Differential regulation of neuropeptide mRNA expression in intrastriatal striatal transplants by host dopaminergic afferents

(enkephalin/substance P/6-hydroxydopamine/haloperidol/*in situ* hybridization)

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ABSTRACT The effects of dopamine-specific manipulations on neuropeptide gene expression in intrastriatal grafts of fetal striatal tissue were studied by quantitative *in situ* hybridization histochemistry, using ³⁵S-labeled oligonucleotide probes. Messenger RNA transcripts for the striatal neuropeptides preproenkephalin (PPE) and preprotachykinin (PPT) were detected in neurons forming discrete patches in the striatal grafts. The relative abundance of PPE and PPT mRNA-expressing neurons within the graft patches (51–54%) was similar to that found in normal caudate-putamen. In specimens with intact dopamine afferents the expression of PPE mRNA in grafted neurons was similar to that found in normal caudate-putamen, whereas the hybridization signal for PPT mRNA was 27% higher in the graft neurons than in the normal caudate-putamen. Removal of host dopaminergic afferents by 6-hydroxydopamine lesions of the ipsilateral mesostriatal dopamine pathway increased the hybridization signal for PPE mRNA both in the grafts (+84%) and in the spared ipsilateral host caudate-putamen (+125%), whereas the PPT signal was reduced by 53% in the grafts and by 51% in the remaining host caudate-putamen. Similarly, chronic treatment of grafted animals with the dopamine receptor antagonist haloperidol (2 mg/kg per day for 10 days) produced a 146% increase in the PPE signal in the grafts and a 175% increase in the intact contralateral caudate-putamen, whereas the signal for PPT mRNA was again decreased by 52% and 51% in the grafts and host caudate-putamen, respectively. These results show that the host nigrostriatal dopamine pathway differentially regulates enkephalin and substance P gene expression within striatal grafts and thereby exerts a tonic functional influence over grafted striatal neurons.

Grafts of fetal striatal tissue, implanted into the ibotenic acid-lesioned caudate-putamen of adult rats, develop into neuron-rich structures having morphological and neurochemical features that resemble, at least in part, those of the normal caudate-putamen (for review, see ref. 1). These grafts have been shown to integrate both anatomically and functionally with the host brain circuitry. In fact, grafted striatal neurons establish synaptic connections with the host globus pallidus (1) and, in turn, receive innervation from the principal striatal afferent systems (i.e., nigral dopaminergic, cortical and thalamic inputs) (1–5). The host dopamine (DA) afferents terminate specifically in those areas (or patches) of the graft that display striatum-like features (e.g., dopamine- and cyclic-AMP-related phosphoprotein, DARPP-32, and calbindin immunoreactivity) (4, 5). These striatum-like patches, which express both striosomal and matrix markers (4, 5), also contain the majority of efferent projecting graft neurons (5). Thus, all the components of a functional nigrostriato-pallidal circuit appear to exist in striatal grafts. In-

deed, data obtained from behavioral experiments examining drug-induced turning behavior in unilaterally lesioned and grafted animals support this idea (6, 7).

In recent years, *in situ* hybridization histochemistry (ISHH) has provided additional possibilities to monitor DA receptor-mediated regulation of striatal neuron function at the cellular level. In particular, enkephalin- and substance P-containing neurons, which represent the two major classes of striatal projection neurons (8, 9), are known to undergo long-lasting changes in response to DA denervation or receptor blockade. DA appears to differentially regulate peptide expression in the caudate-putamen, such that enkephalin-containing striatopallidal neurons are tonically inhibited and striatonigral substance P neurons are tonically activated by the dopamine afferents (8, 9).

In the present investigation, we have used quantitative ISHH to analyze the expression of the neuropeptide mRNAs encoding the enkephalin precursor preproenkephalin (PPE) and the substance P precursor molecule preprotachykinin (PPT) in fetal striatal grafts, in response to manipulations of the host-derived DA innervation of the grafts.

MATERIALS AND METHODS

Lesion and Transplantation Surgery. Nineteen female adult Sprague-Dawley rats (body weight, $\approx$ 250 g; Alab, Stockholm) were used. The animals were anesthetized with Equithesin (3 ml/kg of body weight) during all surgery. All animals received unilateral 14- μ g injections of ibotenate (Sigma; 10 μ g/ μ l in 0.1 M phosphate buffer, pH 7.4) divided over three injection sites in the head of the caudate-putamen as described (5). Seven to 10 days after lesioning, cell suspensions of fetal striatal tissue were prepared from the striatal primordia of rat embryos (gestational day 14–15) (crown-rump length, 12.5–14.5 mm) (5). Approximately 1 million cells (4–5 μ l of suspension) were implanted stereotactically into the lesioned caudate-putamen at two sets of coordinates: (i) A = 0.2, L = 3.0, V = 4.5 and (ii) A = 1.5, L = 2.5, V = 4.7, TB = -2.3.

