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Nicotinic receptors modulating somatodendritic and terminal dopamine release differ pharmacologically

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Abstract

Ascending dopaminergic and noradrenergic neurons possess somatodendritic and terminal nicotinic cholinoceptors in the rat. Each neuronal population expresses mRNA for several types of nicotinic cholinoceptor subunit, including α6 and β3. In superfused rat striatal synaptosomes, epibatidine evoked release of [^{3}H]dopamine with similar efficacy to ACh, whereas nicotine and cytisine were weaker ($70 \pm 6\%$ and $58 \pm 6\%$, respectively). The four agonists were equi-efficacious in evoking [^{3}H]noradrenaline release from hippocampal synaptosomes. Nicotine-evoked synaptosomal release was tetrodotoxin-insensitive. Somatodendritic nicotinic cholinoceptors on dopaminergic neurons were studied using a dendrosomal [^{3}H]dopamine release assay and also in locomotor activity tests. In both assays, nicotine appeared more efficacious than epibatidine. Furthermore, with repeated nicotine exposure, the acute locomotor stimulant response to nicotine increased, whereas the epibatidine response became undetectable. In conclusion, somatodendritic nicotinic cholinoceptors located on dopaminergic neurons appear to differ pharmacologically from those on striatal dopaminergic terminals and hippocampal noradrenergic terminals.

Keywords: Nicotinic receptor; Epibatidine; Somatodendritic; Substantia nigra; Ventral tegmental area; Dendrosome

1. Introduction

Dopaminergic and noradrenergic neurons that innervate the rat forebrain express nicotinic cholinoceptors. Activation of somatodendritic receptors produces depolarization and increased neuronal firing (Egan and North, 1986; Pidoplichko et al., 1997; Sorenson et al., 1998), whereas nicotinic cholinoceptor stimulation local to the terminal increases transmitter release (see Wonnacott, 1997). Although dopaminergic and noradrenergic neurons clearly differ in terms of subunit expression (Le Novère et al., 1996; Wonnacott, 1997) and pharmacology (Clarke and Reuben, 1996; Kulak et al., 1997), the subtypes of nicotinic cholinoceptor expressed by these neurons have not been identified with certainty.

Nigrostriatal and mesolimbic dopaminergic neurons express a virtually identical set of nicotinic cholinoceptor subunits in rat brain (Le Novère et al., 1996; Charpantier et al., 1998). Electrophysiological experiments, employing brief and rapid delivery of agonist, have identified at least two subtypes of somatodendritic nicotinic cholinoceptor on these neurons: a rapidly desensitizing α7-like receptor and a non-α7 receptor containing β2 subunits (Pidoplichko et al., 1997; Picciotto et al., 1998). Nicotinic modulation of dopamine release from axon terminals has been characterized in superfused striatal synaptosomes, and α4β2 or α3β2-containing nicotinic cholinoceptors have been variously proposed to mediate this effect (Grady et al., 1992; Kulak et al., 1997; Kaiser et al., 1998; Luo et al., 1998).

Noradrenergic neurons that innervate the forebrain originate mainly in the locus coeruleus. Here, somatodendritic nicotinic cholinoceptors that have been identified possess a non-α7 pharmacology (Egan and North, 1986). The locus coeruleus is the sole source of noradrenergic afferents to the hippocampus (Aston-Jones et al., 1995), and in hippocampal synaptosomes, nicotinic modulation of noradrenaline release has a pharmacological profile suggestive of α3β4-containing nicotinic cholinoceptors (Clarke and Reuben, 1996; Luo et al., 1998).

Two recent sets of findings, however, call for a re-evaluation. First, quantitative mRNA analysis suggests that in both dopaminergic and noradrenergic neurons, α6 and

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β3 may be the most prevalent subunits (Le Novère et al., 1996). Alpha6 expression has also been detected immunohistochemically (Göldner et al., 1997), and recent evidence suggests that α6-containing nicotinic cholinoceptors may contribute to the locomotor stimulant effect of nicotine (Le Novère et al., 1999). Second, although the results of α6β3 subunit coexpression have not been reported, the α6 subunit has been shown to co-assemble with β2 or β4 subunits to form functional nicotinic cholinoceptors in heterologous expression systems (Gerzanich et al., 1997; Fucile et al., 1998). Pharmacological comparison with nicotinic cholinoceptors expressed by dopaminergic and noradrenergic neurons is limited, since published estimates of agonist efficacy are imprecise and have only been obtained for terminal nicotinic cholinoceptors (Grady et al., 1992; El-Bizri and Clarke, 1994; Clarke and Reuben, 1996).

In superfused synaptosomes, nicotine-evoked striatal dopamine release is reported to be partially (54%) inhibited by tetrodotoxin (Marshall et al., 1996), whereas nicotine-evoked hippocampal noradrenaline release is not (Clarke and Reuben, 1996). These findings raise the possibility that the corresponding nicotinic cholinoceptor subtypes are differentially located at the ultrastructural level (Léna et al., 1993; Wonnacott, 1997). However, subtle procedural differences cannot be ruled out since the results were obtained in different laboratories.

The first goal of the present study was therefore to obtain more precise estimates of agonist efficacy in ascending catecholaminergic neurons, for comparison with recombinant nicotinic cholinoceptors. The second aim was to re-examine whether nicotine-evoked dopamine and noradrenaline release differ in tetrodotoxin sensitivity. The third goal was to perform a pharmacological comparison between somatodendritic and terminal nicotinic cholinoceptors. For this purpose, we used a dendrosomal dopamine release assay (Marchi et al., 1991). Somatodendritic nicotinic cholinoceptors were also characterized less directly in a behavioural assay (locomotor activity).

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Charles River, Canada) were used, weighing 200–250 g (release studies) or 295–325 g (locomotion studies) at the start of the experiment. Rats were housed two per cage in an animal room lit from 7 a.m. to 7 p.m. Food and water were available ad libitum. Subjects were allowed to accommodate to the housing conditions for 4 days after arrival, and were drug-naive. All experiments were approved by the McGill University Animal Care Committee, in accordance with Canadian Council on Animal Care guidelines.

