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Expression of muscarinic acetylcholine and dopamine receptor mRNAs in rat basal ganglia

(*in situ* hybridization/striatum/substantia nigra/D2 dopamine receptor/receptor subtype)

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ABSTRACT **Within the basal ganglia, acetylcholine and dopamine play a central role in the extrapyramidal control of motor function. The physiologic effects of these neurotransmitters are mediated by a diversity of receptor subtypes, several of which have now been cloned. Muscarinic acetylcholine receptors are encoded by five genes (m1–m5), and of the two known dopamine receptor subtypes (D1 and D2) the D2 receptor gene has been characterized. To gain insight into the physiological roles of each of these receptor subtypes, we prepared oligodeoxynucleotide probes to localize receptor subtype mRNAs within the rat striatum and substantia nigra by *in situ* hybridization histochemistry. Within the striatum, three muscarinic (m1, m2, m4) receptor mRNAs and the D2 receptor mRNA were detected. The m1 mRNA was expressed in most neurons (>80%); the m2 mRNA, in neurons which were both very large and rare; and the m4 and D2 mRNAs, in 40–50% of the neurons, one-third of which express both mRNAs. Within the substantia nigra, pars compacta, only the m5 and D2 mRNAs were detected, and most neurons expressed both mRNAs. These data provide anatomical evidence for the identity of the receptor subtypes which mediate the diverse effects of muscarinic and dopaminergic drugs on basal ganglia function.**

The maintenance of a balance between cholinergic and dopaminergic tone within the basal ganglia has long been appreciated as being central to the clinical management of many extrapyramidal motor disorders (1–3). For example, muscarinic antagonists and dopamine agonists have both been used in the treatment of Parkinson disease (2, 3). Unfortunately, both types of drugs exert many untoward side effects (2), particularly in later phases of the disease. The recent discovery of a heterogeneity of muscarinic and dopaminergic receptor subtypes has led to the suggestion that these subtypes may mediate distinct aspects of cholinergic and dopaminergic function. On the basis of pharmacologic data, muscarinic receptors have been divided into three subtypes (M1, M2, and M3) (4) and dopaminergic receptors into two (D1 and D2) (5, 6). Molecular cloning efforts have identified five genetically distinct muscarinic receptor subtypes (m1–m5) (7–10). Functional expression of these genes has indicated a correlation between the genetically and pharmacologically defined subtypes, where the M1 = m1, m4, and m5; the M2 = m2; and the M3 = m3 (11). A dopamine D2 receptor has also recently been cloned (12–15). Because the available pharmacologic tools do not discriminate among all the receptor subtypes, and due to the limited anatomic resolution that receptor autoradiographic procedures allow, we have prepared oligodeoxynucleotide probes to determine which cells within the basal ganglia express each receptor subtype mRNA.¶ These data should provide a rational basis for the development of subtype-selective drugs for the management of movement disorders.