6-Hydroxydopamine Lesion and Haloperidol Treatment. At more than 3 months after grafting, seven animals were subjected to a 6-hydroxydopamine (6-OHDA) lesion of the ascending DA pathway ipsilateral to the transplanted striatum as described (5). These animals were allowed to survive for 4 weeks before sacrifice. The lesions resulted in loss of tyrosine hydroxylase immunostaining in the striatum and tyrosine hydroxylase mRNA in the substantia nigra, as determined from selected sections (data not shown). In another group of animals (n = 6), daily haloperidol injections (Haldol, Janssen; 2 mg/kg per day i.p.) were carried out for 10 days prior to sacrifice, with a 24-hr delay between the last

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Abbreviations: DA, dopamine; ISHH, *in situ* hybridization histochemistry; 6-OHDA, 6-hydroxydopamine; PPE, preproenkephalin; PPT, preprotachykinin.

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FIG. 1. Negative prints of autoradiographic films showing PPE (A–C) and PPT (D–F) mRNA expression in the normal caudate-putamen (CPU) and nucleus accumbens (Acc in D) (on the left) and in the striatal grafts (on the right; indicated by arrowheads) in control (CONTL, A and D), 6-OHDA-lesioned (B and E), or chronic haloperidol (HALOP, C and F)-treated animals, using ISHH. H, intact host caudate-putamen spared by the ibotenate lesion. (Exposure times: PPE film, 4 days; PPT film, 6 days; bars = 1 mm.)

injection and sacrifice. The remaining animals ($n = 6$) served as controls for both the 6-OHDA lesions and the haloperidol treatment.

In Situ Hybridization Histochemistry. The probes used were DNA oligonucleotides (Scandinavian Gene Synthesis, Köp- ing, Sweden) complementary to nucleotides 322–360 of the cloned cDNA sequence for PPE (10) and nucleotides 145–192 of the mRNA sequence for PPT (11). Probes were labeled at the 3' end with [α - 35 S]dATP (>37 TBq/mmol; Amersham) using terminal deoxynucleotidyltransferase (Amersham). All probes used were labeled to specific activities of $>10^9$ cpm/ μ g. Specificity of hybridization was determined by competition experiments in which the 35 S-labeled probe was diluted with either the same unlabeled oligonucleotide or an

unlabeled unrelated oligonucleotide and by Northern analysis (data not shown).

The animals were decapitated under chloral hydrate anesthesia and the brains were immediately frozen on dry ice. Slide-mounted coronal sections (16 μ m) were air dried, fixed in 4% paraformaldehyde/0.1 M phosphate buffer for 10 min, and then rinsed in phosphate-buffered saline (three times, 5 min each) before dehydration. Hybridization was carried out overnight (16–18 h) with ≈ 250 μ l of hybridization mixture [50% formamide (deionized)/4 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate)/1 $\times$ Denhardt's solution/1% sarcosyl/10% dextran sulphate containing sheared and de-natured salmon sperm DNA at 500 μ g/ml, 200 mM dithio-threitol, and 35 S-labeled oligonucleotide at 10^7 cpm/ml] per

FIG. 2. Effect of 6-OHDA lesion or chronic haloperidol treatment on PPE mRNA expression in striatal grafts. (A, C, and E) Darkfield photomicrographs of emulsion autoradiograms from sections through intrastriatal striatal grafts after ISHH with the 35 S-labeled PPE probe. (B, D, and F) High-magnification brightfield views of silver grain accumulation over PPE-expressing neurons in the graft patches after dopamine-specific manipulation. (A and B) Sections through striatal grafts in control untreated rats (CONTL), showing the nonhomogeneous (patchy) distribution of PPE-labeled neurons in the graft. (C and D) Graft sections after a 6-OHDA lesion of the mesostriatal DA pathway (4 weeks survival). (E and F) Sections through striatal grafts after treatment for 10 days with haloperidol (HALOP). Both treatments resulted in increased grain densities in the graft patches of expression and over single neurons in these patches. (Exposure time, 21 days; bars: A, C, and E, 100 μ m; B, D, and F, 20 μ m.)

