antagonist potency of dihydro-β-erythroidine, methyllyaconitine and trimetaphan

The nicotinic antagonist, dihydro-β-erythroidine (DHBE), was tested for its potency to inhibit nicotine-induced [^{3}H]-dopamine and [^{3}H]-NA release. Six assays were performed: in four assays, both responses were tested, and in two, [^{3}H]-NA release only was tested. The nicotine challenge concentrations were 0.16 μM (for [^{3}H]-dopamine) and 5 μM (for [^{3}H]-NA), approximating the EC_{50} values determined in Experiments 1 and 2. In the absence of DHBE, nicotine increased [^{3}H]-dopamine release by 46% and [^{3}H]-NA release by 60%. DHBE was approximately 35 fold more potent in inhibiting the [^{3}H]-dopamine response (IC_{50} 0.032±0.019 μM) than the [^{3}H]-NA response (1.2±0.6 μM) (Figure 7a). Basal [^{3}H]-dopamine release was significantly altered by DHBE, but only at two concentrations (0.03 and 0.3 μM: both 49% increase, P<0.001). Basal [^{3}H]-NA release was unaffected (P>0.2).

The nicotinic antagonist, methyllycaconitine (MLA), was tested in three assays, with [^{3}H]-dopamine and [^{3}H]-NA release tested in parallel (Figure 7b). Nicotine was again given close to its EC_{50} (0.16 μM and 5 μM, respectively). In the absence of antagonist, nicotine increased [^{3}H]-dopamine release by 66% and [^{3}H]-NA release by 50% over baseline. MLA was approximately 25 fold more potent in inhibiting the [^{3}H]-dopamine response (IC_{50} 0.038±0.01 μM) than the [^{3}H]-NA response (1.0±1.4 μM). Basal release of [^{3}H]-dopamine and [^{3}H]-NA was unaffected by MLA (P>0.1 for both).

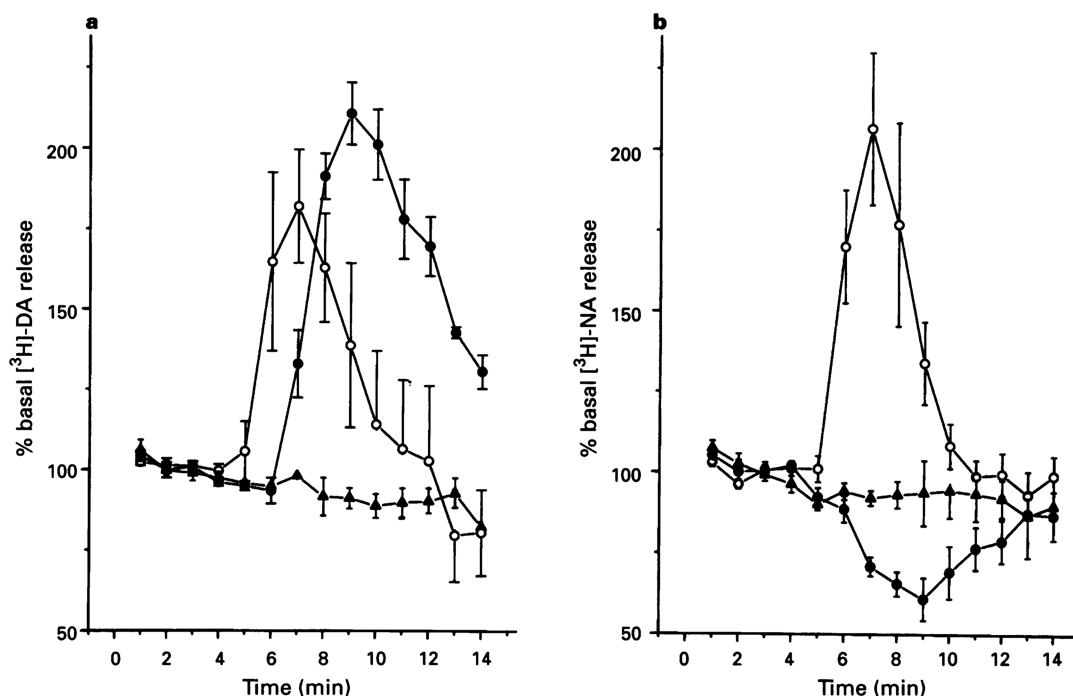

Figure 5 Time course of nicotine- vs. lobeline-evoked release of striatal [^{3}H]-dopamine ([^{3}H]-DA (a) and hippocampal [^{3}H]-NA (b)). After a 5 min basal collection period, a 1 min pulse was given, either of superfusion buffer (▲), nicotine 100 μM (○), or lobeline 100 μM ([^{3}H]-dopamine or 320 μM ([^{3}H]-NA) (●). The vertical axis represents the mean ($\pm$s.e.mean) peak release, calculated as a percentage of basal release (n=4–6 channels).