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MATERIALS AND METHODS

Oligodeoxynucleotide Probes. Three 48-base oligodeoxynucleotide probes for each of the five muscarinic receptor mRNAs were made on an Applied Biosystems automated DNA synthesizer. These probes were designed to hybridize to both the rat and human receptor mRNAs with subtype specificity. To ensure specificity and cross-species hybridization, every probe fulfilled the following two requirements: fewer than 4 base mismatches between the rat and human genes and greater than 20 mismatches between subtypes. The sequences of the muscarinic probes are as follows; bases refer to rat sequences (complete sequences are reported in m1, GenBank accession no. M16406; m2, ref. 16; m3, no. M16407; m4, no. M16409; and m5, ref. 8): m1A, bases 189–236 (5′-TGG TGA TCC CGA TGA AGG CCA CCT GCC AGG GAC CCT TTC CTG GTG CCA-3′); m1B, bases 827–874 (5′-TGA CCT CTC TGA GCT GCT GCT GCT GCC ACC ACC TTT GCC TGG TGT CTC-3′); m1C, bases 1440–1487 (5′-GGC GCT TGG GGA TCT TGC GCC AGC GCC TCT TGT CCC AGC GGC AGA GCA-3′); m2a, bases 45–93 (5′-TCC AGC CAC AAG GAC AAT AAA TAC CAC TTC AAA TGT CTT GTA AGG ACT-3′; m2B, bases 243–290 (5′-TCA CAT ACT ACA GGT CCC AAA GGC CAG TAG CCA ATC ACA GTG TAG AGG-3′); m2c, bases 1126–1173 (5′-AGC CAA GAT TGT CCT GGT CAC TTT CTT TTC CCG GGA TGG TGG AGG CTT-3′); m3A, bases 42–89 (5′-TGA TGT TGG GAA ACA AAG GCG AGG TTG TAC TGT TAC TGT GCA AGG TCA-3′); m3B, bases 1396–1443 (5′-AGA GCA AAC CTC TTA GCC AGC GTG GCC TCC TTG AAG GAC AGA GGT AGA-3′); m3c, bases 1746–1783 (5′-GTG AAA AAT GAC CGA CTG TCT CTG CTG GTA CTG CTG TTT GCG CCT CTT-3′); m4A, bases 686–733 (5′-GCT CTT GAG GAA AGC CAG AGT CTT GGC CTT CTT CTC CTT GGG GCC CTC-3′); m4B, bases 868–915 (5′-CGT TCC TTG GTG TTC TGG GTG GCA CTG CCT GAG CTG GAC TCA TTG GAA-3′); m4C, bases 1683–1730 (5′-TTT GTA GAG CCA CTG CCC ACT CCA GCC ATT GTC CCC CAT CTT CCT GAG-3′); m5A; bases 112–159 (5′-GGA GAT CAT GAC CAA GAC ATT GCC GAC AAT GGT CAT CAG GCT GAC CAC-3′); m5B, bases 1110–1157 (5′-GCT TTT ACC ACC AAT CGC AAC TTA TAG GCA ACA CAC TTC TGA CTC TTG-3′); m5C, bases 1512–1559 (5′-TCT TCT ACT TTT TTC TTT TTC CAC CGG CAG AGA AGA AGC AGC TTA AAG-3′). The three probes to the dopamine D2 receptor and the three probes to the α

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¶The sequences of probes reported in this paper are complementary to sequences that have been deposited in the GenBank data base (accession numbers: m1, M16406; m3, M16407; and m4, M16409).

subunit of transducin have been described previously (14). The rat and human D2 receptor genes have been shown to encode two alternatively spliced forms of the receptors that differ only by an additional 29 amino acids within their third cytoplasmic loop (13, 15). The probes used in our study do not distinguish between these two forms. Mixtures of the three probes for any given receptor mRNA were 3'-end labeled (average tail length 10–15 bases) by using terminal deoxynucleotidyltransferase (Bethesda Research Laboratories) and deoxyadenosine 5'-[α-thio]triphosphate labeled with ^{35}S (>1000 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq).

***In Situ* Hybridizations.** Tissue preparation and *in situ* hybridization procedures were similar to those previously described (17, 18). Male Sprague–Dawley rats (200–250 g, Taconic Farms) were sacrificed by decapitation, and their brains were removed and frozen on dry ice. Twelve- and 6-μm frozen sections were cut and mounted onto gelatin-coated slides. The *in situ* hybridizations were performed with a probe concentration of $\approx 9 \times 10^6$ dpm/50 μl of hybridization buffer [4× SSC (1× = 0.15 M NaCl/0.015 M sodium citrate, pH 7.2), 50% (vol/vol) formamide, 1× Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), sheared single-stranded salmon sperm DNA (ssDNA) at 250 μg/ml, 100 mM dithiothreitol, and 10% dextran sulfate] per slide. After overnight incubation at 37°C in a humid chamber, the slides were washed four times for at least 30 min at 57°C and then twice for at least 45 min at room temperature in 1× SSC. Some of the sections were placed directly against film for 5 weeks, while others were dipped in a photographic emulsion and exposed for various times (for 12-μm sections, the m1 and D2 for 4 weeks, the m2 and m5 for 6 weeks, and the m3 and m4 for 7 weeks; all the 6-μm sections for 12 weeks). Dipped sections were counterstained with 0.2% toluidine blue in water.

