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D_2-Dopamine Receptor-Mediated Inhibition of Cyclic AMP Formation in Striatal Neurons in Primary Culture

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SUMMARY

Dopamine (DA) regulation of intracellular cyclic AMP formation in purified, intact striatal neurons in primary culture was examined. DA (EC_{50}, 3 μM) and vasoactive intestinal polypeptide (VIP; EC_{50}, 10 nM) stimulated cyclic AMP formation by 2- and 5-fold, respectively. In the presence of 0.1 μM forskolin (which was virtually ineffective alone), neurohormone efficacy was augmented; potency was unaffected. In the presence of 0.1 μM SCH 23390, a selective D_1 antagonist, the DA dose-response curve was shifted rightward in a competitive manner. At low concentrations (0.01–1.0 μM), however, DA inhibited basal cyclic AMP formation. The inhibitory effect, but not the shift of the dose-response curve, was blocked by 5 μM *l*-sulpiride, a selective D_2 antagonist. At saturating concentrations of VIP (0.1–1.0 μM), no other neurohormone can further augment cyclic AMP formation. Under these conditions, increasing concentrations of DA resulted in a dose-dependent (IC_{50}, 0.5 μM) inhibition of VIP-stimulated cyclic AMP synthesis. This effect was augmented in the presence of 0.1 μM SCH 23390 and blocked by 5 μM *l*-sulpiride. Sulpiride antagonism was stereospecific, with the *l*-isomer being 30-fold more potent than the *d*-isomer. The rank order of potency for a series of dopaminergic agonists and antagonists at the receptor mediating attenuation of cyclic AMP formation suggests that it is of the D_2 type. Furthermore, both DA and Met-enkephalin inhibition of cyclic AMP formation is lost after exposure of striatal neurons to islet activator protein. These findings suggest that a D_2 receptor mediates the inhibition of intracellular cyclic AMP formation by DA in striatal neurons in primary culture, and may do so by an interaction with the inhibitory guanine nucleotide regulatory protein of adenylate cyclase.

INTRODUCTION

A wide body of evidence suggests the existence of multiple receptive sites for DA in the central nervous system (1). These sites may be generally grouped into two categories (2). The D_1 site is linked to the enzyme adenylate cyclase and mediates the enhanced formation of cyclic AMP. Although these sites may be involved in behavioral patterns such as locomotion (3), their affinities for a series of antagonists do not correlate with the therapeutic efficacy of these drugs in affective disorders of ascending dopaminergic pathways. The D_2 site, whose mechanism of action has remained unclear, appears to be the site of action for neuroleptic drugs and has thus been termed the DA receptor involved in the pathology of psychotic diseases, such as schizophrenia (1). Furthermore, it is likely that D_2 but not D_1 receptors are involved in the regulation of cholinergic neurotransmission in the striatum (4, 5). In the pituitary gland, a D_2 receptor mediates the attenuation of both hormone release and cyclic AMP formation (6–8).

Previous investigations of DA actions in the striatum examining cyclic AMP efflux from tissue slices (5, 9) have suggested a negative coupling of the D_2 receptor to adenylate cyclase. These studies were limited, however, by their inability to (a) observe the D_2 inhibition without prior activation of the D_1 site, (b) determine the cell type (glial or neuronal) for the observed effect, or (c) provide direct evidence for a change in intraneuronal cyclic AMP levels. More recently, a preliminary communication by Onali and co-workers (10) reported that, in homogenates of rat striata, high concentrations of DA resulted in the attenuation of basal adenylate cyclase activity. We have developed a technique whereby the intracellular formation of cyclic AMP can be measured in intact neurons of the striatum, isolated from non-neuronal cell types. The regulation of cyclic AMP formation by dopaminergic

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drugs and other neurohormones endogenous to the striatum was examined.

