Characterization of Nicotinic Agonist-Induced [³H]Dopamine Release from Synaptosomes Prepared from Four Mouse Brain Regions

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ABSTRACT

The inhibition of uptake of [³H]dopamine into synaptosomes prepared from four mouse brain regions was investigated. The inhibition curves demonstrated that in three regions, striatum, nucleus accumbens, and olfactory tubercle, [³H]dopamine was taken up exclusively by dopaminergic terminals. In frontal cortex, however, only a portion of the uptake was into dopaminergic terminals, with a larger amount taken up by noradrenergic terminals, and another small portion by serotonergic terminals. Release studies in frontal cortex indicated that in this region only dopaminergic and noradrenergic terminals are capable of packaging [³H]dopamine in a form allowing vesicle-mediated release; additionally, only the dopaminergic terminals have functional presynaptic nAChRs that, when stimulated by nicotinic agonists, evoke [³H]dopamine release. Agonist-stimulated [³H]dopamine release was characterized from synaptosomes prepared from four mouse brain regions. α-Conotoxin MII was a partial inhibitor of dopamine release in all of the brain regions, which suggests that a minimum of two nicotinic cholinergic receptors (nAChRs) are expressed in the nerve terminals of all four brain regions. No nicotine-induced [³H]dopamine release was detected in any brain region when the synaptosomes were prepared from β2 null mutant mice, which indicates that the β2 subunit is required for all nAChRs mediating this release. Dose-response curves were constructed for seven agonists in each of the brain regions. The pharmacological properties of synaptosomal [³H]dopamine release appear similar across the four brain regions. The results suggest that all four regions express the same nAChRs, although subtle regional differences may exist.

Major dopaminergic pathways originate in the substantia nigra (SN) and the ventral tegmental area (VTA) of the midbrain and project to several forebrain regions via the medial forebrain bundle. The nigrostriatal and mesocortical projections have a distinct topography, both mediolateral and rostrocaudal and, in the reverse orientation, dorsoventral (Moore and Bloom, 1978; Fuxe et al., 1985; Cragg et al., 1997). The density and intraneuronal distribution of varicosities also vary across brain region. Extensive axonal collateralization and dense varicosities are seen in the striatum, nucleus accumbens, and olfactory tubercle, whereas in the frontal cortex infrequent varicosities are seen on single axons (Moore and Bloom, 1978). There are also measurable differences in levels of mRNA and proteins involved in dopamine metabolism (Ciliax et al., 1995; Cragg et al., 1997), as well as in rates of dopamine uptake and release in the various terminal fields (Garris and Wightman, 1994).

It has been suggested that there are two functionally related components of dopamine release: phasic and tonic (Grace, 1991). Phasic release is mediated by the somatodendritic activation and firing of dopaminergic neurons, resulting in dopamine release in the terminal fields. Tonic release is a modulatory release mediated by presynaptic stimulation of dopaminergic terminals that may be important in regulating the responsiveness of the dopaminergic system. In support of this idea, the dopamine transporter null mutant mice, which exhibit chronic elevation of extracellular dopamine at the terminal field, also show decreased responsiveness of the synthesis- and release-regulating autoreceptors (Jones et al., 1999).

Nicotinic cholinergic receptors (nAChRs) are found on dopaminergic neurons at somatodendritic sites as well as pre-
terminals and/or presynaptic sites. Activation of receptors at both sites results in an increase in dopamine release (Clarke and Pert, 1985; Changeux et al., 1996; Role and Berg, 1996; Wonacott, 1997; MacDermott et al., 1999). The physiological significance of these receptors, especially those located presynaptically, remains unresolved (Changeux et al., 1996; Clarke, 1999; Kaiser and Wonacott, 1999). Nicotine-induced increases in dopamine release seen in vivo may occur because presynaptic ionotropic receptors exert an influence on membrane potential and thereby inhibit or facilitate action potentials (Role and Berg, 1996; Wonacott, 1997; MacDermott et al., 1999). In addition, presynaptic nAChRs may be involved in the tonic release of dopamine.

It is well established that activation of presynaptic nAChRs results in an increase in dopamine release (Wonacott, 1997; MacDermott et al., 1999), but the subunit composition of the nAChR subtypes that modulate this dopamine release is still in doubt. Dopaminergic neurons of the SN and VTA express message for numerous nAChR subunits, including α4, α5, α6, α7, β2, β3, and β4 (Klink et al., 2001). As a result, many different types of nAChR might be formed in dopaminergic neurons.

The studies reported herein attempted to characterize, using genetic and pharmacological strategies, nicotinic agonist-induced [3H]dopamine release from synaptosomes prepared from four brain regions that are projection fields for the dopaminergic neurons of the SN/VTA. The results of these studies indicate that at least two nAChR subtypes, likely the same two, are located on the presynaptic nerve terminals in all four brain regions studied.