2.2. Dopamine and noradrenaline release from superfused synaptosomes and dendrosomes

Methods for synaptosomal preparation and measurement of transmitter release were virtually identical to those described in detail elsewhere (Clarke and Reuben, 1996). In each assay, crude synaptosomal (P2) fraction was prepared from dissected striatum and/or hippocampus. Brain tissue was pooled from four rats per assay. The P2 fractions were resuspended (140–150 mg wet weight of original tissue/ml) 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 (striatum) or 0.2 μM [^{3}H]noradrenaline (hippocampus) in SB at 37°C for 10 min.

The superfusion apparatus contained 32 identical channels, each comprising a small polypropylene retention chamber, through which superfusate was pumped at 0.4 ml/min. Each experiment comprised one or more assays. In each assay, data were collected simultaneously from all 32 channels. 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 SB alone or with SB containing antagonist, as appropriate. In experiments 3.1, 3.3 and 3.4, all agonists were tested using SB that contained diisopropylfluorophosphate (DFP) 100 μM and atropine sulphate 1 μM (or 10 μM for ACh 1000 μM), added to inhibit hydrolysis of ACh and to prevent activation of muscarinic cholinoceptors, respectively.

Next, 11 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). 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]noradrenaline, tritium release largely corresponds to unmetabolized transmitter (Rapier et al., 1988; Pittaluga and Raiteri, 1992). Therefore, in the present study, we refer to dopamine or noradrenaline release, as appropriate.

The procedure for measuring dendrosomal release of dopamine was essentially the same as for synaptosomes, with the following modifications. From each of four rats, a block of tissue (wet weight approx. 75 mg) containing the substantia nigra and ventral tegmental area was dissected from a coronal section approximately 2 mm thick. The dendrosomal preparation was suspended in 4 ml buffer (75 mg wet weight of original tissue/ml). Citalopram 0.3 μM

and desipramine 0.1 μM were added to the superfusion buffer in order to selectively inhibit uptake by 5-HT (5-hydroxytryptamine) and noradrenaline transporters, respectively (Kelly et al., 1985; Hyttel, 1994). The dendrosomal suspension was incubated in 0.4 μM [^{3}H]dopamine for 20 min. This higher concentration was adopted in order to increase the signal; dopamine transporters on dendrosomes and striatal synaptosomes are reported to possess similar K_m values (Marchi et al., 1991). The wash period prior to sample collection was 30 min.

2.3. Locomotor activity apparatus and behavioural testing procedures

Twelve clear perspex photocell cages (42 × 25 × 31 cm high) were used. Perforated lids provided ventilation, and a mixture of used and new sawdust covered the bottom of the cage. Two parallel infrared photobeams were located 34 cm apart, 4 cm away from the short sides of the cage, and 3 cm above the cage floor. Photobeam interruptions were registered by an in-house computer program and locomotor activity was measured as the number of times the rat crossed the cage from one beam to the other. Thus, beam breaks measure the number of times the rat crossed the cage. A given rat was always tested in the same photocell cage. At the beginning of each test day, animals were weighed, replaced in their home cages, and allowed to habituate to the locomotor test room for 25–30 min. Each rat was then injected with saline or drug and immediately placed in a photocell cage. Locomotor activity was measured over a period of 60 min, i.e., six consecutive periods of 10 min.

Rats ($n = 12$), initially drug-naive, were handled daily for several days prior to locomotor testing. Each rat then received three tests (Monday, Wednesday, Friday), once each with saline, nicotine (0.4 mg/kg s.c.) and epibatidine (3 μg/kg s.c.), given in a counterbalanced order. At the start of the second week, rats received the same dose of nicotine twice daily for 3 days in their home cages. During the third week, locomotor tests were repeated as before.

2.4. Drugs

Chemicals and suppliers were as follows: 3,4-[ring-2,5,6-^{3}H]dopamine (53.7 or 60 Ci/mmol) and levo-[ring-2,5,6-^{3}H]noradrenaline (59.1 Ci/mmol; New England Nuclear, Boston, MA, USA), acetylcholine HCl, atropine sulphate, (+)-epibatidine L-tartrate, pargyline HCl, tetrodotoxin and veratridine (Research Biochemicals, Natick, MA, USA), S(−)-nicotine ditartrate and DFP (Sigma, St. Louis, MO, USA). Desipramine HCl (Merrell Dow Research Institute) and citalopram HBr (H. Lundbeck, Copenhagen, Denmark) were generous gifts. Other chemicals and reagents were purchased from commercial sources. For superfusion, drugs were dissolved in SB. Systemically administered drugs were dissolved in 0.9% saline and injected at 1 ml/kg. Nicotine tartrate was neutralized to pH 7.2 with NaOH. Solutions were aliquoted and stored at −20°C until use. Doses of nicotine and epibatidine are expressed as free base.

2.5. Data analysis

For each channel, the release occurring in each 1-min collection period was calculated as a percentage of basal