MATERIALS AND METHODS

Agents used in this study were obtained from the following sources: dopamine hydrochloride, apomorphine hydrochloride, norepinephrine bitartrate, serotonin creatinine sulfate, isobutylmethylxanthine, ATP, and cyclic AMP (Sigma Chemical Co., St. Louis, MO); vasoactive intestinal polypeptide and methionine-enkephalin (Peninsula Laboratories, San Carlos, CA); forskolin (Calbiochem-Behring, La Jolla, CA). The following compounds were gifts: (+)- and (−)-butaclamol (Ayerst Laboratories); (+)- and (−)-sulpiride (Delagrange); LY 141865 (Eli Lilly Co.); spiroperidol (Janssen Pharmaceuticals); haloperidol and chlorpromazine (Rhône-Poulenc); CB 154 (Sandoz); SCH 23390 (Schering Corp.); SKF 38393 (Smith, Kline and French); and fluphenazine (Squibb).

Preparation of neurons in primary culture. Primary neuronal cultures were generated essentially as described by Prochiantz *et al.* (11). Briefly, cortical or striatal structures were removed from 15–16-day-old Swiss Albino mouse embryos (Iffa Credo, Lyon, France), mechanically dissociated with a fire-narrowed Pasteur pipette, and plated (1×10^6 cells/ml) in 12-well Costar (Cambridge, MA) culture dishes previously coated with poly-(L-ornithine) (1.5 μg/ml, M_r = 40,000, Sigma). The culture medium was a 1:1 mixture of minimal essential medium and F-12 nutrient (Gibco Europe, Paris, France), and included glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), and Hepes[1] buffer (5 mM), all obtained from Sigma. In the place of serum, a defined hormone and salt mixture that included insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), all from Sigma, and selenium salt ($NaSeO_3$; 30 nM) was added. With this technique, after 6 days, these cultures were immunocytochemically and morphologically defined as virtually purified neurons in nature (11).

Cyclic AMP determination. Intracellular cyclic AMP levels were determined by measuring the conversion of [^{3}H]adenine nucleotide precursors (primarily [^{3}H]ATP) to cyclic [^{3}H]AMP. On the sixth day in culture, neurons were washed and incubated at 37° (5% CO_2-air mixture) with culture media containing 2 μCi/ml [^{3}H]adenine (24 Ci/mmol; Amersham). After 2–3 hr, the cultures were washed and incubated with 0.75–1.00 mM isobutylmethylxanthine and test agents (all in culture media) in a volume of 1 ml for 5 min at 37°. The reaction was terminated by aspiration of the media and addition of 1 ml of ice-cold 5% trichloroacetic acid. Cells were scraped with the aid of a rubber policeman and to the mixture was added 100 μl of cold 5 mM ATP and cyclic AMP. Cellular protein was centrifuged at 5000 × *g* and the supernatant was eluted through sequential chromatography on Dowex and alumina columns (7). Cyclic AMP formation is expressed as:

$$\% \text{ Conversion} = \frac{\text{cyclic } [^3\text{H}]\text{AMP}}{\text{cyclic}[^3\text{H}]\text{AMP} + [^3\text{H}]\text{ATP}} \times 100$$

Values are the means of duplicate determinations (that varied by less than 5%) on at least two separate neuronal culture preparations. When three separate cultures were examined, standard errors were determined.

RESULTS

Preliminary studies of cyclic AMP formation in striatal neurons. In preliminary experiments, it was determined that hormone-stimulated cyclic AMP formation in neurons was linear with time up to 10 min after exposure to agonist (data not shown). Therefore, 5-min incubations were used throughout. Exposure of striatal neurons to 5-HT (EC_{50}, 5 μM), DA (EC_{50}, 3 μM), and VIP (EC_{50}, 10 nM) resulted in a dose-dependent 1.2-, 2-, and 5-fold stimulation of cyclic AMP formation, respectively (Fig. 1A). Forskolin is a ubiquitous activator of cyclic AMP-generating systems in both broken and intact cells, also capable of potentiating hormonal activation of adenylate cyclase. In striatal neurons, forskolin induced up to 30-fold stimulated cyclic AMP formation at 100 μM (Fig. 1B). Furthermore, in the presence of 0.1 μM forskolin, which had virtually no effect on cyclic AMP formation alone, neurohormone efficacy was augmented 2-fold while potency was unaffected. Thus, forskolin effects on striatal neurons are similar to those ascribed to homogenates or intact slices of brain tissue.