**Experimental Procedures**

**Materials.** [3H]Dopamine was obtained from either PerkinElmer Life Sciences (Boston, MA) (3,4-[ring-2,5,6-3H] at 30–60 Ci/mmol) or Amersham Biosciences (Piscataway, NJ) (7,8-[3H] at 40–60 Ci/mmol). HEPES and sucrose were products of Roche Applied Science (Indianapolis, IN). Methylcarbamylcholine chloride, dihydro-β-erythroidine (DHβE), desipramine, and zimelidine were purchased from Sigma/RBI (Natick, MA). Sigma-Aldrich (St. Louis, MO) was the source for the following compounds: nomifensine, GBR12909, (−)-epibatidine hydrochloride, cytosine-1,2,3-triazol-4-yl-β-nicotinylglycine (CTTXMII), (−)-nicotine tartrate, (−)-nicotine(+)-di-p-tolualtrtarate, acetylcholine iodide (ACh), carbachol iodide, (−)-epibatidine hydrochloride, cytosine, tetramethylammonium chloride, atropine sulfate, diisopropylfluorophosphate, bovine serum albumin, ascorbic acid, and pargyline. Mecamylamine was a gift from Merck Sharp & Dohme (Rahway, NJ). α-Conotoxin MII (αCtxMII) was synthesized as described previously (Cartier et al., 1996). All other chemicals were of reagent grade.

**Animals.** Animal care and experimental procedures were approved by the Animal Care and Utilization Committee of the University of Colorado, Boulder. Mice were bred in the specific pathogen-free colony at the Institute for Behavioral Genetics (Boulder, CO). The animals were maintained on a 12-h light/dark cycle (lights on from 7:00 AM to 7:00 PM), and housed five to a cage with same-sex littermates, with free access to food and water. Unless otherwise indicated, females of the C57BL/6J strain, 60 to 90 days of age, were used. β2 nAChR subunit null mutant mice (Picciotto et al., 1995) were bred onto the C57BL/6J strain for six to eight generations. Genotype was determined by polymerase chain reaction analysis of tail DNA (Picciotto et al., 1995).

**Synaptosome Preparation.** Each mouse was sacrificed by cervical dislocation. The brain was removed from the skull and was immediately placed on ice and chilled for 5 min. Coronal slices (1 mm) were taken using glass coverslips and a mouse brain matrix (Activational Systems, Inc., Detroit, MI; currently available from Harvard Apparatus, Holliston, MA). Nucleus accumbens (NA) was dissected from slices in which the anterior commissure was visible (1–2 slices) by use of a 1.2-mm glass capillary tube. Frontal cortex (FC), olfactory tubercle (OT), and striatum (ST) were dissected from the slices using forceps. Diagrams of brain slices were used as a guide (Paxinos and Watson, 1986; Franklin and Paxinos, 1997). Tissue was homogenized (16–20 strokes by hand) in 0.5 ml of 0.32 M sucrose buffered with 5 mM HEPES, pH 7.5. P2 synaptosomal pellets were then prepared from each region by centrifugation at 1000g for 10 min, followed by centrifugation of the resulting supernatant at 12,000g for 20 min. The P2 pellets were resuspended in perfusion buffer (120 mM NaCl, 2.4 mM KCl, 3.2 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM HEPES, pH 7.5, 10 mM glucose, 1 mM ascorbic acid, and 0.01 mM pargyline). The following volumes were used: NA and FC, 0.2 ml; and OT and ST, 0.4 ml.

**Uptake Experiments.** Synaptosomes prepared as described for the release experiments were diluted 5- to 15-fold with perfusion buffer to maintain uptake in a linear range (below 10% of input counts per minute). GBR12909 was dissolved in 50% ethanol/50% perfusion buffer in siliconized tubes and subsequently diluted into perfusion buffer using siliconized tubes and pipette tips. Nomifensine, desipramine, and zimelidine were dissolved directly into perfusion buffer before dilution. Aliquots of synaptosomes (90 µl) were added to appropriate concentrations of transport inhibitors (10 µM, ranging from 3 nM to 1 mM) and incubated at 37°C for 5 min before addition of 0.5 µCi of [3H]dopamine (final concentration 0.1 µM). After an additional 5 min of incubation at 37°C each sample was diluted with 0.5 ml of cold buffer and filtered over type AE glass fiber filters (Gelman Sciences, Ann Arbor, MI) with suction. Filters were collected after two more washes with 0.5 ml of cold buffer. They were added to each filter, and radioactivity was determined by scintillation counting (1600TR liquid scintillation spectrometer; Packard Instrument Co., Downer’s Grove, IL). Instrument counting efficiency was 45%. Two types of controls were used. One control used samples containing no inhibitors. These samples were incubated at 37°C to determine 100% activity for each synaptosomal preparation. The other controls also did not include inhibitors; these were incubated on ice and were used to determine nonspecific uptake, which was subtracted from each sample to determine specific uptake.

**Perfusion and Release.** The perfusion procedure has been described previously (Grady et al., 1997a). Briefly, synaptosomes were incubated at 37°C in perfusion buffer for 10 min before addition of 100 nM [3H]dopamine (1 µCi for every 0.2 ml of synaptosomes). The synaptosomal suspension was incubated for an additional 5 min. Aliquots of synaptosomes (80 µl) were distributed onto filters and perfused at 0.6 ml/min for 10 min before fractions were collected. For experiments using ACh as agonist, the synaptosomes were treated with 10 µM diisopropylfluorophosphate to inhibit acetylcholinesterase. Diisopropylfluorophosphate and [3H]dopamine were added at the same time during the last 5 min of the uptake procedure. Atropine (1 µM) was added to the perfusion buffer to inhibit muscarinic receptors. For experiments using αCtxMII, 0.1% bovine serum albumin was added to the perfusion buffer. Fractions were collected every 30 s, and radioactivity was determined by scintillation counting (1600TR liquid scintillation spectrometer; Packard Instrument Co.) after addition of EconoSafe (Sigma/RBI, Mt. Prospect, IL). Instrument counting efficiency was 45%.