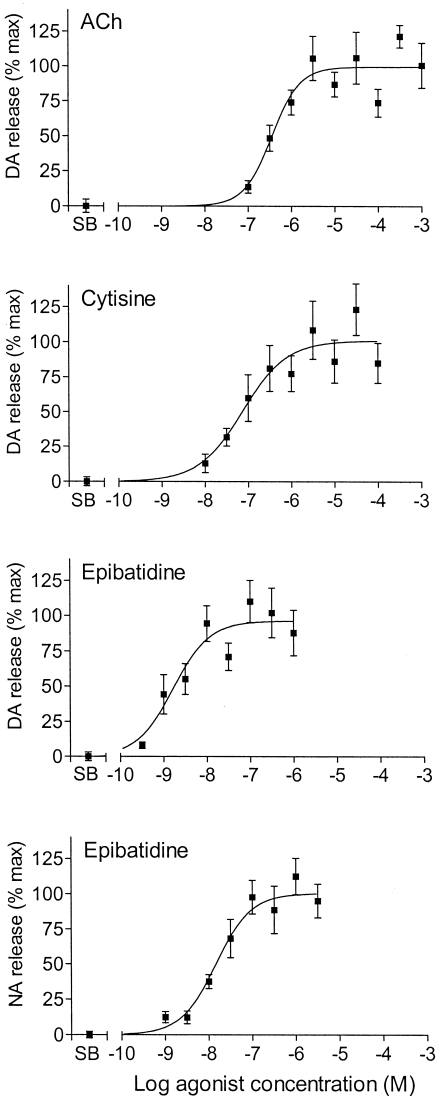

Fig. 1. Effects of nicotinic agonists on release of striatal dopamine and hippocampal noradrenaline from superfused synaptosomes. Synaptosomes were superfused with superfusion buffer (SB) for 35 min prior to administration of a 1-min pulse of the agonist or SB (controls). Agonists were tested in separate groups of 2–3 assays. The vertical axis represents the mean (±S.E.M.) peak release, calculated as a percentage of basal release and then normalized such that the mean response to SB was 0% and the mean response to the three or four highest concentrations was 100% ($n = 6–11$ channels). Non-normalized values of maximal release were 165% for ACh, 156% for cytisine, 198% and 205% for epibatidine.

Table 1
EC_{50} values[a] for evoked striatal [^{3}H]dopamine and hippocampal [^{3}H]noradrenaline release.[b] These data were obtained from superfused synaptosomes made from rat striatum and hippocampus, preloaded with [^{3}H]dopamine and [^{3}H]noradrenaline, respectively

	Acetylcholine (μM)	Nicotine (μM)	Epibatidine (nM)	Cytisine (μM)
DA	0.36 (0.16–0.81)	*0.16 (0.11–0.22)*	1.7 (0.6–4.7)	0.075 (0.024–0.23)
NA	*29 (17–52)*	*5.6 (2.8–11)*	15 (8–29)	*7.4 (3.5–15)*

[a] Estimate and 95% confidence interval.
[b] Values in italics were calculated using data from Clarke and Reuben (1996).

release, determined from a 5-min baseline. Drug-evoked release from synaptosomes was taken as the peak value occurring within each channel in the four samples immediately following agonist challenge. For dendrosomal release, the peak value was calculated from samples 7–11, but since agonist-evoked release appeared less transient and was superimposed upon a descending baseline, the time-averaged release (''area'') was determined for the same period.

Concentration–response data were normalized within each assay such that the mean response to SB was 0% and the mean response to the three or four highest concentrations was 100%. Data were then fitted by non-linear regression analysis (Prism program, Graphpad Software, San Diego, CA) using a three-parameter logistic equation in which only the minimum (i.e., SB) value was held constant. Other drug effects were examined by analysis of variance, using commercial software (Systat 7.0, SPSS, Chicago, IL, USA). Extreme outliers, as identified by the Systat program, were excluded from analysis. Multiple comparisons with a single control group were made with Dunnett's test; other multiple comparisons were made by Student's t test with Bonferroni's correction (Glantz, 1992). Probability values are two-tailed except where noted. All mean values are expressed as ± S.E.M.

3. Results

3.1. Concentration-dependent effects of nicotinic agonists on striatal [^{3}H]dopamine release and hippocampal [^{3}H]noradrenaline release

The purpose of this experiment was to determine concentration–effect curves for the four agonists under study (nicotine, acetylcholine, epibatidine, cytisine) in the two synaptosomal release assays, unless previously determined (Clarke and Reuben, 1996). Acetylcholine, epibatidine and cytisine were tested individually for their ability to evoke striatal [^{3}H]dopamine release (Fig. 1). In this and subsequent experiments, ACh hydrolysis was inhibited and muscarinic receptors were blocked (see Methods). Epibatidine was also tested for its ability to release hippocampal [^{3}H]noradrenaline (Fig. 1). EC_{50} values are shown in Table 1. Based on the present results and on previous findings (Clarke and Reuben, 1996), supramaximal agonist concentrations were chosen for subsequent experiments, as fol-

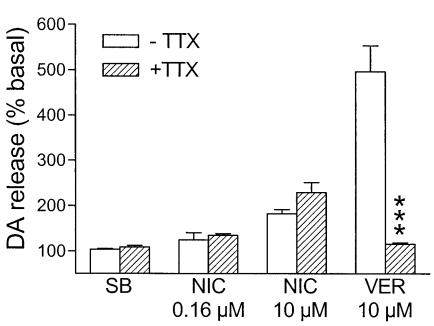

Fig. 2. Effect of tetrodotoxin on nicotine- and veratridine-evoked release of striatal dopamine (DA). Tetrodotoxin (TTX) 1.5 μM was present in the superfusion buffer (SB) for 35 min before as well as during agonist challenge. In control channels, tetrodotoxin was omitted. Synaptosomes were challenged with SB, nicotine 0.16 μM (EC_{50}) or 10 μM (maximal concentration), or with the voltage-gated sodium channel activator veratridine (10 μM), acting as a positive control ($n = 5–21$ channels per condition). Tetrodotoxin virtually eliminated the massive increase in dopamine release evoked by veratridine whereas nicotine-evoked release was not significantly affected. $^{***}P < 0.001$ vs. agonist alone (Bonferroni *t*-tests).

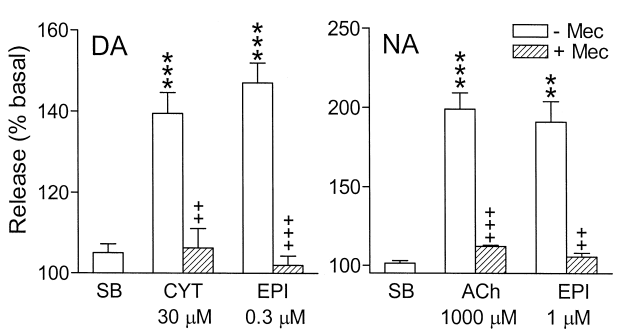

Fig. 3. Effects of mecamylamine on dopamine and noradrenaline release evoked by supramaximal concentrations of nicotinic agonists. Mecamylamine 10 μM was present in the superfusion buffer (SB) for 35 min before as well as during agonist challenge. In control channels, the antagonist was omitted. Synaptosomes were challenged with SB, cytisine (CYT), epibatidine (EPI), or acetylcholine (ACh). The vertical axis represents the mean (± S.E.M.) release, calculated as a percentage of basal release ($n = 8–11$ channels for DA, 3–7 channels for NA). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. SB, $^{++}P < 0.01$, $^{+++}P < 0.001$ vs. agonist alone (Bonferroni *t*-tests).