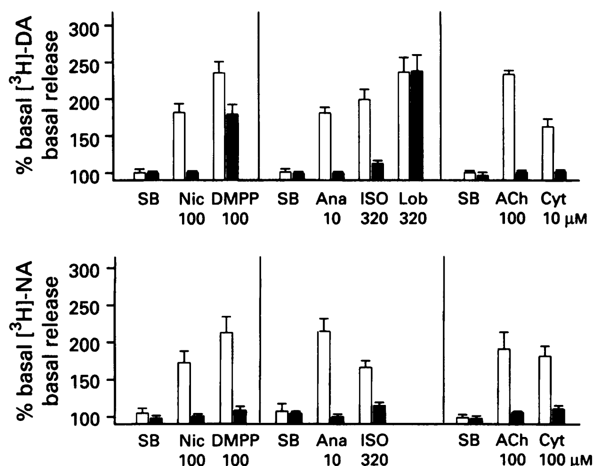

Figure 6 Effects of a high concentration of mecamylamine on agonist-evoked release of striatal [^{3}H]-dopamine ([^{3}H]-DA) (a) and hippocampal [^{3}H]-NA (b). Synaptosomes were superfused with buffer either with (solid columns) or without (open columns) mecamylamine 10 μM, prior to challenge with superfusion buffer (SB) or agonist: nicotine (Nic), 1,1-dimethyl-4-phenylpiperazinium (DMPP), anatoxin-a (Ana), isoarecolone (Iso), lobeline (Lob), acetylcholine (ACh), and cytisine (Cyt). The vertical axis represents the mean ($\pm$s.e.mean) release, calculated as a percentage of basal release (n=5–8 channels, except for DMPP-evoked [^{3}H]-dopamine release where n=13).

Trimetaphan was tested in a similar manner (Figure 7c). In the absence of antagonist, nicotine increased [^{3}H]-dopamine release by 32% and [^{3}H]-NA release by 34%. Unlike DHBE and MLA, trimetaphan inhibited evoked [^{3}H]-dopamine and [^{3}H]-NA release with similar potency (IC_{50} approx. 20 μM) (Figure 7c). Basal release of [^{3}H]-dopamine and [^{3}H]-NA was unaffected by trimetaphan ($P>0.1$ for both).

Experiment 6: Antagonism by mecamylamine and chlorisondamine

In order to compare the effects of mecamylamine and chlorisondamine between the two preparations, the antagonists were given at IC_{50} values previously determined against nicotine-induced [^{3}H]-dopamine release (0.3 and 1.6 μM, respectively) (El-Bizri & Clarke, 1994). Maximal concentrations of nicotine were used (10 μM for dopamine, 100 μM for NA release); both antagonists act insurmountably (El-Bizri & Clarke, 1994; Experiment 8).

Mecamylamine inhibited the dopamine and NA responses to a similar extent (by 38% and 42%, respectively). In contrast, chlorisondamine inhibited the NA response more than the dopamine response (by 79$\pm$10% vs 35$\pm$6%, respectively: t-test/Bonferroni: $P<0.005$). Basal release of [^{3}H]-dopamine and [^{3}H]-NA was not significantly altered by either antagonist ($P>0.1$ for each).

Experiment 7: Effects of antagonists on release evoked by high K^+

Antagonists were also tested for possible non-nicotinic inhibition of release. Each antagonist was applied in a high concentration in the superfusion buffer for 35 min before as well as during challenge with high K^+ (12 mM). No significant alteration of K^+-evoked [^{3}H]-dopamine or [^{3}H]-NA release occurred (main effect of Antag $P>0.2$ for either response; Table 1).

Experiment 8: Surmountable vs. insurmountable antagonism

Dihydro-β-erythroidine inhibits nicotine-induced release of striatal [^{3}H]-dopamine in a surmountable fashion, whereas

mecamylamine and chlorisondamine act insurmountably in the same assay (El-Bizri & Clarke, 1994). In order to determine whether the same mode of block occurred in the [^{3}H]-NA release assay, these three antagonists were tested at an intermediate concentration against nicotine administered near its EC_{50} (5 μM) and at a high concentration (100 μM). Blockade by DHBE (1.6 μM) was overcome by the high concentration of nicotine, whereas that by chlorisondamine (0.5 μM) and mecamylamine (0.4 μM) was not (Figure 8a–c). Methyllycaconitine (MLA) was tested in a similar manner in both the [^{3}H]-dopamine and [^{3}H]-NA release assays. In both assays, inhibition by MLA (0.046 and 2.1 μM, respectively) was surmounted by the high concentration of nicotine (Figure 8d,f). Trimetaphan (10 μM) exerted an insurmountable block in the [^{3}H]-NA release assay (Figure 8e) and possibly in the [^{3}H]-dopamine assay (Figure 8g).

Experiment 9: Effect of tetrodotoxin on nicotine-evoked [^{3}H]-NA release

Tetrodotoxin (TTX) was applied to hippocampal synaptosomes in a high concentration (1 μM), and was present in the superfusion buffer for 35 min before as well as during agonist challenge. In control channels, TTX was omitted. Synaptosomes (11–12 channels per condition) were challenged with SB, nicotine 5 μM, or with the voltage-gated sodium channel activator, veratridine (10 μM), acting as a positive control. TTX virtually eliminated the massive increase in NA release evoked by veratridine (529±22% vs. 119±3% of basal release). In contrast, nicotine-evoked release was non-significantly increased by TTX (137±5% vs. 151±9% of basal release; t=1.43, d.f. 21). TTX reduced basal NA release by 21%.