**Data Analysis.** All curve-fit parameters and errors were calculated using the nonlinear curve-fitting algorithm of SigmaPlot 5.0 for DOS (Jandel Scientific, San Rafael, CA). Release data were plotted as counts per minute versus fraction number (usually 15 fractions were collected; Fig. 2). Fractions collected immediately before and after the agonist- or potassium-induced peak were used to calculate baseline as a single exponential decay. The calculated baseline was subtracted from the data obtained when agonists were added. Those fractions that exceeded baseline by 10% or more were summed to
give total released counts per minute. For many experiments, the data (counts per minute released above baseline) were normalized to baseline to give units of release [counts per minute - baseline]/baseline (Grady et al., 1997a, 2001). Thus, 1 unit of release is twice the baseline release. Dose-response data were fit to the Hill equation (Grady et al., 2001) to calculate EC50 values. Transient and persistent release parameters (Table 3) were calculated by fitting the data to a double-exponential decay equation (Grady et al., 1997a). IC50 values for inhibition of uptake (Table 2) were calculated by fitting the data to single-site inhibition (uptake = U0/(1 + [An]/IC50), where U0 is uninhibited uptake and [An] is the antagonist concentration), to single-site partial inhibition (uptake = U0/(1 + [An]/IC50) + C), where C is the amount of uptake not able to be inhibited, or to double-site inhibition (uptake = U0/(1 + [An]/IC50) + U0/(1 + [An]/IC50*)), where * indicates the second site.

Results

Before the release studies could be done, a series of studies were carried out that attempted to identify the neuronal classes that might take up [3H]dopamine in the four brain regions. These studies were prompted by the observation that dopamine is a substrate for a number of monoamine transporters. Indeed, the dopamine and norepinephrine transporters have similar Km (or Ki) values for dopamine (Pacholczyk et al., 1991; Giros and Caron, 1993). Consequently, the effects of several monoamine transport inhibitors on [3H]dopamine uptake and release were investigated. Table 1 lists published transporter antagonist K values, determined using the cloned transporters. Data from Table 1 indicate that 1) at nanomolar concentrations nomifensine, commonly used as a dopamine transporter blocker, will likely block both the dopamine and norepinephrine transporters and actually may be somewhat more potent at the norepinephrine transporter; 2) desipramine should be quite selective for the norepinephrine transporter; 3) zimelidine should show selectivity for the serotonin transporter; and 4) GBR12909 should show moderate selectivity for the dopamine transporter, although it may have some activity at the norepinephrine transporter.

Figure 1 and Table 2 present results of experiments that assessed inhibition of [3H]dopamine uptake by nomifensine, desipramine, zimelidine, and GBR12909 into synaptosomes prepared from mouse brain regions. Simple and similar inhibition curves were obtained for the striatum, olfactory tubercle, and nucleus accumbens. All of the inhibitors totally blocked specific [3H]dopamine uptake into striatum, olfactory tubercle, and nucleus accumbens. The data obtained in these three regions fit single-site inhibition equations. The rank order (GBR12909 > nomifensine > desipramine > zimelidine) and potency values (Table 2) for the four antagonists are comparable in striatum, olfactory tubercle, and nucleus accumbens. In contrast, the data obtained using frontal cortex were more complex. The nomifensine and desipramine data were best described by a single partial inhibition model, whereas the zimelidine and GBR12909 data were best fit by two-site equations. The rank order and potencies of the inhibitors differed between frontal cortex and the other three regions.

Previous studies from our laboratory have shown that nicotinic agonists stimulate an increase in [3H]dopamine release from mouse striatal synaptosomes, which is dependent on external Ca2+ and is blocked by competitive antagonists, such as DHβE, and the noncompetitive antagonist mecamylamine (Grady et al., 1997a). Figure 2 presents the results of experiments that evaluated the Ca2+ dependence and inhibitor sensitivity (DHβE and mecamylamine) of [3H]dopamine release from synaptosomes prepared from the four brain regions. The results shown in Fig. 2A demonstrate that no nicotine-stimulated release was seen in any brain region in a Ca2+-free medium. Both of the nicotinic antagonists blocked release, but the results were slightly different. DHβE (10 μM) totally blocked nicotine-stimulated [3H]dopamine release in all four of the brain regions (Fig. 2B), whereas 10 μM mecamylamine treatment allowed a transient increase in release, which was followed by total blockade (Fig. 2C). These results indicate that the [3H]dopamine

### Table 1

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Km (nM)</th>
<th>Km (nM)</th>
<th>Km (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nomifensine</td>
<td>6.0</td>
<td>8.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Desipramine</td>
<td>12,000</td>
<td>4.0</td>
<td>567–1680</td>
</tr>
<tr>
<td>Zimelidine</td>
<td>25,000</td>
<td>3.0</td>
<td>382</td>
</tr>
<tr>
<td>GBR12909</td>
<td>17</td>
<td>133</td>
<td>3900</td>
</tr>
</tbody>
</table>

a Data from Giros et al. (1992) for rat dopamine transporter expressed in Ltk− cells.
b Data from Pacholczyk et al. (1991) for human norepinephrine transporter expressed in HeLa cells.
c Data from Blakeley et al. (1991) and Hoffman et al. (1991) for rat serotonin transporter expressed in HeLa cells or in CV-1 cells.
IC50 values from inhibition curves