Cell Counting and Colocalizations. Positive cells were evaluated by inspection, with a minimum of 4–5 times more grains observed over positive than negative cells. In an attempt to limit the counting of nonneuronal cell populations, only those cells that had a diameter >5 μm were included. Because the receptor mRNAs were unevenly distributed to the striatum, all cell counts were performed along a 200-μm-wide (medial to lateral) and 1.6- to 2.0-mm-long (dorsal to ventral) column in the center of the caudate-putamen at the coronal level shown in Fig. 1. For the substantia nigra, pars compacta, only cells in the medial aspect of this structure were analyzed. Two independent mRNAs were localized to the same cell by performing *in situ* hybridizations to consecutive 6-μm sections. Cells were visualized by using the IMAGE program (W. Rasband, National Institutes of Health) run on a Macintosh II. Images were printed onto transparencies and overlays were analyzed visually. Only cells which could be reliably visualized on both sections (>80% of their respective Nissl-stained cell bodies overlapped) were counted.

RESULTS

Oligodeoxynucleotide probe specificity was verified by three criteria. First, each of the three probes to a given receptor

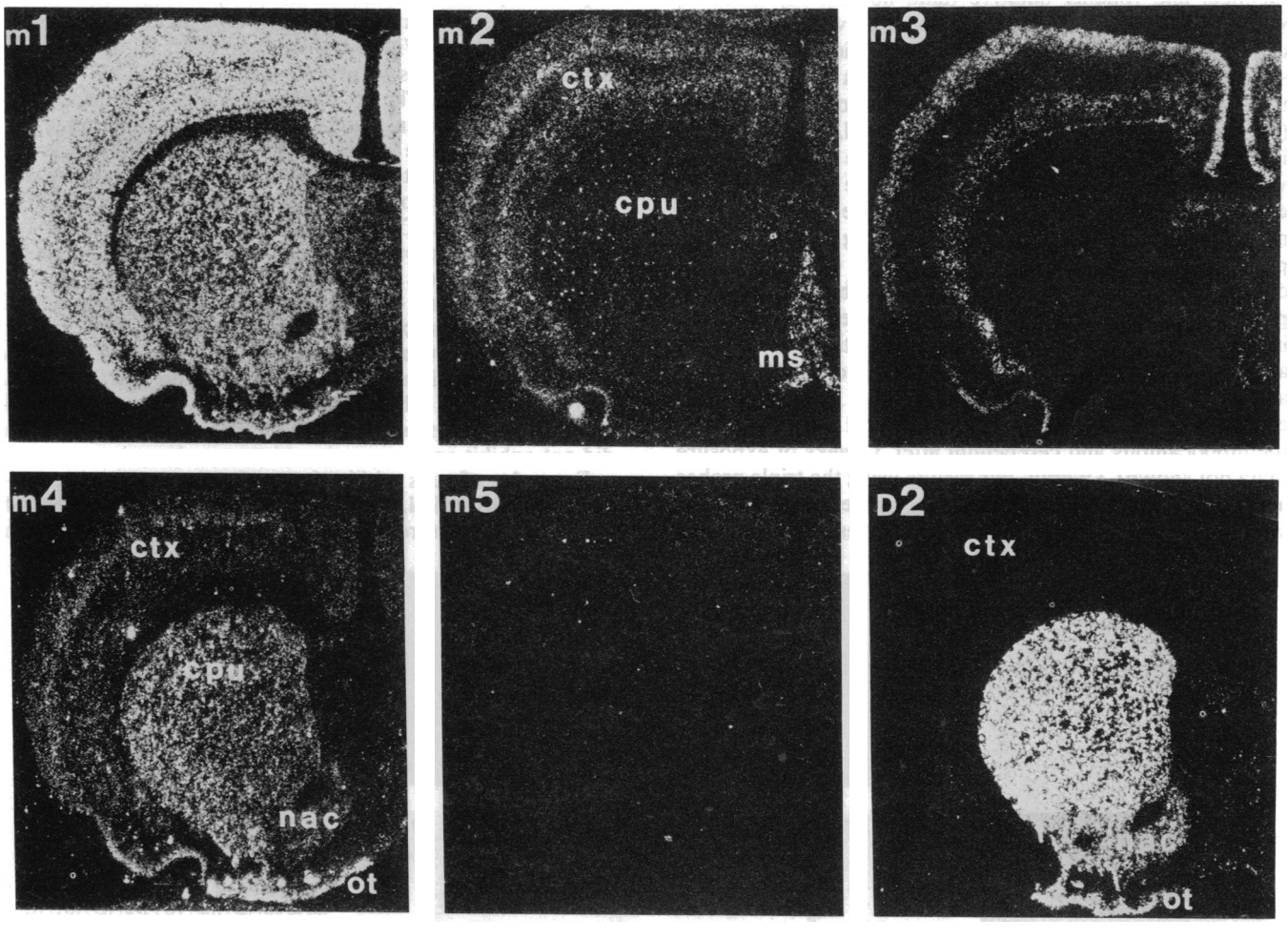

FIG. 1. Expression of the muscarinic and dopamine receptor mRNAs in the rat forebrain. mRNA was autoradiographically localized by exposure of 12-μm coronal sections to x-ray film. In the dark-field photomicrographs the positive regions appear white. All the coronal sections were taken from approximately the same anatomic level in the brain. (×8.) ctx, Cerebral cortex; cpu, caudate-putamen; ms, medial septum; nac, nucleus accumbens; ot, olfactory tubercle.

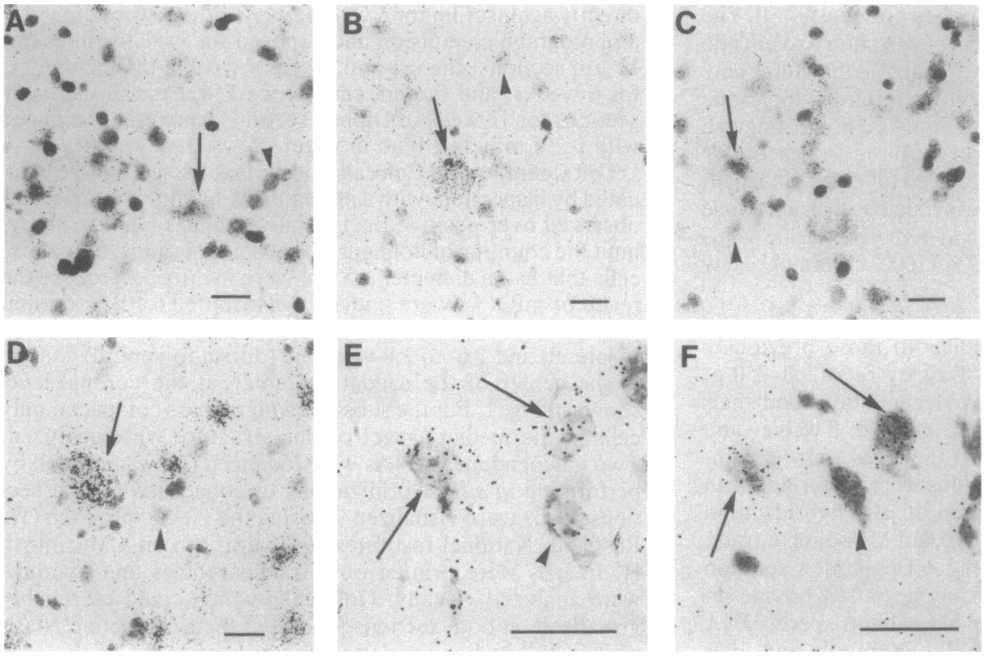