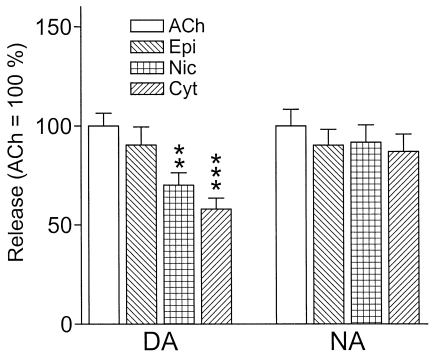

Fig. 4. Efficacy comparison of nicotinic agonists in evoking release of striatal dopamine (DA) and hippocampal noradrenaline (NA). Supramaximal agonist concentrations were as follows (dopamine, noradrenaline assays): nicotine 10, 100 μM; ACh 100, 1000 μM; epibatidine 0.3, 1 μM; and cytisine 30, 100 μM. Agonists were tested in parallel. Evoked release was normalized such that the mean response to SB (i.e., no agonist) was 0% and the mean response to ACh was 100% ($n = 20–21$ channels per condition). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. ACh (Dunnett's test).

lows (dopamine, noradrenaline assays): nicotine 10, 100 μM; ACh 100, 1000 μM; epibatidine 0.3, 1 μM; and cytisine 30, 100 μM.

3.2. Effect of tetrodotoxin on nicotine-evoked striatal [^{3}H]dopamine release

Tetrodotoxin was applied to striatal synaptosomes in a high concentration (1.5 μM), and was present in the superfusion buffer for 35 min before as well as during agonist challenge. In control channels, tetrodotoxin was omitted. Synaptosomes were challenged with SB, nicotine 0.16 and 10 μM (EC_{50} and supramaximal concentrations, respectively), or with the voltage-gated sodium channel activator veratridine (10 μM) acting as a positive control. Release evoked by nicotine 0.16 and 10 μM was non-significantly increased by tetrodotoxin. In contrast, tetrodotoxin virtually eliminated the massive increase in dopamine release evoked by veratridine (Fig. 2; Bonferroni $t(10) = 6.6$, $P < 0.001$). Tetrodotoxin also produced a small (13%) but significant reduction in basal dopamine release ($t(93) = 2.75$, $P < 0.01$; data not shown).

3.3. Blockade of agonist effects in synaptosomes by mecamylamine

The purpose of this experiment was to test whether release evoked by supramaximal agonist concentrations

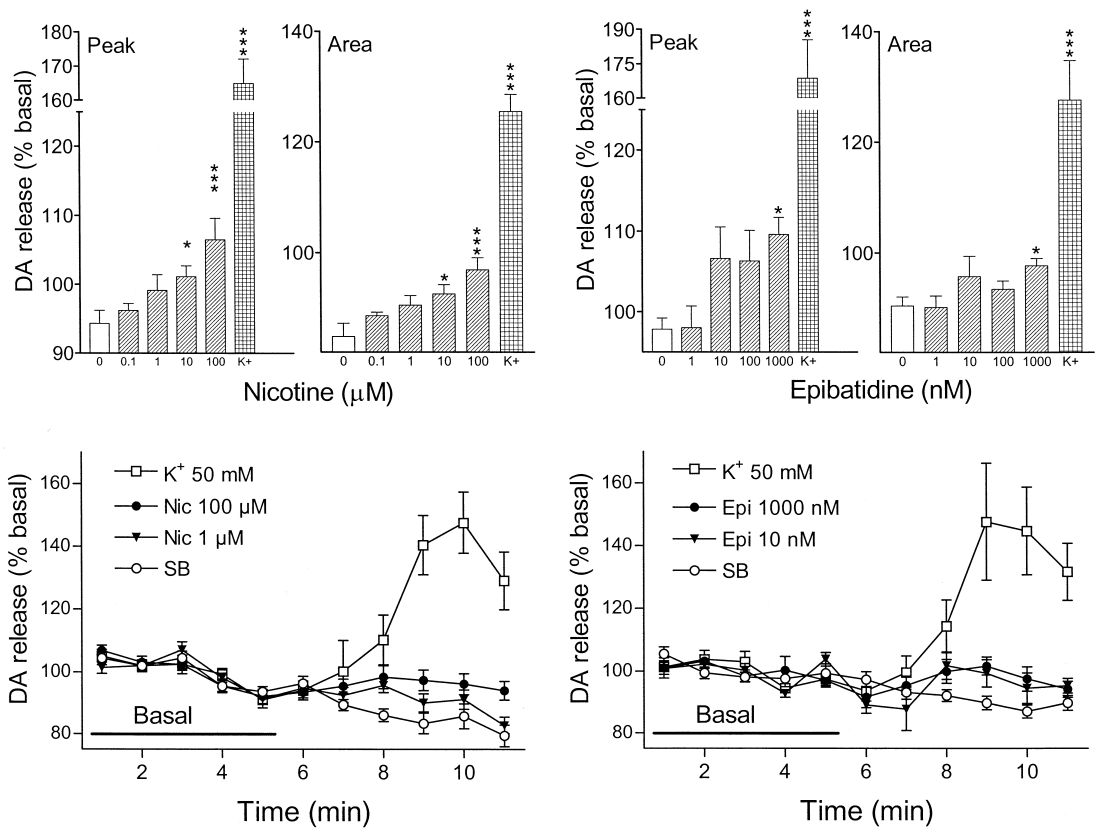

Fig. 5. Dopamine release from superfused dendrosomes evoked by nicotine, epibatidine and high K^+. Dendrosomes were loaded with [^{3}H]dopamine and superfused with buffer (SB) for 30 min prior to collection of 1 min samples. After a basal release period, a 1-min pulse of nicotine (left), epibatidine (right), K^+ or SB was delivered. For a given agonist, all conditions were tested in parallel. The vertical axis represents the mean (±S.E.M.) release, calculated as a percentage of basal release ($n = 8–15$ channels per condition). The time course of release is shown in the lower panel, with certain agonist concentrations omitted for clarity. The peak and area measures (upper panels) refer respectively to the maximal and mean values of release occurring among samples 7–11 for each channel. Both nicotinic agonists produced a small, prolonged, and concentration-related increase in transmitter release. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. SB alone (Dunnett's tests, one-sided).