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Striatum</th>
<th>Olfactory Tubercle</th>
<th>Nucleus Accumbens</th>
<th>Frontal Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nomifensine</td>
<td>188 ± 10</td>
<td>135 ± 15</td>
<td>125 ± 6</td>
<td>43 ± 4 (85%)</td>
</tr>
<tr>
<td>Desipramine</td>
<td>26,000 ± 2,400</td>
<td>20,000 ± 7,000</td>
<td>9,800 ± 1,900</td>
<td>3.4 ± 2.0 (70%)</td>
</tr>
<tr>
<td>Zimelidine</td>
<td>32,000 ± 3,400</td>
<td>22,000 ± 2,300</td>
<td>19,000 ± 3,700</td>
<td>55 ± 155 (20%)</td>
</tr>
<tr>
<td>GBR12909</td>
<td>2.5 ± 0.5</td>
<td>4.3 ± 1.0</td>
<td>3.5 ± 0.7</td>
<td>7.0 ± 1.1 (20%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>218 ± 43</td>
<td>(80%)</td>
</tr>
</tbody>
</table>

release measured from synaptosomes prepared from all four brain regions occurs by exocytotic release that is stimulated by activation of one or more nAChR subtypes.

The uptake inhibitor studies suggest that [3H]dopamine uptake in striatum, olfactory tubercle, and nucleus accumbens is virtually all into dopaminergic terminals, whereas uptake into frontal cortex synaptosomes may occur in a combination of dopaminergic, noradrenergic, or serotonergic terminals. Consequently, the effects of treatment with the transporter inhibitors during uptake on subsequent [3H]dopamine release were determined from synaptosomes that had been prepared from frontal cortex. The synaptosomes were incubated with [3H]dopamine in the presence of various inhibitors or combinations of inhibitors. These inhibitors were not present during the release phase of the experiment. Figure 3A presents the uptake data; these data are not corrected for nonspecific uptake, estimated to be 15% of control from samples incubated on ice. Figure 3B presents the effects of the various inhibitors on K+-stimulated [3H]dopamine release, and Fig. 3C presents the effects of the inhibitors on nicotine-stimulated [3H]dopamine release.

The open columns in Fig. 3 present the effects of treatment of frontal cortex synaptosomes with 100 nM desipramine + 100 nM zimelidine. These drugs were used to prevent uptake into synaptosomes from noradrenergic and serotonergic terminals. The 100 nM zimelidine concentration is only twice
the IC$_{50}$ for the mouse serotonin transporter (Table 2) and may not have completely blocked dopamine uptake into serotonergic nerve terminals. A higher concentration was not used because this would have blocked some uptake into dopaminergic nerve terminals. Therefore, most of the uptake should occur in dopaminergic synaptosomes. Uptake of $[^3$H]dopamine measured in the presence of the desipramine/zimelidine combination was 52 ± 6% of control (48 ± 6% inhibition; Fig. 3A), potassium-stimulated release was 39 ± 9% of control (61 ± 9% inhibition; Fig. 3B), and nicotine-stimulated release was 82 ± 17% (19 ± 17% inhibition; Fig. 3C). Thus, even though the desipramine/zimelidine treatment inhibited $[^3$H]dopamine uptake by approximately 50%, little or no decrease in nicotine-stimulated dopamine release occurred.

The diagonally striped bars in Fig. 3 show the results of experiments where 100 nM GBR12909 was added to block uptake into dopaminergic nerve terminals from the frontal cortex. Under this condition, uptake and release should be limited to nondopaminergic terminals. GBR12909 treatment resulted in relatively small changes in $[^3$H]dopamine uptake and K$^+$-stimulated release. Uptake was 80 ± 3% of control (20 ± 3% inhibition; Fig. 3A) and K$^+$-stimulated release was 80 ± 7% of control (20 ± 7% inhibition; Fig. 3B) using GBR12909-treated synaptosomes. In contrast, nicotine-stimulated release was only 17 ± 3% of control (83 ± 3% inhibition; Fig. 3C), indicating almost all nicotine-stimulated release was associated with the dopaminergic population.

The cross-hatched bars in Fig. 3 illustrate the effects of 10 μM nomifensine, which was used to block uptake into both dopaminergic and noradrenergic terminals of frontal cortex. Presumably, any uptake that was measured after nomifensine treatment is nonspecific or occurs in serotonergic nerve terminals. Uptake occurred when nomifensine was added during the uptake phase of the experiment; uptake was 38 ± 1% of control (62 ± 1% inhibition; Fig. 3A). Of this uptake, about 15% is nonspecific, and based on the results of inhibition of uptake by zimelidine, 20% is into serotonergic terminals (Fig. 1; Table 2). However, K$^+$-stimulated release was virtually eliminated; release was 4 ± 2% of control (96 ± 2% inhibition; Fig. 3B). Nicotine-stimulated release was also changed dramatically; release was 4 ± 2% of control (96 ± 2% inhibition; Fig. 3C). Thus, it may be that $[^3$H]dopamine was taken up into serotonergic nerve terminals, but was not taken up into synaptic vesicles, and was not able to be released by either K$^+$ or nicotine.