Discussion

Nicotine directly releases noradrenaline from hippocampal terminals

In the present study, the use of synaptosomes and the quite rapid superfusion make it virtually certain that the effects observed were the result of direct drug actions on the NA terminal, reflecting alterations of tramsmitter release and not

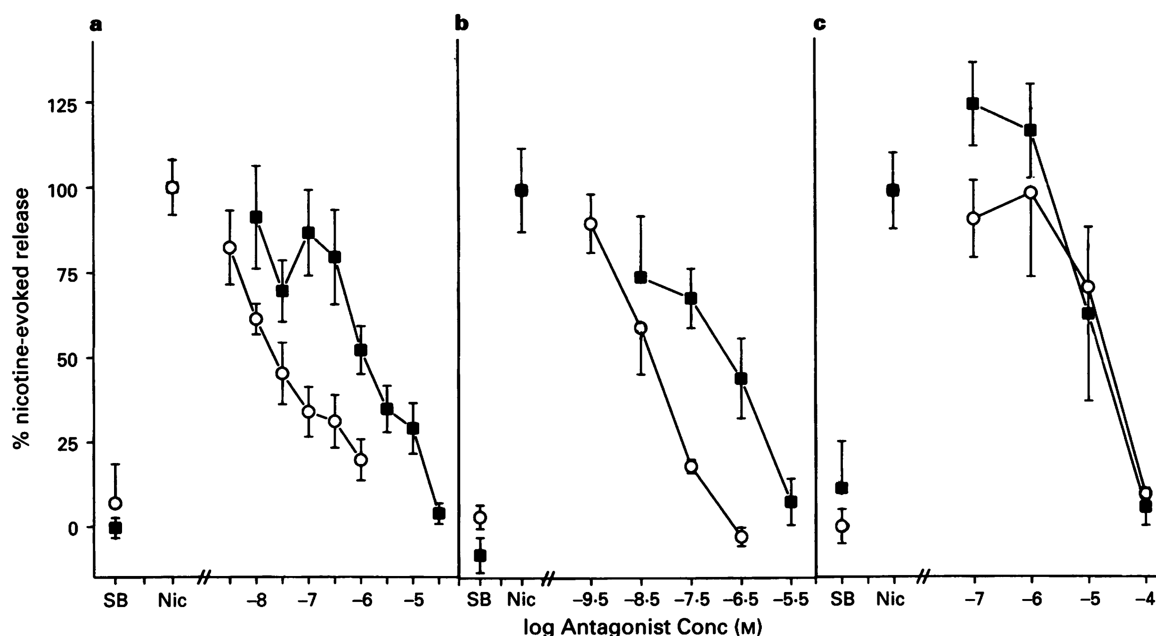

Figure 7 Effects of nicotinic antagonists on nicotine-evoked release. Release of striatal [^{3}H]-dopamine (○) and hippocampal [^{3}H]-NA (■) was assayed in parallel. (a) Dihydro-β-erythroidine; (b) methyllycaconitine; (c) trimetaphan. Antagonists were present in the SB during superfusion prior to challenge with nicotine (0.16 μM for [^{3}H]-dopamine and 5 μM for [^{3}H]-NA). Control channels were superfused with buffer alone and challenged with buffer (SB) or nicotine (Nic). The vertical axis respresents the mean (±s.e.mean) peak release, calculated as a percentage of basal release and normalized (n=4–6 channels for SB, 18–36 for nicotine alone, 6–12 for nicotine with antagonist).

Table 1 K^+-evoked [^{3}H]-dopamine and [^{3}H]-NA release in the presence of nicotinic antagonists (Experiment 7)

	SB	DHBE	MLA	TRIM	CHL	MEc
[^{3}H]-dopamine		1 μM	1 μM	100 μM	10 μM	10 μM
	141±3	153±7	133±6	132±4	145±8	142±10
[^{3}H]-NA		30 μM	30 μM	100 μM	10 μM	10 μM
	193±6	205±16	224±24	225±14	212±25	208±9

Drug challenge comprised a pulse of K^+ (12 mM). Evoked release is expressed as the mean±s.e.mean peak effect, as a percentage of basal release (n=4–10 channels for [^{3}H]-dopamine, 7–19 channels for [^{3}H]-NA). For abbreviations, see text.