FIG. 2. Cellular localization of receptor mRNAs within the striatum. mRNA was localized autoradiographically in emulsion-dipped coronal sections. All the panels are bright-field photomicrographs, in which the autoradiographic grains appear dark. (Scale bars all represent 20 μm.) Twelve-micrometer sections are illustrated for m1 (*A*), m2 (*B*), m4 (*C*), and D2 (*D*). For the colocalizations of m4 (*E*) and D2 (*F*) mRNAs in the same cells, 6-μm sections were used. Arrows indicate positive cells, and arrowheads indicate negative cells. In *E* and *F*, only positive cells are represented on both sections.

was used individually for *in situ* hybridizations, and each showed an identical pattern of labeling. When a mixture of the three probes was used, the distribution of the signal was identical and roughly additive (data not shown). Second, when the mixture of the three probes for a given receptor was used for Northern blots, the probes hybridized to mRNA species with an anatomic pattern analogous to the results of the *in situ* hybridizations. The m1 [3.1-kilobases (kb)], m2 (7.1-kb), m3 (4.7-kb), and m4 (3.6-kb) mRNAs were all seen in the cortex and hippocampus, yet only the m1 and m4 were visualized in striatum (data not shown). The m5 has yet to be detected by Northern blot analysis. The sizes of these mRNAs agree well with those seen using different single probes (19, 20). The D2 probes have been described previously (17). Third, to test for background hybridization, three probes to the α subunit of transducin, a protein not expressed in brain, were used for *in situ* hybridizations to sections adjacent to those utilized for the receptor mRNAs. No appreciable background was seen in any brain region included in this study, and only a slight signal was observed in the hippocampus and cerebellum after 5 weeks of exposure (data not shown). Overall, our results using the triple probes are entirely consistent with previous studies on the expression of muscarinic receptor subtype genes using different single probes (19, 20). The major difference is an increase in sensitivity and decrease in autoradiographic times.