The horizontally striped bars in Fig. 3 present the effects of the GBR12909/zimelidine combination. GBR12909 (100 nM) and 100 nM zimelidine were added to block uptake into dopaminergic and serotonergic terminals of frontal cortex. Under these conditions, uptake should occur primarily in synaptosomes derived from noradrenergic nerve terminals. Uptake was reduced to 66 ± 4% of control (34 ± 4% inhibition; Fig. 3A) by GBR/zimelidine treatment. Potassium-stimulated release was decreased to 78 ± 9% of control (22 ± 9% inhibition; Fig. 3B), and nicotine-stimulated release was 14 ± 3% (86 ± 3% inhibition; Fig. 3C). These results indicate that $[^3$H]dopamine is taken up into noradrenergic nerve terminals and that the dopamine can be released by K$^+$-induced depolarization, but not by activation of an nAChR.

Uptake of $[^3$H]dopamine and K$^+$-stimulated $[^3$H]dopamine release under conditions of partial transporter inhibition is highly correlated (Fig. 4; $r = 0.96$). The regression line has an intercept of 33.4% uptake, indicating that not all of the $[^3$H]dopamine taken up is able to be released by 20 mM K$^+$. Uptake and nicotine-stimulated release did not correlate ($r = 0.11$; correlation not shown).

αCtxMII partially blocks agonist-stimulated dopamine release from rat (Kulak et al., 1997; Kaiser et al., 1998) and mouse (Grady et al., 1997a, 2001) striatal synaptosomes. This toxin was used to determine whether a similar inhibition pattern is seen in synaptosomes prepared from the four brain regions. Figure 5 presents the results of these experiments. All regions showed a partial inhibition of nicotine-stimulated release of $[^3$H]dopamine by 30 nM αCtxMII, a concentration that blocks maximally (IC$_{50}$ of 2 nM; Grady et al., 1997b). The effect of αCtxMII was not significantly different between regions (striatum, 47 ± 4% inhibition; olfactory tubercle, 39 ± 2% inhibition; nucleus accumbens, 55 ± 3% inhibition; and frontal cortex, 41 ± 9% inhibition). All are significantly different from control by paired t test or by t test for difference from a hypothetical population (100%) ($P < 0.05$ and $P < 0.01$, respectively).

Nicotine-stimulated $[^3$H]dopamine release from mouse striatal synaptosomes is totally eliminated by the β2 null mutation (Whiteaker et al., 2000a; Grady et al., 2001). Figure 6 presents the results of experiments where $[^3$H]dopamine release was measured using synaptosomes derived from the four brain regions. Synaptosomes were prepared from wild-type (+/+), heterozygous (+/−), and homozygous null mutant (−/−) mice. Release was measured after stimulation with 20 mM K$^+$ and 10 μM nicotine. Normal K$^+$-evoked release was seen in the β2 null mutant mice, whereas nicotine-stimulated release was undetectable. Nicotine-stimulated release was decreased from that seen in the +/- mice in all regions in mice heterozygous for the β2 subunit. The percentage of decrease from the wild-type mice was some-

![Fig. 4. Correlation of uptake of $[^3$H]dopamine and potassium-stimulated release under conditions of partial inhibition of uptake in synaptosomes prepared from frontal cortex. The data (as percentage of control) are from Fig. 3, A and B.](image-url)
what variable among regions and ranged from 28 ± 7% for olfactory tubercle to 41 ± 5% for frontal cortex; however, the differences among regions were not significant.

Dose-response curves for l-nicotine-stimulated dopamine release were constructed for each brain region (Fig. 7A). Nicotine produced concentration-dependent increases in dopamine release in all four brain regions. EC\textsubscript{50} values for release were similar across the four brain regions, but differences in maximal release were seen. The greatest agonist-stimulated release compared with baseline was found in the olfactory tubercle, whereas nucleus accumbens and striatum were of similar magnitude and frontal cortex was considerably lower (the ratio of efficacy for OT/ST/NA/FC = 1.00:0.67:0.57:0.33). In comparison, K\textsuperscript{+}-stimulated release normalized to baseline was similar for all regions (Fig. 6). Figure 7B shows data for cytisine-stimulated release. Cytisine is a partial agonist in all regions, although it had relatively more activity in frontal cortex.

Fig. 5. Effect of αCtxMII on [3H]dopamine release from four brain regions. Synaptosomes from each region were perfused with buffer ± 30 nM αCtxMII for 5 min before a 1-min exposure to 1 μM l-nicotine. Release normalized to baseline (units) is shown as mean ± S.E.M. for seven experiments. The effect of αCtxMII is significant for all regions (P < 0.05).

Fig. 6. Effect of the β2 null mutant genotype on [3H]dopamine release. Synaptosomes were prepared from all four brain regions of mice of each of the β2 null mutant genotypes (females, 60–180 days of age). Release of [3H]dopamine was evoked by a 30-s exposure to either 10 μM l-nicotine or 20 mM potassium. Data are expressed as normalized release (units) ± S.E.M. for n = 5 (+/+), n = 6 (+/−), and n = 4 (−/−). By one-way analysis of variance, both the +/− and −/− genotypes were significantly different from the other two genotypes for nicotine-stimulated release [striatum, F(2,12) = 155.6, P < 0.0001; olfactory tubercle, F(2,12) = 76.7, P < 0.0001; nucleus accumbens, F(2,12) = 39.7, P < 0.0001; and frontal cortex, F(2,12) = 24.0, P < 0.0002]. For potassium-stimulated release, the +/− genotype was significantly lower than the +/+ genotype only for frontal cortex [F(2,12) = 5.89, P < 0.02] and did not differ from the −/− genotype. The −/− genotype did not differ in potassium-stimulated release from the +/+ for any brain region. Baselines and counts per minute remaining on filters did not differ with genotype.

regression lines were in the 0.85 to 0.87 range, likely because the EC\textsubscript{50} values for the most potent agonist, epibatidine, was lower when striatum was the source of the synaptosomes (3–5-fold; Table 3).