was mediated via nicotinic cholinoceptors. Agonists were tested in the presence vs. absence of 10-μM mecamylamine, a nicotinic receptor antagonist that acts in an insurmountable fashion in both assays (El-Bizri and Clarke, 1994; Clarke and Reuben, 1996). Mecamylamine did not significantly alter basal release of either dopamine ($t(52) = 1.13$) or noradrenaline ($t(29) = 0.52$).

In the dopamine release assay, responses to epibatidine 0.3 μM and cytisine 30 μM were completely antagonized (Fig. 3). Analogous results have been obtained previously for supramaximal concentrations of nicotine and acetylcholine (Clarke and Reuben, 1996). In the noradrenaline release assay (Fig. 3), responses to epibatidine 1 μM were blocked, as previously shown for nicotine and cytisine (Clarke and Reuben, 1996), whereas a small (11%) residual response to acetylcholine 1000 μM was seen ($P < 0.001$).

3.4. Agonist efficacy in striatal and hippocampal synaptosomes

In order to determine efficacy relative to acetylcholine, the four agonists were tested in parallel at supramaximal concentrations (Fig. 4). Evoked release of dopamine and noradrenaline were each tested in a set of three assays. Within each assay, evoked release was normalized such that the mean response to SB was 0% and the mean response to acetylcholine was 100%.

In the striatal dopamine release assay (Fig. 4), nicotine and cytisine were significantly less efficacious than acetylcholine ($70 \pm 6\%$ and $58 \pm 6\%$, respectively, Dunnett's test: $P < 0.01$ and $P < 0.001$). Epibatidine ($90.3 \pm 9.2\%$) did not differ significantly from acetylcholine. In contrast, in the noradrenaline release assay, agonist efficacies were all similar, and none differed significantly from acetylcholine (Fig. 4). Efficacies relative to acetylcholine were as follows: nicotine, $92 \pm 9\%$; epibatidine, $90 \pm 8\%$; and cytisine $87 \pm 9\%$.

3.5. Effects of nicotine and epibatidine on [^{3}H]dopamine release from dendrosomes

Nicotinic agonists were found to evoke [^{3}H]dopamine release from dendrosomes, but the effect was considerably smaller than that from synaptosomes. Several attempts were made to increase nicotinic responses, but none proved useful. These included the following manipulations intended to increase the releasable pool of [^{3}H]dopamine: incubation with 50 mM K^+ or 100 μM amphetamine prior to loading with [^{3}H]dopamine; pretreating rats and synaptosomes with the dopamine synthesis inhibitor α-methyl-*para*-tyrosine (300 mg/kg i.p., 100 μM, respectively); low K^+ (1.2 mM) in the SB; and shorter wash times (10 and 20 min) prior to the agonist pulse. Other ineffective manipulations included addition of the dopamine uptake blocker nomifensine (1 μM) to the SB, and continuous bubbling of SB with a mixture of 95% O_2 and 5% CO_2.

Both nicotine and epibatidine increased [^{3}H]dopamine release in a concentration-dependent manner (Fig. 5). The nicotine effect did not reach a clear plateau within the range tested (0.1–100 μM), and thus an EC_{50} value could not be estimated. Epibatidine appeared to attain a maximum effect at 10 or 100 nM, and a half-maximal effect

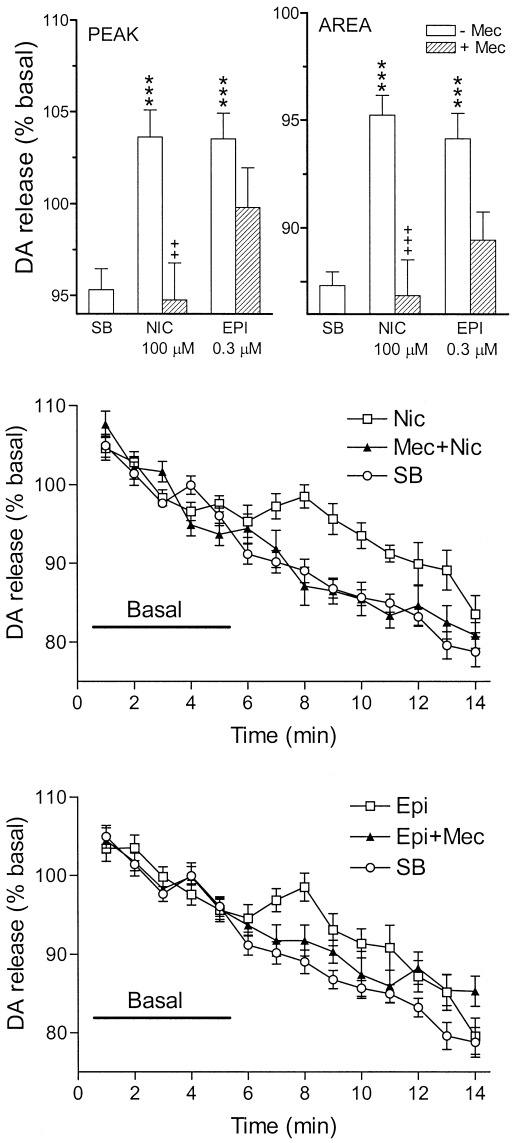

Fig. 6. Effects of mecamylamine on dendrosomal dopamine release evoked by high concentrations of nicotine and epibatidine. Where present, mecamylamine 10 μM was given before as well as during agonist challenge. After a basal release period, a 1-min pulse of nicotine (100 μM), epibatidine (300 nM), or SB was delivered. All conditions were tested in parallel. The vertical axis represents the mean (±S.E.M.) release, calculated as a percentage of basal release ($n = 21–27$ channels per condition). The time course of release is shown in the lower panels. The Peak and Area measures (upper panel) refer respectively to the maximal and mean values of release occurring between the 7th and 11th samples. $^{***}P < 0.001$ vs. SB, $^{++}P < 0.01$, $^{+++}P < 0.001$ vs. agonist alone (Bonferroni *t*-tests).

between 1 and 10 nM. Responses to high K^+ (50 mM), tested in parallel, were greater than either agonist (Fig. 5).