The only structures within the basal ganglia observed to express dopamine and muscarinic receptor mRNAs were the striatum, the substantia nigra, pars compacta, and the subthalamic nucleus. Within the striatum (Fig. 1), the m1, m2, m4, and D2 mRNAs were detected, while the m3 and m5 mRNAs were not. The m1 mRNA was observed throughout the caudate-putamen, nucleus accumbens, and olfactory tubercle. Within the caudate-putamen, m1 mRNA was expressed in a lateral-to-medial gradient. The m2 mRNA was found in a few cells located mainly in the lateral aspects of the caudate-putamen. The m4 and D2 mRNAs, like the m1 mRNA, were expressed throughout the caudate-putamen, nucleus accumbens, and olfactory tubercle. Unlike the m1 mRNA, the D2 and m4 mRNAs showed a medial-to-lateral gradient of expression. The m1 and the m4 mRNAs, but not the D2 mRNA, were seen in the islands of Calleja [this structure was misidentified in our earlier report on D2 mRNA (17)]. Within the subthalamic nucleus only the m3 and m4 mRNAs were expressed, while the entopeduncular nucleus did not exhibit any expression (data not shown).

Examples of cells within the caudate-putamen which express receptor mRNAs are shown in Fig. 2. The m1 and m4 mRNAs were expressed in medium-sized (10–15 μm in

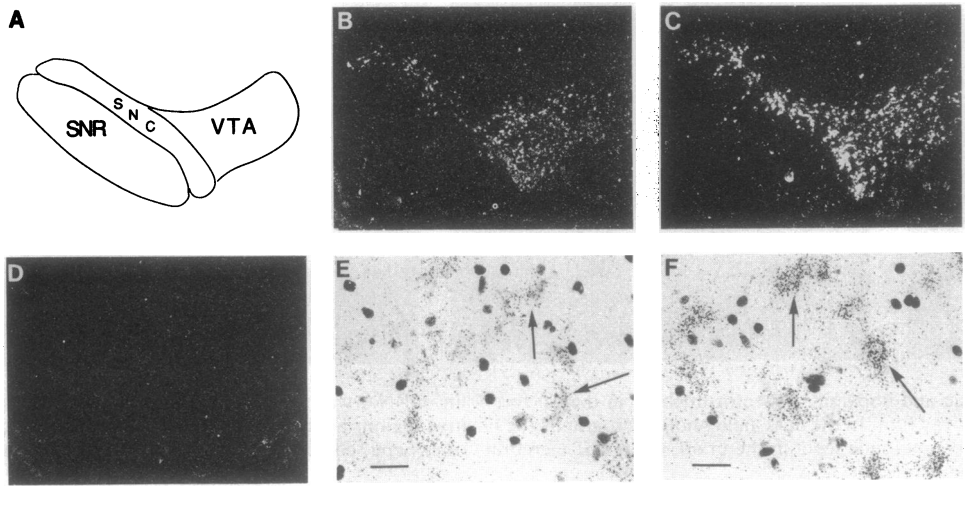

FIG. 3. Localization of muscarinic and dopamine receptor mRNAs in the mesencephalon. mRNA was localized autoradiographically in emulsion-dipped sections. (*A*) Sketch of the anatomic structures shown in the dark-field photomicrographs of 12-μm coronal sections of *B–D*. SNR, substantia nigra, pars reticulata; SNC, substantia nigra, pars compacta; VTA, ventral tegmental area. (*B*) m5. (*C*) D2. (*D*) m1. (*E* and *F*) Higher-power bright-field photomicrographs of m5 (*E*) and D2 (*F*). Arrows indicate positive cells. (Scale bars represent 20 μm.)

diameter) neurons, with little or no expression in the rare large (>20 μm) neurons. The m2 mRNA was expressed almost exclusively in the large neurons, while D2 mRNA was expressed in both the medium-sized and large neurons. On a per cell basis, the D2 mRNA was more abundant than the muscarinic receptor mRNAs. There was no expression of any receptor subtype mRNA in glial-like cells (<5 μm), but because of the histologic limitations of fresh frozen tissue, precise cellular morphology was difficult to assess.