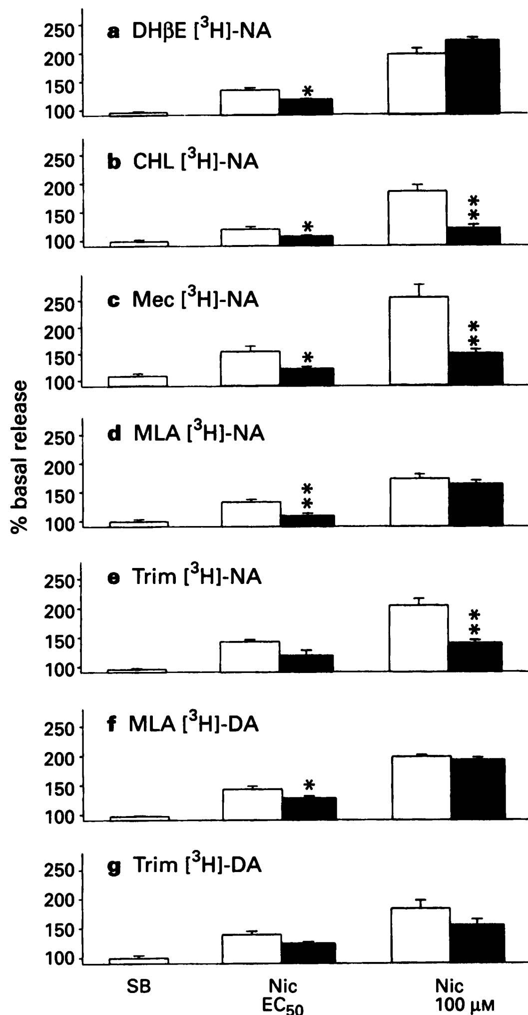

Figure 8 Surmountable vs. insurmountable antagonism of nicotine-evoked release. Synaptosomes were superfused with an intermediate concentration of antagonist (solid column) or with SB alone (open columns), and challenged with either SB or nicotine. Nicotine was given near its estimated EC_{50} (0.16 μM for [^{3}H]-dopamine ([^{3}H]-DA) 5 μM for [^{3}H]-NA) or at a high concentration (100 μM). (a) [^{3}H]-NA: dihydro-β-erythroidine 1.6 μM; (b) [^{3}H]-NA: chlorisondamine 0.5 μM; (c) [^{3}H]-NA: mecamylamine 0.4 μM; (d) [^{3}H]-NA: methyllycaconitine 2.1 μM; (e) [^{3}H]-NA: trimetaphan 10 μM; (f) [^{3}H]-DA: methyllycaconitine 0.046 μM; (g) [^{3}H]-DA: trimetaphan 10 μM. The vertical axis represents the mean ($\pm$s.e.mean) peak release, calculated as a percentage of basal release and normalized ($n=4$ channels for SB, 6–7 for nicotine). $*P<0.05$, $**P<0.01$ different from nicotine alone (Bonferroni t test).

reuptake. The present analysis therefore provides strong evidence that nicotine acts directly on noradrenergic terminals of the hippocampus via nicotinic AChRs.

In early *in vitro* studies, high concentrations of nicotine were used that probably produced effects that were not mediated by nicotinic AChRs (Balfour, 1973; Arqueros *et al.*, 1978). In two subsequent studies, both employing hippocampal slices, lower concentrations of nicotine were found to evoke NA release (Snell & Johnson, 1989; Sacaan *et al.*, 1995). In the first of these, a direct action of nicotine on noradrenergic terminals was not tested for. In the most recent study, NA release evoked by nicotinic agonists was largely blocked by tetrodotoxin, implicating a probable indirect action requiring axonal conduction. However, this conclusion is weakened by a recent report that tetrodotoxin may also inhibit synaptosomal transmitter release evoked by a nicotinic agonist (Soliakov *et al.*, 1995).

The latter finding prompted our final experiment, in which nicotine-evoked NA release from hippocampal synaptosomes was shown to be unaffected by tetrodotoxin. This observation supports the suggestion made by Sacaan *et al.* (1995) that their evoked NA release was substantially indirect in origin. Certain other features distinguish their nicotinic response from the response described here in synaptosomes. In their preparation, DMPP was more potent than cytisine and nicotine. In addition, the antagonist DHBE appeared ineffective; however, this drug acts in a surmountable fashion (El-Bizri & Clarke, 1994; Experiment 8).

Certain agonists evoked transmitter release via a nicotinic mechanism

A number of nicotinic agonists induced [^{3}H]-dopamine and [^{3}H]-NA release by a mechanism that was completely mecamylamine-sensitive (Experiment 4). Although mecamylamine is commonly regarded as a selective nicotinic antagonist, some antagonist activity has been reported at NMDA-type glutamate receptors (Olney *et al.*, 1987; Snell & Johnson, 1989; Clarke *et al.*, 1994a). In our striatal assay, we have been unable to detect NMDA-evoked [^{3}H]-dopamine release (Clarke & Reuben, 1995b); hence the complete abolition of nicotinic agonist-evoked [^{3}H]-dopamine release by mecamylamine most probably reflects an entirely nicotinic mechanism.

In the hippocampus, the interpretation of mecamylamine sensitivity is less straightforward. As in the striatal assay, mecamylamine was used at a concentration (10 μM) which had no clear effect against NMDA responses in mesencephalic cell culture (Clarke *et al.*, 1994a). However, in the hippocampus, mecamylamine appears unusually potent, in that it is reported to inhibit NMDA-induced [^{3}H]-NA release in the low micromolar range (Snell & Johnson, 1989). This action was observed in a slice preparation, where indirect actions of NMDA were not excluded. Indeed, in our synaptosomal assay of [^{3}H]-NA release, the maximal effect of NMDA is only one quarter that of nicotine, even under conditions that favour NMDA receptor activation (unpublished results). In conclusion, the observed sensitivity to mecamylamine suggests that most agonists under study evoked [^{3}H]-dopamine and [^{3}H]-NA release largely or exclusively via nicotinic AChRs located on terminals in striatum and hippocampus, respectively.