In order to assess relative efficacy, high concentrations of nicotine (100 μM) and epibatidine (300 nM) were directly compared in the presence or absence of a high concentration of mecamylamine (10 μM). Basal release did not differ in the presence vs. absence of mecamylamine (mean ± S.E.M., respectively: 793 ± 46 vs. 776 ± 34 DPM/min; $t(113) = 0.31$). In the absence of antagonist, the agonists produced effects of similar magnitude that lasted approximately 7 min (Fig. 6). Mecamylamine blocked nicotine-evoked release, but the apparent reduction of epibatidine-evoked release did not reach significance after the Bonferroni correction had been made (peak and area measures: $P > 0.6$ and $P > 0.06$, respectively).

3.6. Effects of nicotine and epibatidine on locomotor activity

For comparison, doses of nicotine (0.4 mg/kg s.c.) and (+)-epibatidine (3 μg/kg s.c.) were chosen that have previously been found to produce maximal locomotor stimulation (Clarke and Kumar, 1983; Menzaghi et al., 1997). In drug-naive rats, the locomotor stimulant effects of nicotine and epibatidine tend to be obscured early in the session by a depressant effect (Clarke and Kumar, 1983; Menzaghi et al., 1997). Drug effects were therefore analyzed from 20–60 min post-injection. Two-way analysis of variance (ANOVA) was performed on the drug–saline difference scores. This analysis revealed that the relative effects of nicotine vs. epibatidine depended on when the drugs were tested, i.e., before vs. after the period of repeated nicotine injections (AGONIST × BLOCK interaction: $F(1,11) = 21.1$, $P < 0.001$). Thus, prior to subchronic treatment (Fig. 7), nicotine tended to increase activity more than epibatidine, but this difference was not significant ($t(11) = 2.06$, $P > 0.05$). After the subchronic treatment (Fig. 7), the nicotine effect was increased ($t(11) = 2.39$, $P < 0.05$) whereas an epibatidine effect was no longer seen except during the first 10 min.

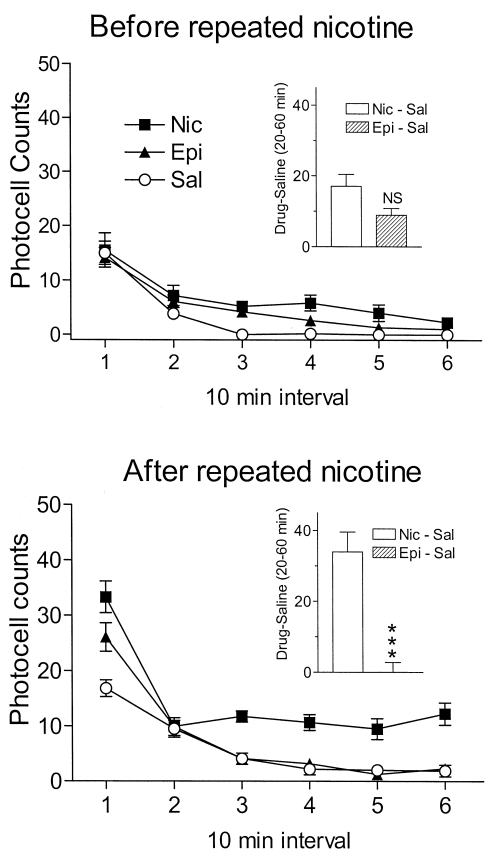

Fig. 7. Effects of nicotine and epibatidine on locomotor activity. Each rat ($n = 12$) was tested for 60 min immediately after s.c. administration of nicotine (0.4 mg/kg), (+)-epibatidine (3 μg/kg) and saline, in a counterbalanced order. These three tests were repeated after a period of twice-daily nicotine injections. In drug-naive animals (upper panel), nicotine tended to stimulate activity more than epibatidine, but this difference was not significant (inset: NS) when analyzed during the stimulatory phase (20–60 min). After repeated nicotine treatment, the stimulant effect of nicotine was increased during the 20–60 min period but that of epibatidine was absent. $^{***}P < 0.001$ vs. nicotine (t-test).

4. Discussion

4.1. Novel aspects of the present study

The present study provides several novel findings. More precise estimates of agonist efficacy were obtained for nicotine-evoked dopamine and noradrenaline release from synaptosomes. The dopamine response was shown to be tetrodotoxin-insensitive. Dopamine release evoked by nicotinic cholinoceptor stimulation was demonstrated for the first time in a dendrosomal dopamine release assay. Comparison of synaptosomal and dendrosomal release revealed pharmacological differences. Lastly, nicotine was shown to be strikingly more effective than epibatidine in stimulating locomotor activity in nicotine-experienced rats.

4.2. Nicotinic agonist efficacy in synaptosomes

Agonist efficacy has been previously assessed in synaptosomal dopamine and noradrenaline release assays (Grady et al., 1992; El-Bizri and Clarke, 1994; Clarke and Reuben, 1996). However, published estimates were only qualitative, and epibatidine was not included in the comparison. We can now confirm quantitatively that cytisine is less efficacious than acetylcholine in releasing striatal dopamine (El-Bizri and Clarke, 1994), but equi-efficacious in releasing hippocampal noradrenaline (Clarke and Reuben, 1996). The present results additionally reveal that nicotine is significantly less efficacious than acetylcholine in the dopamine release assay, whereas epibatidine is virtually equi-efficacious to acetylcholine in both assays.