The m5 and D2 were the only receptor mRNAs observed in the substantia nigra, pars compacta, and ventral tegmental area (Fig. 3). Within these regions the mRNAs were present in nearly all of the large cells. These cells have previously been shown to be dopaminergic (18, 21). The globus pallidus, as well as the substantia nigra, pars reticulata (Fig. 3*C*) had an occasional cell that expressed the D2 mRNA. Similar to its higher levels of expression in the striatum and other brain regions, the D2 mRNA was more abundant than the m5.

Cell counts in the caudate-putamen revealed that the m1, m2, m4, and D2 mRNAs were expressed in 81%, 3.5%, 42%, and 46% of the neurons, respectively (Table 1). Of the cells expressing D2 mRNA, all cells expressed m1 mRNA and 37% expressed m4 (Table 2). There was a discrepancy in the frequency of cells that expressed the m1 mRNA between the 12-μm and 6-μm sections (81% and 99%, respectively). The smaller, perhaps glial, cells not expressing the m1 mRNA are not large enough to be frequently represented in two consecutive thin sections. Also, cells not expressing any mRNA are more difficult to visualize in thin sections. Examples of two coincidental cells that expressed both the m4 and D2 mRNAs are shown in Fig. 2 *E* and *F* (arrows). Also shown are noncoincident negative cells (arrowheads). Within the substantia nigra, pars compacta, the m5 and the D2 mRNAs were colocalized, with nearly all cells expressing both mRNAs.

DISCUSSION

Using probes based on the sequences of five muscarinic receptors (7–10) and a dopamine D2 receptor (12–15), we used *in situ* hybridization histochemistry to localize these receptor mRNAs in the rat basal ganglia. Overall, these receptor mRNAs are expressed on distinct yet partially overlapping neuronal populations. These data provide evidence for the identities of the receptor subtypes that are involved in different aspects of the cholinergic/dopaminergic interactions that form the basis of the clinical management of extrapyramidal motor dysfunction.

Convergence of cholinergic and dopaminergic neurotransmission within the basal ganglia occurs in the striatum. The striatum receives a topographically organized dopaminergic projection from cells in the substantia nigra, pars compacta, and the ventral tegmental area (21). The cholinergic innervation of the striatum is intrinsic and originates from a small number (<3%) of the large neurons (22, 23). Another potential, although less likely, site of interaction between acetylcholine and dopamine is in the substantia nigra. The pedunculopontine tegmental nucleus, which contains cholinergic cells, has been reported to have reciprocal connections with the substantia nigra and other structures of the basal ganglia (24). More recent studies, however, have failed to identify a significant cholinergic innervation of these extrapyramidal structures. Rather, a population of noncholinergic cells admixed with the cholinergic cells form the extrapyramidal connections (25).

Table 1. Percentage cells expressing receptor mRNAs in caudate-putamen

mRNA	% cells	
	Brain A	Brain B
m1	78 ± 6	84 ± 8
m2	3.6 ± 4	3.4 ± 4
m4	40 ± 8	45 ± 7
D2	45 ± 5	47 ± 8

Sections were 12 μm. All values are means of data collected from individual fields ± SD (n = number of fields); 323–445 cells in 13–16 fields were counted.

Table 2. Percentage of cells in caudate-putamen (CPU) and substantia nigra, pars compacta (SNC), which coexpress receptor mRNAs

Region	mRNA	% express	% coexpress
CPU	m1	99 ± 2	100 ± 0
	D2	43 ± 9	
	m4	44 ± 12	37 ± 16
	D2	48 ± 9	
SNC	m5	99 ± 2	99 ± 2
	D2	100 ± 0	

Sections were 6 μm. Coexpression values are percentages of D2-positive cells which express the respective muscarinic receptor mRNA. Data are presented as in Table 1; 90–113 cells in 5–8 fields were counted for each determination.