Studies done with nAChRs expressed in cell lines and oocytes have demonstrated that subunit composition also influences maximal response (R\textsubscript{max}), or efficacy (Lindstrom et al., 1996; Wang et al., 1996). Therefore, the R\textsubscript{max} for each agonist was also determined. These data are compared in Fig. 9. The rank order of release from the four brain regions was similar for all seven of the test agonists. Olfactory tubercle had greater release normalized to baseline than the other regions for all agonists. Frontal cortex had lower release than the other regions for all of the agonists tested, except methylcarbachol. Compared with acetylcholine, cytisine was a partial agonist in all regions, and methylcarbachol was a partial agonist in all regions, although it had relatively more activity in frontal cortex.

A second experiment compared the amount of agonist-
stimulated release (as counts per minute) to the amount of synaptosomal protein. In these experiments, 10 µM L-nicotine was used to stimulate release. The ratio of efficacy normalized to baseline was OT/ST/NA/FC = 1.00:0.71:0.76:0.25, but when normalized to synaptosomal protein, the ratio was OT/ST/NA/FC = 1.00:0.74:0.98:0.05. Thus, when compared on the basis of protein frontal cortex has considerably less activity than the other regions.

The agonist-stimulated [3H]dopamine release from striatal synaptosomes has been shown to have two components, which have been called transient and persistent (Grady et al., 1997a). Figure 10 shows the results of experiments where transient and persistent release were measured in the four brain regions. As is evident in the figure, nicotine produced a rapid increase in dopamine release, which decreased in the continued presence of nicotine in all four brain regions. The decrease in release was biphasic and could be separated into two components, transient and persistent, by fitting the data to a double-exponential equation. Several parameters were calculated from these data: maximal transient release (VT), maximal persistent release (VP), rate of desensitization of the transient release (DT), rate of desensitization of the persistent release (DP), and the ratio of persistent to transient response (VT/VP). These parameters, derived from the curve fits, are presented in Table 4. The pattern is similar for all four regions. The rank order of VT from synaptosomes of olfactory tubercle, nucleus accumbens, striatum, and frontal cortex is similar to the maximal release seen in the experiments reported in Fig 9. When the VP/VT was compared across the brain regions small differences were uncovered; the striatum appears to have a slightly higher proportion of persistent response than the other regions. The desensitization rates for both the DT and DP are significantly different (P < 0.05, t test) in the nucleus accumbens than in the striatum. The rate of desensitization of the persistent release process is also significantly different when olfactory tubercle and striatum are compared (P < 0.05).

Discussion

The experiments presented herein demonstrate that nictinic agonists stimulate Ca2+-dependent [3H]dopamine release from synaptosomes prepared from the four brain regions, consistent with release from synaptic vesicles via an influx of Ca2+. Release is blocked by both DHβE and mecamylamine, which suggests that release is evoked by activating nAChRs. Release is modulated by a minimum of two nAChRs because a maximally effective concentration of αCtxtMII blocked less than 60% of the release in each of the brain regions. No agonist-stimulated release was detectable using synaptosomes from β2 null mutants. Thus, β2 subunits are obligatory components of all the presynaptic nAChRs that modulate dopamine release. The pharmacological properties of [3H]dopamine release were nearly identical in the four brain regions, suggesting that the same nAChR subtypes, in approximately the same ratio, mediate nicotine-stimulated [3H]dopamine release from presynaptic nerve terminals of each of the four regions.

The uptake of [3H]dopamine was characterized using inhibitors that have some selectivity for the three major monoamine transporters (dopamine, norepinephrine, and serotonin). When striatum, nucleus accumbens, and olfactory tubercle were analyzed, all the antagonists produced complete inhibition, which fit single-site models. In these three regions, GBR12909 was the most potent inhibitor of [3H]dopamine uptake. The IC50 values for uptake inhibition in these three brain regions are similar to published Ki values for inhibition of the dopamine transporter, suggesting that [3H]dopamine uptake occurred only in dopaminergic neurons in striatum, nucleus accumbens, and olfactory tubercle.

Transporter-mediated [3H]dopamine uptake into synaptosomes obtained from the frontal cortex is clearly different from the other regions. Major differences include 1) GBR12909 and zimelidine inhibition curves were biphasic, 2) desipramine and nomifensine were partial inhibitors, and 3) the rank order of potency of the antagonists was different. Approximately 15 to 20% of the [3H]dopamine is taken up into dopaminergic terminals as shown by the finding that 15 to 20% of total [3H]dopamine uptake could be accounted for by the high-affinity portion of the GBR12909 inhibition curve. Similarly, the difference between the maximum inhibition by nomifensine (85%), which blocks both the dopamine and norepinephrine transporters, and desipramine (70%), which blocks only the norepinephrine transporter, is 15%. As much as 70% of the [3H]dopamine may be transported into noradrenergic terminals because the high-affinity inhibition by desipramine and the low-affinity portion of the GBR12909 curve are approximately 70%. The remainder of the [3H]dopamine may have been taken up into synaptosomes derived from serotonergic nerve terminals because low concentrations of zimelidine, a somewhat selective inhibitor of the serotonin transporter, inhibited 15 to 20% of uptake. This conclusion is supported by the finding that nomifensine, which does not bind to the serotonin transporter, totally failed to block 15% of the uptake.