Comparisons between agonist-evoked [^{3}H]-dopamine and [^{3}H]-NA release

Tests with nicotinic agonists revealed differences between the two assays and between individual drugs. Nicotine produced a similar maximal effect in the two tissues, but was considerably more potent in releasing [^{3}H]-dopamine than [^{3}H]-NA. (+)-Anatoxin-a is a potent nicotinic agonist in both the periphery and the CNS (Swanson *et al.*, 1992; Thomas *et al.*, 1993; Soliakov *et al.*, 1995). Like nicotine, it potently inhibits [^{3}H]-nicotine and [^{125}I]-α-bungarotoxin binding sites in rat brain (Wonnacott *et al.*, 1992). However, it only partially mimics the nicotine discriminative stimulus, and its effects on locomoter activity differ from those of nicotine (Stolerman *et al.*, 1992). In our assays, (+)-anatoxin-a resembled nicotine in terms of its maximal effect, in its higher potency for [^{3}H]-dopamine vs. [^{3}H]-NA release, and in sensitivity to mecamylamine. It is important to note that although both nicotine and anatoxin-a acted more potently in the [^{3}H]-dopamine release assay, a submaximal K^+ stimulus appeared equi-effective in the two assays. This indicates that the greater agonist potency seen in the [^{3}H]-dopamine release assay reflects a difference at the level of nicotinic AChRs rather than in the coupling of depolarization to release mechanisms.

The synthetic nicotinic antagonist, isoarecolone, mimics certain central actions of nicotine but not others. Thus, it inhibits [^{3}H]-nicotine binding to rat brain, produces a nicotine-like cue, but fails to mimic locomotor stimulant actions of

nicotine (Reavill *et al.*, 1987; 1990b; Whiteaker *et al.*, 1995). Isoarecolone and nicotine also produce somewhat different behavioural effects in monkeys (Buccafusco *et al.*, 1995). Recently, isoarecolone was shown to release [^{3}H]-dopamine from rat striatal and cortical synaptosomes in a concentration-dependent and mecamylamine-sensitive manner (Whiteaker *et al.*, 1995). In the present study, isoarecolone increased release of both striatal [^{3}H]-dopamine and hippocampal [^{3}H]-NA, and both effects were blocked by mecamylamine. The results obtained in striatal tissue were very similar to those of Whiteaker *et al.* (1995). In this tissue, isoarecolone appeared at least as efficacious as nicotine (Figure 3c). In contrast, isoarecolone is reported to be less efficacious than nicotine in releasing [^{3}H]-dopamine from rat frontal cortex (Whiteaker *et al.*, 1995).

Lobeline and DMPP exerted non-nicotinic actions

Two nicotinic drugs, lobeline and DMPP, exerted actions that were not blocked by mecamylamine. Lobeline possesses a complex pharmacological profile. Like nicotine, it possesses a high affinity for [^{125}I]-α-bungarotoxin, [^{3}H]-nicotine, and [^{125}I]-dihydro-β-erythroidine binding sites (Schmidt, 1977; Marks & Collins, 1982; Williams & Robinson, 1984) in rodent brain. It also inhibits [^{3}H]-MK-801 binding, suggesting additional activity at NMDA-type glutamate receptors (Aizenman *et al.*, 1991). Lobeline fails to mimic nicotine in tests of conditioned place preference (Fudala & Iwamoto, 1986), discriminative stimulus properties (Romano *et al.*, 1981; Reavill *et al.*, 1990a), and locomotor activity (Stolerman *et al.*, 1995), and receptor binding upregulation (Bhat *et al.*, 1991). These differences are probably not due to inadequate central penetration (Reavill *et al.*, 1990a; Bhat *et al.*, 1991). Possibly the only evidence that lobeline can activate brain nicotinic AChRs is a report that lobeline-evoked [^{3}H]-dopamine release from mouse striatal synaptosomes is partially mecamylamine-sensitive (Grady *et al.*, 1992). The present experiments, conducted in rats, appear consistent with the suggestion that lobeline releases dopamine from cytosolic stores via a non-nicotinic mechanism (Sakurai *et al.*, 1982). This is not a 'tyramine-like' action, since [^{3}H]-NA release was not enhanced, but rather reduced by an unknown mechanism.

DMPP is a classic ganglion stimulant that is commonly used as a nicotinic agonist in CNS tissue. However, in the periphery, DMPP does not mimic nicotine in all of its actions (Romano, 1981; Holbach *et al.*, 1977). Previously, we observed that striatal [^{3}H]-dopamine release evoked by a high concentration of DMPP (100 μM) was only partially dependent on external calcium, and exceeded the maximal effect of nicotine (El-Bizri & Clarke, 1994); here, we show that this response was only partially inhibited by mecamylamine. At a lower concentration of DMPP (10 μM), evoked [^{3}H]-dopamine release appears largely mecamylamine-sensitive (El-Bizri & Clarke, 1994).

An analogous non-nicotinic action of DMPP did not contribute to the [^{3}H]-NA response, since the latter was entirely mecamylamine-sensitive. In this assay, DMPP and nicotine were equally efficacious. The present results contrast with observations in rat hippocampal slices, where even low concentrations of DMPP released [^{3}H]-NA in a mecamylamine-insensitive manner and to a much greater degree than nicotine (Snell & Johnson, 1989). It is clear that comparisons between DMPP and nicotine should be treated cautiously, particular if sensitivity to nicotinic antagonists is not demonstrated.

Comparisons between nicotinic antagonists

The nicotinic antagonists tested in the present study were active against nicotine-evoked release in both assays. No antagonist inhibited release evoked by a high concentration of K^+, confirming that the observed inhibition was not due to an effect on transmitter release mechanisms downstream of the nicotinic AChR.