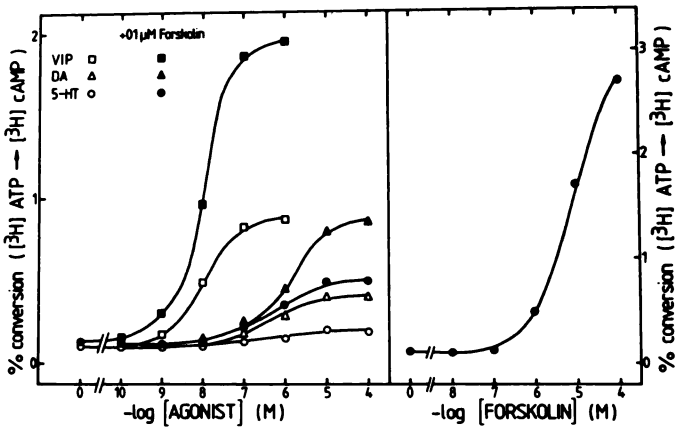

FIG. 1. *Effects of 5-HT, DA, VIP, and forskolin on cyclic AMP formation in striatal neurons*

Striatal neurons were exposed to increasing concentrations of (A) 5-HT (○, ●), DA (△, ▲), or VIP (□, ■) in the absence (open symbols) and presence (closed symbols) of 0.1 μM forskolin, and (B) forskolin (●). Cyclic AMP formation was determined as described in Materials and Methods.

Stimulation of the D_1 site on striatal neurons by DA resulted in a 3.5-fold increase in cyclic AMP levels; half-maximal stimulation (EC_{50}) was obtained with 3 μM DA (Fig. 2A). In the presence of 0.1 μM SCH 23390, a reported selective D_1 antagonist (12), the dose-response curve for DA was shifted to the right as expected. However, at low DA concentrations (0.1–1.0 μM), a significant reduction (35–40%) of basal levels of cyclic AMP formation was observed. When 5 μM *l*-sulpiride, a selective D_2 antagonist was added, the inhibition at low concentrations was not observed, while the shift of the dose-response curve due to SCH 23390 was still visible, although less pronounced.

VIP is a potent stimulator of adenylate cyclase in neurons. In addition, VIP displays a unique property among stimulating hormones whereby saturating concentrations activate the enzyme to such a great extent that no other hormones may further augment enzyme activity in neuronal cell homogenates (13). We found a similar effect on cyclic AMP formation in intact striatal neurons; VIP stimulated cyclic AMP formation 15–20-fold (EC_{50}, 10 nM). At 100 nM VIP (near maximal dose), DA did not further activate but inhibited VIP-stimulated cyclic AMP formation by 25–30%; half-maximal inhibition (IC_{50}) was obtained with 0.5 μM DA (Fig. 2B). In the presence of 0.1 μM SCH 23390, DA inhibition was

[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; VIP, vasoactive intestinal peptide; DA, dopamine; IAP, islet activator protein; 5-HT, 5-hydroxytryptamine.

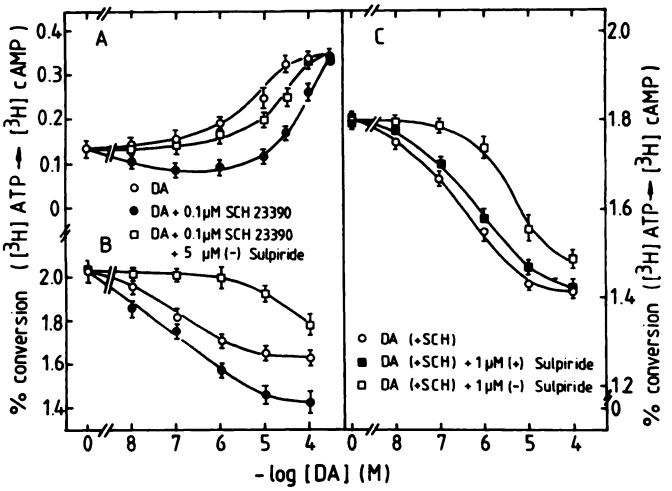

FIG. 2. *Dopamine effects on basal (A) and VIP-stimulated (B, C) cyclic AMP formation in striatal neurons*