The finding that [3H]dopamine is taken up by a number of transporters in frontal cortex suggested that release might occur from more than dopaminergic nerve terminals when cortical synaptosomes were used. Therefore, release was investigated where the synaptosomes were treated with trans-
port inhibitors during the uptake phase of the experiment. Four conditions were selected to isolate the dopaminergic, the nondopaminergic, the serotonergic, and the noradrenergic terminals. [3H]Dopamine releasable by K⁺/H11001 exposure, which stimulates synaptic vesicle-mediated release by membrane depolarization, allowing Ca²⁺ influx through voltage-sensitive Ca²⁺ channels, is not dependent on nAChRs. K⁺-stimulated release was highly correlated with uptake (r = 0.96), but the regression line has an intercept of 33.4% uptake, indicating that this portion cannot be released by exposure to 20 mM K⁺. Approximately 15% of this unreleasable pool can be accounted for by nonspecific uptake. A possible explanation for the remainder is that although [3H]dopamine taken up into dopaminergic and noradrenergic terminals was released by 20 mM K⁺, the portion taken up by serotonergic terminals (15%) was not. Perhaps [3H]dopamine cannot be properly packaged in synaptic vesicles in serotonergic termi-

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Striatum</th>
<th>Olfactory Tubercle</th>
<th>Nucleus Accumbens</th>
<th>Frontal Cortex</th>
</tr>
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<tr>
<td>ACh</td>
<td>0.24 ± 0.03</td>
<td>0.28 ± 0.04</td>
<td>0.32 ± 0.10</td>
<td>0.29 ± 0.05</td>
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<td>CARB</td>
<td>2.46 ± 0.72</td>
<td>3.71 ± 0.92</td>
<td>2.17 ± 0.49</td>
<td>2.78 ± 0.49</td>
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<td>CYT</td>
<td>0.017 ± 0.002</td>
<td>0.019 ± 0.004</td>
<td>0.022 ± 0.008</td>
<td>0.032 ± 0.018</td>
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<tr>
<td>EPI</td>
<td>0.00017 ± 0.00017</td>
<td>0.00076 ± 0.00027</td>
<td>0.00080 ± 0.00027</td>
<td>0.00059 ± 0.00048</td>
</tr>
<tr>
<td>L-NIC</td>
<td>0.017 ± 0.02</td>
<td>0.16 ± 0.01</td>
<td>0.13 ± 0.02</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>MeCARB</td>
<td>0.19 ± 0.03</td>
<td>0.19 ± 0.06</td>
<td>0.16 ± 0.08</td>
<td>0.26 ± 0.09</td>
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<tr>
<td>TMA</td>
<td>8.64 ± 1.59</td>
<td>7.52 ± 1.21</td>
<td>8.88 ± 1.28</td>
<td>6.69 ± 3.05</td>
</tr>
</tbody>
</table>

CARB, carbachol iodide; CYT, cytisine; EPI, (+)-epibatidine; L-NIC, l-nicotine; MeCARB, methylcarbamylcholine chloride; TMA, tetramethylammonium chloride.

Fig. 8. Correlation of EC₅₀ values for agonist-stimulated [3H]dopamine release from various brain regions. EC₅₀ values (micromolar) were determined from data fit to the Hill equation for seven agonists: acetylcholine, carbachol, cytisine, epibatidine, l-nicotine, methylcarbachol, and tetramethylammonium. Correlation coefficients for comparisons to striatum (shown) were 0.99 (ST versus NA), 1.00 (ST versus FC), and 0.99 (ST versus OT). Slopes ranged from 0.85 to 0.87 and intercepts from −0.05 to 0.01. Correlation coefficients for other comparisons between regions (NA versus FC, NA versus OT, and FC versus OT) were all 0.99 to 1.00, slopes ranged from 1.01 to 1.02 and the range of intercepts was −0.01 to 0.04.

Fig. 9. Comparison of maximum release by eight agonists in four brain regions. R_max values of data presented in Figs. 7 and 8 fit to the Hill equation are compared for the four brain regions.

Fig. 10. Comparison of nicotine-evoked transient and persistent [3H]dopamine release in four brain regions. All synaptosomes were exposed to 10 μM l-nicotine for 10 min from 2.5 to 12.5 min of perfusion. Data points shown are means of seven to eight experiments. Curves shown are fits of the data to a first-order double-exponential decay.
nals. The pattern of release of [3H]dopamine by nicotine, however, was not correlated with the amount of uptake \( (r = 0.08) \). When uptake into dopaminergic terminals was blocked, nicotine-stimulated release was nearly eliminated, but when uptake into noradrenergic and serotonergic terminals was blocked, nicotine-stimulated release was not altered. These results indicate that most of the agonist-stimulated dopamine release originated from dopaminergic terminals and suggest that functional presynaptic nAChRs are located on dopaminergic terminals but not noradrenergic terminals in cortex.