The profile of agonist efficacy observed in the synaptosomal dopamine release assay is quantitatively similar to that reported in striatal slices (Sullivan et al., 1994; Sacaan

et al., 1995), but diverges from other in vitro responses mediated by native nicotinic cholinoceptors in rat and mouse brain. These responses include: striatal 5-HT release (Reuben and Clarke, 1999), hippocampal acetylcholine release (Wilkie et al., 1993), hippocampal type II responses (Alkondon and Albuquerque, 1995), thalamic Type II responses (Zoli et al., 1998), midbrain and thalamic rubidium flux (Lauffer and Hucho, 1982; Marks et al., 1996), γ-aminobutyric acid (GABA) release (Lu et al., 1998), and noradrenaline release from fetal rat (Gallardo and Leslie, 1998). In almost all cases, cytisine was considerably less efficacious than in the present study.

In the hippocampal noradrenaline release assay, all four agonists including cytisine possessed high efficacy. To our knowledge, only two central responses mediated by native nicotinic cholinoceptors have demonstrated a comparable efficacy profile: hippocampal acetylcholine release (Wilkie et al., 1993) and electrophysiological (type IA) responses (Alkondon and Albuquerque, 1995). In other respects, however, these responses are different from nicotine-evoked noradrenaline release. Thus, the acetylcholine release assay is associated with ''bell shaped'' agonist dose–response curves, and type IA-associated nicotinic cholinoceptors are much less sensitive to agonists.

4.3. Tetrodotoxin sensitivity in synaptosomes

It has been proposed that blockade by the sodium channel blocker tetrodotoxin can serve to distinguish neurotransmitter release mediated by ''preterminal'' vs. ''presynaptic'' nicotinic cholinoceptors (Léna et al., 1993). However, partial tetrodotoxin sensitivity has previously been observed in the synaptosomal dopamine release assay (54% — Marshall et al., 1996) as well as in certain other assays (Marks et al., 1995), indicating that tetrodotoxin sensitivity may also reflect proximity of exocytotic release mechanisms to nicotinic cholinoceptors located on the same terminal (Wonnacott, 1997). The contrasting observation that nicotine-evoked noradrenaline release from hippocampal synaptosomes was entirely tetrodotoxin-insensitive (Clarke and Reuben, 1996) suggested that nicotinic cholinoceptors modulating dopamine and noradrenaline release might occupy different locations at their respective terminals. However, in the present study, we found nicotine-evoked dopamine release to be entirely tetrodotoxin-insensitive, even though tetrodotoxin blocked the effect of the sodium channel activator veratridine tested in parallel. Thus, the difference in tetrodotoxin sensitivity most likely reflects an as yet unidentified procedural difference between laboratories.

4.4. Dendrosomal release of dopamine

Somatodendritic nicotinic cholinoceptors on dopaminergic neurons have been studied in vitro in electrophysiological experiments in which membrane depolarization and firing rate have been the main measures (Pidoplichko et al., 1997; Picciotto et al., 1998; Sorenson et al., 1998). Somatodendritic release of dopamine, which in freely-moving animals is correlated with neuronal activity (Kalivas and Duffy, 1991), can be measured in a dendrosomal preparation (Marchi et al., 1991). In the present study, care was taken to avoid artefact. Thus, uptake of [^{3}H]dopamine by noradrenaline and 5-HT elements was pharmacologically inhibited during loading, and a monoamine oxidase inhibitor (pargyline) was used to inhibit metabolism of [^{3}H]dopamine. Moreover, re-uptake of [^{3}H]dopamine was minimized by using superfusion, and accordingly, responses to nicotine were found to be unaffected by the addition of the dopamine reuptake blocker nomifensine. These precautions virtually assure that nicotine augmented dopamine overflow by increasing release and not by inhibiting reuptake.

An advantage of the dendrosomal release assay is its procedural similarity to the synaptosomal release assay; tissue preparation, superfusion conditions, and time course of agonist application are comparable. Dendrosomes resemble synaptosomes in several known respects: they migrate similarly upon differential and continuous density gradient centrifugation (Hefti and Lichtensteiger, 1978), and depolarization-evoked release is dependent to a similar extent on extracellular Ca^{2+} (Marchi et al., 1991) and is likely to occur at least partly via vesicle-mediated exocytosis (Nirenberg et al., 1996). A major limitation is that responses to high K^+ (Marchi et al., 1991; present study) to nicotine and to epibatidine were all much smaller than in the synaptosomal assay, despite several attempts to increase the size of these effects. It should also be noted that the nicotinic cholinoceptor population revealed in this assay may represent only a subset of somatodendritic nicotinic cholinoceptors on dopamine cells. For example, additional nicotinic cholinoceptor subtypes may be preferentially desensitized or inactivated in our dendrosomal preparation.

In dendrosomes, epibatidine-evoked dopamine release was found to be only partially inhibited by a high concentration of mecamylamine, an insurmountable and broad-spectrum nicotinic antagonist (El-Bizri and Clarke, 1994; Clarke and Reuben, 1996). A mecamylamine-insensitive action of epibatidine has also been observed in a striatal 5-HT release assay, albeit at a higher agonist concentration (Reuben and Clarke, 1999). Taking sensitivity to mecamylamine into account, nicotine was at least as efficacious as epibatidine in stimulating nicotinic cholinoceptors that control somatodendritic release of dopamine. This result implies that somatodendritic and terminal nicotinic cholinoceptors differ pharmacologically.