A, neurons were exposed to increasing concentrations of DA, alone (○) or together with 0.1 μM SCH 23390 (●), or together with 0.1 μM SCH 23390 and 5 μM *l*-sulpiride (□). B, in the presence of 100 nM VIP, neurons were exposed to increasing concentrations of DA alone (○), or together with 0.1 μM SCH 23390 (●), or together with 0.1 μM SCH 23390 and 5 μM *l*-sulpiride (□). C, stereospecificity of sulpiride antagonism of dopaminergic inhibition. In the presence of 100 nM VIP and 0.1 μM SCH 23390, neurons were exposed to DA alone (○), or together with 1 μM *l*-sulpiride (□) or together with 1 μM *d*-sulpiride (■). Values are the means of three separate cultures ± standard error.

more pronounced (35–40%); however, the IC_{50} was unchanged. The selective D_2 antagonist *l*-sulpiride shifted the inhibitory DA dose-response curve to the right, in a competitive manner. Furthermore, sulpiride antagonism of DA-induced inhibition was stereospecific (Fig. 2C), with the *l*-isomer being 30-fold more potent than the *d*-isomer.

Effects of dopaminergic agonists and antagonists. A variety of dopaminergic drugs were examined for their ability to act as agonists or antagonists at the D_2 receptor mediating attenuation of cyclic AMP formation. The rank order of potency for antagonists (Table 1) correlated quite closely with those for the inhibition of [^{3}H]spiroperidol binding to striatal membranes (14) as well as with blockade of the D_2 receptor in the pituitary (7). Furthermore, specific D_2 agonists such as bromocryptine (IC_{50}, 5 nM) and LY 141865 (15) (IC_{50}, 50 nM) were 100 and 10 times more potent than DA, respectively, in inhibiting VIP-induced cyclic AMP formation (Fig. 3). Apomorphine (IC_{50}, 0.2 μM) was slightly more potent than DA, while the adrenergic neurotransmitter norepinephrine was more than 100-fold weaker. SKF 38393, a specific D_1 site agonist, was virtually ineffective at the D_2 receptor mediating inhibition of cyclic AMP production. Once again, the rank order of potency correlated very well with inhibition of [^{3}H]spiroperidol binding (14) and the D_2 receptor in the pituitary (7).

Islet activator protein effects on the inhibition of cyclic AMP formation in neurons. The stimulation and inhibition of adenylate cyclase appear to be mediated by distinct guanine nucleotide regulatory proteins N_s and N_i, respectively (16–18). A common β subunit appears to be

TABLE 1

Comparison between affinities (K_i apparent) of DA antagonists on DA attenuation of cyclic AMP formation and [^{3}H]spiroperidol binding in striatum and DA inhibition of adenylate cyclase in the pituitary gland

Antagonist	Striatum		Pituitary
	Cyclic AMP formation[a]	[^{3}H]Spiroperidol binding[b]	Adenylate cyclase[c]
	nM		
Butyrophenone			
Haloperidol	11.8	1.4	10.0
Spiroperidol	1.5	0.1	0.8
Phenothiazine			
Fluphenazine	20.0	2.8	16.0
Chlorpromazine	88.0	10.0	64.0
Benzamide			
(−)-Sulpiride	67.0	120.0	47.0
(+)-Sulpiride	2000.0	3000.0	2000.0
Miscellaneous			
(+)-Butaclamol	4.6	1.4	7.5
(−)-Butaclamol	4000.0	3000.0	5000.0

[a] Values [calculated as previously described (7)] are from this study and represent the means of duplicate determinations on two separate cultures.

[b] Values are taken from Leysen *et al.* (14) except for (+)-sulpiride, taken from Seeman (1).

[c] Values are taken from Enjalbert and Bockaert (7).