\( \alpha \text{CtxMII} \) totally blocks \( \alpha 3\beta 2 \) (Cartier et al., 1996), \( \alpha 3\beta 2\beta 3 \) (Luo et al., 2000), and several subtypes containing \( \alpha 6 \) subunits (Kuryatov et al., 2000) expressed in oocytes. \( \alpha \text{CtxMII} \) selectively inhibits some nAChRs found at somatodendritic sites on dopaminergic neurons (Klink et al., 2001), and on presynaptic nerve terminals in the striatum (Grady et al., 1997b; Kulak et al., 1997; Kaiser et al., 1998; Whiteaker et al., 2000b). \( \alpha \text{CtxMII} \) treatment of synaptosomes produced a partial inhibition of nicotine-stimulated [3H]dopamine release from all four regions. This partial inhibition of similar magnitude may mean that all four brain regions contain a similar ratio of a minimum of two nAChRs, one that is sensitive to \( \alpha \text{CtxMII} \) and one nAChR that is not.

Pharmacological analyses of nAChRs expressed in oocytes have demonstrated that changing subunit composition often results in profound changes in agonist potency (Luetje and Patrick, 1991; Role and Berg, 1996; Wang et al., 1996; Kuryatov et al., 2000). The potencies of seven agonists were highly correlated across the four brain regions. Thus, it may be that the dopaminergic nerve terminals in the four brain regions express the same receptors. This result, obtained with mouse, agrees with the observations that the potency for release of [3H]dopamine from rat striatal and cortical slices by four nicotinic agonists (Puttfarcken et al., 2000), and from rat striatal and frontal cortical synaptosomes by two agonists (Whiteaker et al., 1995) are highly correlated.

Comparing agonist efficacies (maximal response) may also be useful in determining receptor subtypes that modulate a response (Lindstrom et al., 1996; Wang et al., 1996; Whiteaker et al., 2000a). In a comparison of maximal release, five compounds appeared to be full agonists in all regions and two, cytisine and methylcarbachol, were partial agonists. Across regions, epibatidine appears to be relatively less efficacious in nucleus accumbens than in striatum, and methylcarbachol relatively more efficacious in frontal cortex. Overall, however, the patterns are similar, and indicate no major nAChR subtype differences.

Relative to the other regions, the \( R_{\text{max}} \) values for frontal cortex may be underestimated because a component of the baseline may be derived from noradrenergic or serotonergic terminals. We attempted to resolve this problem with experiments that used desipramine and zimelidine to selectively block uptake. The baselines from these release experiments were reduced to 71 ± 11% of control, but this decrease was insufficient to qualitatively affect the results; frontal cortex release relative to baseline was still lower than the other regions for all agonists other than methylcarbachol, perhaps indicating that nAChRs are more sparsely represented on dopaminergic terminals in frontal cortex compared with other regions.

When regional nicotine-stimulated [3H]dopamine release was compared with amount of synaptosomal protein, frontal cortex clearly had less dopamine release. Other studies have yielded results that indicate that nicotine-stimulated dopamine release is lower in frontal cortex than in other brain regions such as striatum and nucleus accumbens. Microdialysis measurements of nicotine-stimulated dopamine release from three regions of rat brain showed that release in response to nicotine from frontal cortex was considerably less than release from nucleus accumbens or striatum (Marshall et al., 1997). A synaptosomal release study from rat also showed lower nicotine-stimulated release in frontal cortex than striatum (Whiteaker et al., 1995). All of these results are consistent with the neuroanatomy of dopaminergic terminals, indicating that terminals are sparser in frontal cortex than other regions (Moore and Bloom, 1978).

The maximal release results also show that agonist-stimulated [3H]dopamine release was higher in olfactory tubercle than other regions. This is not reflected in binding studies where olfactory tubercle had somewhat lower [3H]epibatidine and [125I]\( \alpha \text{CtxMII} \) binding than nucleus accumbens or striatum (Whiteaker et al., 2000b). Perhaps a greater percentage of nAChRs in the olfactory tubercle is on dopaminergic terminals than in other regions.

The profile of transient and persistent release stimulated by L-nicotine was similar, but not identical, for all four regions. The rate constants for desensitization of transient release were slightly higher in nucleus accumbens than in other regions and the rate of decrease of the persistent response was slightly higher for both nucleus accumbens and olfactory tubercle. Ratios of persistent to transient release showed that striatum may have a somewhat higher relative amount of persistent release.

In conclusion, it is likely that at least two different functional nAChRs, which differ in sensitivity to \( \alpha \text{CtxMII} \) and include the \( \beta 2 \) subunit, are expressed on dopaminergic terminals found in the four brain regions. Pharmacological comparisons failed to provide indisputable evidence that different subtypes are in the four regions, but minor differences in agonist efficacy, in the kinetics of transient and persistent release, and relative amounts of persistent release were

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Comparison of transient and persistent dopamine release in four brain regions by curve fit method</th>
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<tbody>
<tr>
<td><strong>Value</strong></td>
<td><strong>Transient Release (( V_T ))</strong></td>
</tr>
<tr>
<td>NA</td>
<td>3.36 ± 0.08</td>
</tr>
<tr>
<td>FC</td>
<td>1.44 ± 0.05</td>
</tr>
<tr>
<td>ST</td>
<td>2.16 ± 0.04</td>
</tr>
<tr>
<td>OT</td>
<td>4.37 ± 0.07</td>
</tr>
</tbody>
</table>
found. These results suggest that all four brain regions express the same, or very similar, nAChRs, the ratios of which may vary slightly across the four brain regions.

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References


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