The nicotinic antagonist, dihydro-β-erythroidine (DHBE), antagonizes many central nicotinic actions, and potently inhibits high-affinity [^{3}H]-ACh, [^{3}H]-nicotine and [^{125}I]-α-bungarotoxin binding to rodent brain (Schwartz & Kellar, 1983; Rapier *et al.*, 1990). Consistent with a competitive mode of action, DHBE surmountably inhibited nicotine-evoked release of both striatal [^{3}H]-dopamine (El-Bizri & Clarke, 1994) and hippocampal [^{3}H]-NA (Experiment 8). Since the degree of inhibition produced by a competitive antagonist depends on the agonist concentration, DHBE was tested against the approximate EC_{50} of nicotine in each tissue. These experiments suggest that DHBE has a 35 fold higher affinity for nicotinic AChRs mediating [^{3}H]-dopamine release than [^{3}H]-NA release. This observation accords with electrophysiological evidence suggesting that DHBE can usefully distinguish putative nicotinic AChR subtypes, when differential sensitivity to nicotine is taken into account (Mulle *et al.*, 1991).

Methyllycaconitine (MLA) is a nicotinic antagonist that possesses a particularly high affinity for brain nicotinic AChRs that are sensitive to α-bungarotoxin (Ward *et al.*, 1990; Alkondon *et al.*, 1992). Thus, MLA inhibits [^{125}I]-α-bungarotoxin binding at low nanomolar concentrations (Ward *et al.*, 1990; Alkondon *et al.*, 1992), and inhibits α-toxin-sensitive nicotinic responses at sub-nanomolar concentrations (Alkondon *et al.*, 1992). In contrast, blockade of α-toxin-insensitive responses tested to date has required micromolar concentrations of MLA (Drasdo *et al.*, 1992). In experiment 8, MLA exerted a surmountable block of nicotine-evoked [^{3}H]-dopamine and [^{3}H]-NA release, consistent with evidence from radioligand binding and functional studies suggesting that MLA acts competitively (Drasdo *et al.*, 1992; Alkondon *et al.*, 1992). In the same experiment, MLA blocked nicotine-evoked [^{3}H]-dopamine release with an IC_{50} of 0.038 μM, a value considerably lower than that reported by others ($1-5$ μM; Drasdo *et al.*, 1992); the higher IC_{50} value obtained in the latter study may reflect the use of a higher agonist concentration. Experiment 5 also showed that MLA, like DHBE, inhibited nicotine-evoked [^{3}H]-NA release less potently than [^{3}H]-dopamine release.

The ganglion blocker, trimetaphan, antagonizes certain nicotinic responses when applied directly to the rat CNS (Caulfield & Higgins, 1983; Dar *et al.*, 1993; Mitchell, 1993). Not all responses are reported to be blocked (Khan *et al.*, 1994), and evidence from avian brain suggests that trimetaphan can serve to distinguish nicotinic AChR subtypes (Weaver *et al.*, 1994). However, in the present study, trimetaphan inhibited both NA and dopamine responses with similar potency. Trimetaphan acts surmountably in peripheral tissues and in avian brain (Brown, 1980; Weaver *et al.*, 1994). However, it only weakly inhibits [^{3}H]-nicotine binding to rat brain (Loiacono *et al.*, 1993) and manifested an insurmountable action, at least in the [^{3}H]-NA release assay (Experiment 8).

Two other insurmountable nicotinic antagonists, mecamylamine and chlorisondamine, were tested less extensively. Mecamylamine appears to distinguish two types of central nicotinic responses in mice, with a potency difference of at least one order of magnitude (Collins *et al.*, 1986). However, no major potency difference was seen (Experiment 6). Chlorisondamine has rarely been tested in CNS preparations *in vitro* (Izenwasser *et al.*, 1991; Marks *et al.*, 1993; El-Bizri & Clarke, 1994); here we show that chlorisondamine inhibits nicotine-evoked [^{3}H]-NA release. At the single concentration tested, chlorisondamine produced a greater degree of block in the [^{3}H]-NA assay than in the [^{3}H]-dopamine assay. This difference is consistent with the existence of different receptor populations, but since chlorisondamine is a putative nicotinic AChR channel blocker, this result could simply reflect different rates of spontaneous channel openings between the two tissues (El-Bizri & Clarke, 1994).

The identity of nicotinic AChRs mediating striatal dopamine release

The subunit composition of nicotinic AChRs mediating dopamine release is not clear. However, based on the reported

anatomical pattern of α subunit mRNA, it is likely that α4 and α5 predominate in nigrostriatal dopaminergic neurones; the α3 subunit appears weakly expressed, and the α7 subunit not at all (Wada *et al.*, 1989; 1990; Séguéla *et al.*, 1993). Message for β2 subunit is strongly expressed (Wada *et al.*, 1989), whereas β4 mRNA has not been detected (Dineley-Miller & Patrick, 1992).

Identification and localization of nicotinic AChR protein has been hampered by a paucity of subtype-selective probes. In the rat, dopaminergic terminals appear to possess high-affinity [^{3}H]-nicotine binding sites (Schwartz *et al.*, 1984; Clarke & Pert, 1985) which are likely to include α4 and β2 subunits (Whiting & Lindstrom, 1988; Flores *et al.*, 1992).