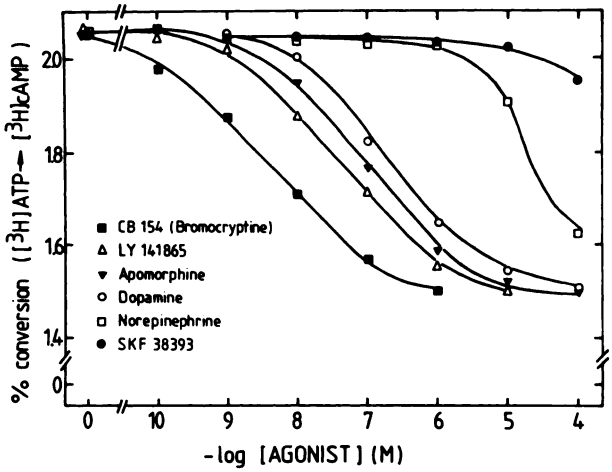

FIG. 3. *Effects of DA agonists on VIP-stimulated cyclic AMP formation*

In the presence of 100 nM VIP and 0.1 μM SCH 23390, neurons were exposed to increasing concentrations of bromocryptine (■), LY 141865 (△), apomorphine (▼), DA (○), norepinephrine (□), or SKF 38393 (●). Conditions are as described in the legend to Fig. 1. Values are the means of duplicate determinations on separate cultures that varied less than 5%.

the link and, therefore, the effector of the equilibrium between the two systems (19). The IAP from *Bordetella pertussis* selectively ADP ribosylates and inactivates N_i (20). IAP has been reported to uncouple inhibition mediated by hormone receptors in several tissues (21, 22). Thus, the effect of IAP pretreatment on opiate receptor- (23, 24) as well as D_2-mediated inhibition of cyclic AMP formation was examined. Incubation of striatal neurons with IAP (10 μg/ml) overnight resulted in a loss of the ability of Met-enkephalin to inhibit both forskolin- and VIP-stimulated cyclic AMP formation (Fig. 4). In fact,

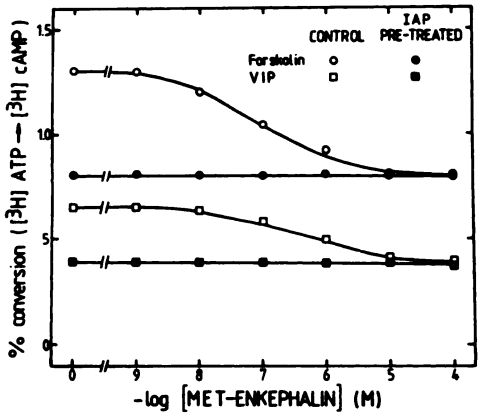

FIG. 4. *Effect of islet activator protein on Met-enkephalin inhibition of forskolin-(10 μM) and VIP- (100 nM) stimulated cyclic AMP formation*

Neurons were incubated overnight with vehicle (100 mM phosphate buffer, 2 M urea, pH 7.4; ●), or IAP (10 μg/ml; ○) for 18 hr. After this period, control and pretreated neurons were exposed to increasing concentrations of Met-enkephalin. The incorporation of [^{3}H]adenine into the [^{3}H]adenine nucleotide precursor pool as well as basal levels of cyclic AMP formation in pretreated cells were unchanged relative to control. Values are the means of duplicate determinations on two separate culture preparations.

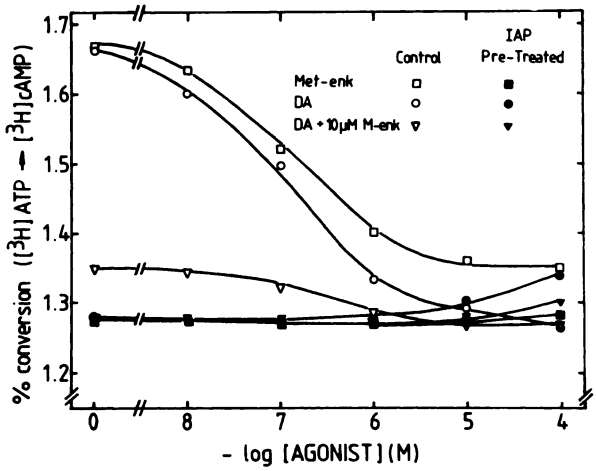

FIG. 5. *Effect of pretreatment with islet activator protein on dopamine and Met-enkephalin inhibition of VIP-stimulated cyclic AMP formation*

Neurons were treated as described in the legend to Fig. 4. Control and pretreated neurons were exposed to 100 nM VIP and increasing concentrations of dopamine (○, ●), Met-enkephalin (□, ■), or dopamine together with 10 μM Met-enkephalin (▽, ▼). Values are the means of duplicate determinations of two separate culture preparations.