4.5. Locomotor activity

Stimulation of somatodendritic nicotinic cholinoceptors was also assessed in tests of locomotor activity. The rationale for this assay is as follows. Convergent evidence suggests that the locomotor stimulant effect of nicotine is

mediated via an activation of mesolimbic dopaminergic neurons (Louis and Clarke, 1998). Both locomotor stimulation and increased mesolimbic dopamine outflow appear to depend on nicotinic cholinoceptor stimulation in the dopamine cell body region rather than in the terminal field (Benwell et al., 1993; Corrigall et al., 1994; Nisell et al., 1994). Although it was recently proposed that mesolimbic activation may be indirect, deriving from stimulation of α7 nicotinic cholinoceptors located on glutamatergic or aspartatergic afferents (Schilström et al., 1998a,b), this suggestion appears unlikely for several reasons. First, dopamine cells strongly express somatodendritic nicotinic cholinoceptors which can be stimulated in vitro at behaviourally-relevant concentrations (Pidoplichko et al., 1997; Sorenson et al., 1998; present study). Second, the finding that nicotinic stimulation of dopamine transmission is dependent upon concomitant NMDA receptor stimulation in the VTA need not imply a presynaptic action of nicotine (Schilström et al., 1998a). Third, the notion of mediation by α7 nicotinic cholinoceptors relies upon the administration of potentially non-selective concentrations of methyllycaconitine (Schilström et al., 1998b). Fourth, α7 nicotinic cholinoceptors tend to desensitize rapidly upon agonist exposure (Seguela et al., 1993).

In the present study, parallel tests revealed that in rats that were initially drug-naive, a maximally effective dose of nicotine was at least as effective as an equivalent dose of epibatidine. This result is consistent with previous but less direct drug comparisons (Menzaghi et al., 1997). Since epibatidine, in contrast to nicotine, appears to increase locomotion partly through a non-nicotinic mechanism (Menzaghi et al., 1997), it is likely that nicotine is more efficacious than epibatidine in increasing locomotor activity via somatodendritic nicotinic cholinoceptors.

The acute locomotor stimulant effect of nicotine is enhanced after repeated administration (Clarke and Kumar, 1983; present study). To our knowledge, previous locomotor effects with epibatidine have been restricted to drug-naive subjects. In the present study, the locomotor stimulant effect of epibatidine was found to be virtually absent in nicotine-experienced rats. The basis for this divergence is not clear, although a pharmacokinetic mechanism is unlikely, given that rats chronically trained to discriminate nicotine (0.4 mg/kg s.c.) remained responsive to epibatidine (Damaj et al., 1994).

Taken together, the results from both the dendrosomal release assay and locomotor tests suggest that nicotine is more efficacious than epibatidine at somatodendritic receptors on dopaminergic neurons. To the best of our knowledge, this pharmacological profile has not been previously reported in native or recombinant nicotinic cholinoceptors.

4.6. Identity of nicotinic cholinoceptor subtypes mediating catecholamine release in synaptosomes and dendrosomes

It has been proposed that striatal dopamine release is modulated at least partly by presynaptic α4β2 or α3β2-containing nicotinic cholinoceptors, or both (Grady et al., 1992; Kulak et al., 1997; Kaiser et al., 1998; Luo et al., 1998). However, certain observations cannot readily be reconciled with this. First, recombinant rat α4β2 and α3β2 nicotinic cholinoceptors appear 1–3 orders of magnitude less sensitive to nicotine or acetylcholine (Colquhoun and Patrick, 1997; Sabey et al., 1999). Second, neither nicotinic cholinoceptor attribution adequately accounts for the observed effects of cytisine and neuronal bungarotoxin in the striatal dopamine release assay (Grady et al., 1992; El-Bizri and Clarke, 1994; present study). For example, cytisine appears to require β4-containing nicotinic cholinoceptors for appreciable agonist activity (Luetje and Patrick, 1991; Papke and Heinemann, 1994; Zwart et al., 1994; Colquhoun and Patrick, 1997), whereas expression of β4 message has not been detected in dopaminergic neurons (Dineley-Miller and Patrick, 1992). The efficacy of cytisine determined in the present study (58%) is higher than the figure of 16% reported for putative α4β2 nicotinic cholinoceptors mediating Rb^+ efflux from thalamic synaptosomes (Marks et al., 1996). This observation suggests that α4β2 nicotinic cholinoceptors, if present on dopamine terminals, are probably not functionally predominant. If other nicotinic cholinoceptor subunits play a role in modulating release, α6 and β3 are obvious candidates on account of their strong expression in dopamine cells (Le Novère et al., 1996). The agonist potency and efficacy profile associated with the dopamine release assay does not resemble that of the only recombinant α6-containing nicotinic cholinoceptors characterized to date (i.e., chicken α6 + chicken or human β4) (Gerzanich et al., 1997; Fucile et al., 1998).

The pharmacological characteristics of nicotine-evoked hippocampal noradrenaline release suggest that the response is mediated, at least in part, by α3β4-containing nicotinic cholinoceptors (Clarke and Reuben, 1996; Luo et al., 1998). The results of the present efficacy comparison are consistent with this conclusion. However, the involvement of α6 and β3 subunits, which also appear strongly expressed in these neurons (Le Novère et al., 1996), cannot be ruled out.

The present findings indicate that somatodendritic and terminal nicotinic cholinoceptors on dopaminergic neurons are pharmacologically distinct. How does this conclusion relate to the occurrence of high-affinity [^{3}H]nicotine binding sites at both somatodendritic and terminal levels of dopaminergic neurons (Clarke and Pert, 1985)? Although it has been proposed that [^{3}H]nicotine binding sites represent a single (α4β2) nicotinic cholinoceptor subtype (Whiting and Lindstrom, 1987; Zoli et al., 1998), present evidence cannot exclude the possibility that in restricted brain regions, other subunits (e.g., α6, β3) contribute. Indeed, recent behavioural evidence suggests that α6-containing nicotinic cholinoceptors participate in nicotine-induced locomotion (Le Novère et al., 1999) that likely occurs via a somatodendritic action on mesolimbic dopa-

minergic neurons (see Section 4.5). Immunohistochemical studies have revealed the presence of α4 subunits on the outer cell membrane of dopamine dendrites (Sorenson et al., 1998); it remains to be established whether other subunits such as α6 are similarly located. In future studies, the high efficacy of nicotine relative to epibatidine may provide a useful clue to the subtype identity of somatodendritic nicotinic cholinoceptors.

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