Cholinergic and dopaminergic terminals within the striatum contain presynaptic muscarinic and dopaminergic receptors, respectively, that inhibit neurotransmitter release (26). The observation of D2 mRNA in the dopaminergic neurons of the mesencephalon is consistent with this gene encoding the dopamine D2 receptors, which presynaptically control dopamine release in the striatum (26–28) and inhibit the firing of dopamine neurons in the substantia nigra, pars compacta (29). The finding of m2 mRNA in the large striatal neurons, which are likely to be cholinergic, is consistent with this gene encoding the muscarinic receptor, which presynaptically controls acetylcholine release. These large neurons are probably cholinergic because their size, abundance, and distribution are consistent with those known to contain choline acetyltransferase (ChoAT) by immunocytochemical methods (22, 23), as well as those that express ChoAT mRNA (unpublished observation). Further, the m2 mRNA is the muscarinic receptor subtype mRNA that is expressed in cholinergic neurons in other regions of the rat brain (unpublished observations). Finally, cholinergic drugs inhibit the release of acetylcholine in various brain regions with a M2 pharmacology (30), and binding studies have demonstrated the presence of receptors with a M2 pharmacology in the striatum (31, 32).

Muscarinic receptors directly stimulate and indirectly inhibit the release of dopamine from striatal terminals (33). Since dopaminergic neurons express m5 mRNA, it is likely that this receptor mediates the direct stimulation of dopamine release. In support of this possibility, muscarinic receptors which stimulate dopamine release have a M1-like pharmacology (30, 34). The muscarinic receptor subtype which indirectly inhibits dopamine release is not known; however, a role for γ-aminobutyric acid (GABA) neurons has been suggested in this response (33). Since the majority of striatal neurons synthesize GABA, and since the m1 and m4 receptors are so widely distributed in the striatum, GABA neurons express the m1 and m4 receptors.

Receptor binding studies have indicated that dopamine D2 receptors are synthesized by neurons located within the striatum (35, 36). Since the D2 mRNA is expressed by cells within the striatum, and the distribution of the mRNA is in general agreement with the topography of binding sites, this gene is likely to encode these postsynaptic receptors. Because the large, presumably cholinergic, striatal neurons express D2 mRNA, and since D2 receptors directly inhibit the release of acetylcholine from striatal nerve terminals (26, 37), it is likely that this gene encodes for these receptors. This gene has also recently been shown to be expressed in enkephalinergic neurons within the striatum (38).

Determining the site at which muscarinic antagonists enhance dopamine-mediated psychomotor behavior is particularly important, as this is the site at which antimuscarinic drugs are likely to exert their therapeutic activity in the treatment of Parkinson disease (39). The m5 receptor is excluded, because the active site is known to be postsynaptic with respect to dopaminergic neurons (40) and because blockade of m5 receptors is likely to inhibit rather than enhance dopaminergic neurotransmission. That leaves the m1, m2, and m4 receptors as likely candidates. Trihexyphenidyl, a muscarinic antagonist which is widely used in the treatment of Parkinson disease, is more potent at m1 and m4 receptors than at the other muscarinic receptors (unpublished observations). If the active site is colocalized with the D2 receptor in the striatum, one would expect that it would exert an opposing cellular effect to the D2 receptor. Since both the m1 and m4 receptors are colocalized with D2 receptors in the striatum, the m1 receptor is the more likely candidate because, unlike the m2 and m4 receptors, it mediates such an opposing effect in transformed cells.

It is clear that different subtypes of muscarinic receptors exert opposing effects on nigrostriatal dopaminergic function, and thus subtype-selective drugs may allow for a more selective manipulation of the function of this pathway. Our data suggest the m1 receptor is the most likely site at which nonselective muscarinic antagonists enhance dopamine-mediated psychomotor behavior. Unfortunately, this receptor may be a poor candidate for future drug development efforts because it is also concentrated in the cerebral cortex and hippocampus, brain regions likely to mediate the adverse cognitive effects of muscarinic antagonists. On the other hand, selective blockade of m5 receptors is likely to inhibit dopaminergic function. Thus, antagonists of this receptor may have clinical utility in the treatment of disorders characterized by an elevated dopaminergic tone (schizophrenia and Tourette syndrome). Since the m5 receptor has a very limited expression in nondopaminergic neurons, m5-selective antagonists are likely to induce fewer untoward side effects.

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