IAP pretreatment resulted in a reduction of both forskolin- and VIP-induced cyclic AMP formation, analogous to the maximal opiate effect in untreated cells. DA inhibition of VIP-stimulated cyclic AMP formation was similarly affected by IAP (Fig. 5). These findings suggest that D_2 receptor inhibition may be mediated by N_i, the inhibitory guanine nucleotide regulatory protein of adenylate cyclase. In order to determine the possible relationship between DA and opiate receptors on striatal neurons, DA inhibition of cyclic AMP formation was examined in the presence of 10 μM Met-enkephalin. Under these conditions, DA elicited only a very slight further inhibition of VIP-stimulated cyclic AMP formation. The lack of additivity in DA and Met-enkephalin attenuation of cyclic AMP formation suggests that the D_2 and opiate receptors may be present on the same neuronal cell type in the striatum (Fig. 5).

DISCUSSION

While actions at D_2 DA receptors by neuroleptics correlate with their classical behavioral and clinical effects, the mechanism(s) by which such effects may be mediated have not been determined. Based on the results obtained in this study, we propose that D_2 receptor activation results in the attenuation of cyclic AMP formation in striatal neurons. The rank order of potency for dopaminergic agonists and antagonists (Table 1, Fig. 3) at this DA receptor on striatal neurons correlates very well with binding studies carried out with D_2-selective ligands (1, 14). The ability of IAP to uncouple D_2-mediated inhibition of cyclic AMP formation is in accord with this toxin's ability to modify interactions between the D_2 receptor and N_i in the striatum, as determined by binding studies which demonstrated a selective decrease in agonist affinity (25). Similar uncoupling of D_2 and N_i in the pituitary after treatment with IAP has been recently reported (22, 26). Taken together, these findings provide evidence for a direct relationship between the D_2 receptor and adenylate cyclase. It is of interest that incubation of striatal neurons with IAP resulted in a reduction of maximal hormone (VIP) response (Figs. 4 and 5). While in many tissues treatment with IAP has been noted to potentiate the response at hormone receptors positively linked to adenylate cyclase (27), in other tissues, such as the pituitary (26) and S49 wild-type cells (28), maximal hormone-stimulated adenylate cyclase was reduced after treatment with IAP. These findings suggest that a tissue specificity for N_i, or IAP interaction with N_i, may exist (28).

The pharmacology of the D_2 receptor attenuating cyclic AMP formation in striatal neurons is quite similar to that for the D_2 receptor that inhibits adenylate cyclase activity in the pituitary gland [Table 1; (14)]. In the pituitary, there is strong evidence for a role for cyclic AMP in the regulation of hormone release (7, 8, 22). However, it is still uncertain whether D_2 receptor-mediated inhibition of cyclic AMP formation is directly related to dopaminergic control of striatal cholinergic transmission (29). While the pharmacological responses are virtually identical, manipulation of cyclic AMP levels did not clearly alter cholinergic transmission in intact animals (4) or in striatal slices (5). Although these questions may be better answered with purified neuronal cell populations, it is possible that D_2 receptor activation results in other intracellular effects. One such effect may be the mobilization of calcium pools via inositol phospholipid hydrolysis, a mechanism that has been attributed to neurotransmitter receptors not positively linked to adenylate cyclase (30). In fact, it has recently been reported that some of the receptors whose activation results in inositol phospholipid hydrolysis also inhibit adenylate cyclase; muscarinic receptors (31) or angiotensin receptors (32) are of this class. This suggests a possible interaction between the two mechanisms or

further subclasses of these receptors. In addition, while our findings confirm a functional postsynaptic site for the D_2 receptor on striatal neurons, the precise role and mechanisms of action of DA receptors on presynaptic terminals innervating the striatum remain unclear.

The similarity between the effects at postsynaptic D_2 and opiate receptors on adenylate cyclase in the striatum is particularly striking, since both receptors also mediate inhibition of acetylcholine release (33). Furthermore, our findings (Fig. 5) suggest that these receptors may be found on the same cell type. The interrelationship between D_2 and opiate receptors in the regulation of intracellular (cyclic AMP and calcium) and subsequent physiological (acetylcholine release) events in the striatum may provide insights into the pathological mechanisms of central nervous system disorders. Isolated striatal neurons in primary culture may provide a suitable model, and a combined pharmacological and physiological analysis of neurotransmitter, cyclic nucleotide, and calcium effects on neuronal function is currently underway.

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