Attempts to relate pharmacology to subunit composition have relied to an important extent on the analysis of pairwise combinations of nicotinic AChR subunits expressed either in *Xenopus* oocytes or in mammalian cell lines. However, few if any recombinant nicotinic AChRs studied so far have been found to match native receptors in terms of pharmacology and channel properties (Alkondon & Albuquerque, 1993; Papke, 1993; Covernton *et al.*, 1994). Analyses of this sort are complicated by several factors, including possible differences in post-translational modification, and by evidence that at least some neuronal nicotinic AChR subtypes comprise three or more types of subunit (Vernallis *et al.*, 1993 ; Conroy & Berg, 1995).

The pharmacology of nicotine-evoked striatal [^{3}H]-dopamine release in the rat does not clearly correspond to any single combination of nicotinic AChR subunits so far described in oocytes. The nicotinic agonist, cytisine, is active in this assay (El-Bizri & Clarke, 1994), suggesting the involvement of β4 subunits (Luetje & Patrick, 1991; Papke & Heinemann, 1994), despite the lack of detected message for this subunit. However, the maximal effect of cytisine is somewhat less than that of nicotine or ACh, at least in the rat (Experiment 4; El-Bizri & Clarke, 1994), implying that β2 subunits may also play a role (Luetje & Patrick, 1991; Papke & Heinemann, 1994). Nicotine-evoked dopamine release is blocked by neuronal bungarotoxin (Schulz & Zigmond, 1989); among recombinant receptors tested to date, the latter acts most potently at those comprised of α3β2 subunits, but other pairwise subunit combinations are also inhibited, including α4β2 (Luetje *et al.*, 1990; Papke *et al.*, 1993).

In the present study, anatoxin-a released [^{3}H]-dopamine with high potency. The EC_{50} value (50 nM) is similar to that previously obtained in a similar preparation (Soliakov *et al.*, 1995). It also resembles that observed in recombinant chick α4β2 receptors (Thomas *et al.*, 1993), but this does not exclude mediation by other receptor subtypes. In addition, chronic administration of anatoxin-a is reported to upregulate [^{3}H]-nicotine binding sites in a manner correlated with nicotine-evoked [^{3}H]-dopamine release from fat striatal synaptosomes (Rowell & Wonnacott, 1990). This suggests that anatoxin-a evokes dopamine release by acting at least in part on nicotinic AChRs containing α4 and β2 subunits. Lobeline, in contrast, acted in an entirely mecamylamine-insensitive manner, consistent with its lack of reported activity at recombinant chick α4β2 receptors (Court *et al.*, 1994).

Dopamine release evoked by isoarecolone was virtually abolished by mecamylamine (Experiment 4), indicating probable mediation by nicotinic AChRs. However, isoarecolone lacks agonistic activity at recombinant chick α4β2 receptors (Court *et al.*, 1994). Although a species difference cannot be ruled out, this result raises the possibility that isoarecolone acts on a different subtype of nicotinic AChRs present on dopaminergic terminals.

The identity of nicotinic AChRs mediating hippocampal noradrenaline release

The hippocampus of the rat derives its entire NA innervation from the locus coeruleus, whose neurones are virtually all noradrenergic (Aston-Jones *et al.*, 1995). Within the locus coeruleus, α3 subunit mRNA is very abundant; in contrast to the substantia nigra, little or no α4, α5, or α7 signal has been detected (Wada *et al.*, 1989; 1990; Séguéla *et al.*, 1993). Message for β2 and β4 subunits both appear moderately abundant (Wada *et al.*, 1989; Dineley-Miller & Patrick, 1992). No information is available from radioligand and antibody studies concerning the identity of possible nicotinic AChR subtypes associated with noradrenergic terminals. This information would be important, since mRNA levels do not reliably indicate relative protein abundance, nor do they indicate where on the neurone these receptor subunits might be located.

Nevertheless, the available non-pharmacological evidence is consistent with the notion that NA terminals possess nicotinic AChRs containing α3 subunits and either β2 or β4 subunits, or both. Pairwise combinations of α3β2 and α3β4 subunits have been characterized in *Xenopus* oocytes by several authors, whereas only preliminary data are available for α3β2β4 receptors (Colquhoun *et al.*, 1993). Comparison with this literature, despite its inherent problems, indicates that the nicotinic AChRs modulating hippocampal NA release bear a close pharmacological resemblance to recombinant α3β4 receptors and not to α3β2 receptors:

(1) *Potency of ACh* In Experiment 1, ACh yielded an EC_{50} of 27 μM. Values obtained for α3β4 receptors expressed in oocytes range from approximately 20 to 50 μM (Cachelin & Jaggi, 1991; Luetje *et al.*, 1993; Cachelin & Rust, 1995), whereas tested under similar conditions, α3β2 receptors yielded an EC_{50} of 350 μM (Cachelin & Jaggi, 1991). Nevertheless, α3β4 receptors expressed in a mammalian cell line are also quite insensitive to ACh (EC_{50} 200 μM) (Wong *et al.*, 1995).

(2) *Agonist relative potency* Experiment 1 revealed that ACh, nicotine, DMPP and cytisine acted with similar potency; this lack of divergence appears to fit best the α3β4 combination expressed in oocytes (Luetje & Patrick, 1991). Whilst nicotine, cytisine and DMPP were virtually indistinguishable in potency, ACh was somewhat less potent. This pattern is again broadly consistent with the α3β4 combination expressed in oocytes (Cachelin & Jaggi, 1991; Luetje & Patrick, 1991; Connolly *et al.*, 1992; Wong *et al.*, 1995; Covernton *et al.*, 1994) and in HEK cells (Wong *et al.*, 1995). It should be noted that accurate comparison is hampered by the absence of full dose-response curves in most oocyte studies. Nevertheless, comparison between nicotine and ACh argues against the involvement of α3β2 receptors in our [^{3}H]-NA release assay (Connolly *et al.*, 1992; Luetje *et al.*, 1993).

(3) *Agonist efficacy* In the [^{3}H]-NA release assay, ACh, nicotine, cytisine and DMPP all appeared to act as full agonists. The high efficacy of cytisine is particularly informative, since it implies the participation of β4 subunits, either alone (Luetje & Patrick, 1991; Papke & Heinemann, 1994) or in combination with β2 subunit (Colquhoun *et al.*, 1993). The similar efficacy observed for ACh, nicotine and cytisine is also consistent with data obtained from α3β4 recombinant nicotinic AChRs expressed in mammalian cells (Wong *et al.*, 1995). The similar efficacy of DMPP and ACh in our assay is hard to evaluate, since data obtained from recombinant α3β4 receptors appear contradictory (Cachelin & Jaggi, 1991; Wong *et al.*, 1995).

(4) *Antagonist potency* The IC_{50} values of mecamylamine and trimetaphan obtained in our [^{3}H]-NA release assay appear consistent with data from oocyte-expressed α3β4 receptors (Cachelin & Rust, 1995). Nevertheless, based on the limited data available from heterologous expression systems, our antagonist data when taken alone would not exclude involvement of other subunit combinations including α3β2 (Connolly *et al.*, 1992). Recently, (+)-tubocurarine was reported to increase agonist-induced activation of nicotinic AChRs in oocytes (Cachelin & Rust, 1994). This potentiation, which occurred only at very low concentrations of both drugs, appears to distinguish β4-containing nicotinic AChRs from β2-containing

nicotinic AChRs. We have attempted to test for a comparable enhancement of NA release, but have found that the use of agonist concentrations considerably below the EC_{50} is not feasible, given the variability inherent in this sort of assay.

The rat superior cervical ganglion provides a further point of comparison, since its neurones appear to express only α3, α7, β2 and β4 message in moderate to high abundance, with α4 and α5 mRNA detected in some studies but not others (Rust *et al.*, 1994; Mandelzys *et al.*, 1994; Zoli *et al.*, 1995). In one study, cytisine was reported to act more potently than ACh, nicotine and DMPP, but full dose-response curves were not obtained (Covernton *et al.*, 1994). In this study, similar results were obtained by these authors using oocyte-expressed α3β4 receptors; responses to DMPP provided the only appreciable difference, and in view of the results of Experiment 4, it is regrettable that DMPP responses were not tested for nicotinic AChR mediation. The results of a second, more recent study resemble our own in several important respects (Mandelzys *et al.*, 1995): no α7-mediated responses were detected; cytisine was equipotent with DMPP, and slightly more potent than ACh; cytisine and ACh were equally efficacious, although DMPP was somewhat less.

Nicotinic receptors in the brain are known to be widely expressed and heterogeneous. However, few nicotinic responses have been extensively characterized in pharmacological terms, and at present it is unclear whether the nicotinic AChRs modulating hippocampal NA release are also associated with any other neuronal population. To date, only two other brain nicotinic responses have been tentatively attributed to α3β4 receptors. The first, observed in medial habenula, appears to differ from our NA release response in terms of sensitivity to mecamylamine and DHBE, and also differs from oocyte-expressed α3β4 receptors in terms of channel properties (Mulle *et al.*, 1991). The second, observed in cultured hippocampal neurones, has been less thoroughly characterized (Alkondon & Albuquerque, 1993). Other pharmacologically characterized nicotinic responses in the CNS clearly differ from the NA release response (Mulle *et al.*, 1991; Vidal & Changeux, 1993).

Possible pharmacological and physiological significance

The demonstration of presynaptic nicotinic AChRs on hippocampal noradrenergic terminals should provide a useful addition to existing *in vitro* pharmacological assays. The present study also provides information that may aid the delineation of nicotinic AChR subtypes with respect to subunit composition and pharmacological properties.

The *in vivo* pharmacological significance of such receptors is less clear. Although subcutaneous administration of nicotine stimulates hippocampal NA release in freely-moving animals, this effect appears to derive mainly or exclusively from a somatodendritic action (Mitchell, 1993). Possibly, the presynaptic receptors require higher concentrations of nicotine to be activated. Alternatively, it is conceivable that presynaptic receptors would enhance transmitter release more effectively if the drug were delivered in a pulsatile fashion, thus avoiding prolonged membrane depolarization which would tend to inactivate voltage-gated ion channels located at the terminal. The physiological significance of nicotinic AChRs located on hippocampal NA terminals is uncertain, since it remains to be determined whether they are innervated by ACh.

We thank Drs Ellis Cooper and Stephen Arneric for helpful suggestions. The following individuals and companies kindly donated drugs: Dr I.P. Stolerman, Dr M.H. Benn, Ciba-Geigy, Hoffman-La Roche, H. Lundbeck A/S, and Merck and Company. This work was supported by the Medical Research Council of Canada. P.B.S.C. holds a Scholarship from the Fonds de la Recherche en Santé du Québec.

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(Received July 19, 1995
Revised September 15, 1995
Accepted October 12, 1995)