FIG. 3. Effect of 6-OHDA lesions or chronic haloperidol treatment on PPT mRNA expression in striatal grafts. (A, C, and E) Darkfield photomicrographs of emulsion autoradiograms from sections through intrastriatal striatal grafts after ISHH with the ^{35}S -labeled PPT probe. (B, D, and F) High-magnification brightfield views of silver grain accumulation over grafted striatal neurons. (A and B) Sections through control untreated grafts (CONTL), demonstrating the patchy distribution of PPT-labeled graft neurons. (C and D) Striatal graft sections from 6-OHDA-lesioned rats. (E and F) Striatal graft sections from rats treated with haloperidol for 10 days (HALOP). Note the reduction in PPT hybridization signal in the patches and the decrease in grain density over single grafted neurons after both treatments. (Exposure time, 28 days; bars: A, C, and E, 100 μm ; B, D, and F, 20 μm .)

slide at 42°C in a humid chamber. Washing was carried out in $1\times$ SSC at 55°C (four times, 15 min each) with the final wash beginning at 55°C and cooling to room temperature. The slides were exposed to autoradiographic film (βmax , Amersham) at -20°C for 2–7 days and then dipped in liquid emulsion (LM-1, Amersham) (1:1 dilution in distilled H_2O) and exposed for 2–5 weeks at -20°C .

Quantitation of mRNA Levels. Quantitative analyses were performed, at both the macroscopic (autoradiographic film analysis) and the microscopic (grain counting) level. Analysis of the autoradiographic film was performed using the software program IMAGE (Wayne Rasband, National Institute of Mental Health, Bethesda, MD) on a Macintosh IICI. Optical densities measured from the hybridized sections were converted to relative units of radioactivity using a ^{14}C standard (Amersham) coexposed on the same film. In most cases, at least four sections from the striatal graft per animal were measured with the tissue background, as determined from the corpus callosum (12), subtracted. Statistical analyses between the treatment groups were performed using one-way analysis of variance (ANOVA) followed by Scheffé's *F* test.

For microscopic analysis, grain density per cell and percentage of labeled cells were measured using an IBAS 2000 image analysis system. Using a magnification of $250\times$, the silver grains overlying medium-sized neurons (15- to 20- μm diameter) within the graft patches of expression or in the normal striatum were measured in pixels (1 grain is equivalent to 1.5–2 pixels). Background levels were determined by

measurement over the surrounding neuropil and were subtracted from the grain area calculated for each cell. Neurons were considered labeled if they expressed three times the background in grain area (13).

RESULTS

Distribution of PPE and PPT mRNA. Neurons expressing PPE or PPT mRNA in the forebrain were found most abundantly in the striatum and olfactory tubercle and, to a lesser extent, in the cortex and septum (14, 15). The expression of PPE and PPT mRNAs within the fetal striatal grafts implanted into the previously excitotoxic lesioned striatum was confined to clusters of neurons forming discrete patches (Figs. 1–3). In the film autoradiograms, the patches positively hybridizing for PPE covered an average of 36% of the total cross-sectional area of the graft, whereas the PPT signal covered a significantly smaller area, $\approx 22\%$. Although the total number of cells expressing PPE or PPT mRNA in the graft, as shown by the emulsion-dipped sections, was significantly lower than that in the intact striatum, the density of cells positively labeled within the patches of expression was similar to that in the intact striatum (51–54%; Table 1). The level of PPE mRNA expression in single neurons was not different between the grafted and normal striatal neurons, although the hybridization signal for PPT mRNA in grafted striatal neurons was 27% higher than that in the normal striatum (Table 1).

Table 1. Quantitation of grain density in striatal graft patches and host caudate-putamen after hybridization with PPE and PPT probes

Probe	Area	Grain area, pixels per cell (% labeled cells)		
		Control	6-OHDA lesioned	Haloperidol treatment
PPE	Graft	34.1 ± 3.2 ($54.0 \pm 1.9\%$)	$62.8 \pm 4.3^{**}$ ($59.7 \pm 1.9\%$)	$83.8 \pm 3.0^{**}$ ($58.0 \pm 2.0\%$)
	Caudate-putamen	34.4 ± 1.8 ($54.5 \pm 2.8\%$)	77.4 ± 4.5 ($60.8 \pm 2.7\%$)	$94.6 \pm 5.1^{**}$ ($57.5 \pm 1.2\%$)
PPT	Graft	$41.2 \pm 3.6^\dagger$ ($50.8 \pm 6.0\%$)	$19.5 \pm 2.3^{**}$ ($38.2 \pm 4.3\%$)	$20.0 \pm 1.7^{**}$ ($32.4 \pm 3.8\%$)
	Caudate-putamen	32.4 ± 3.7 ($40.4 \pm 5.5\%$)	15.8 ± 0.6 ($30.0 \pm 2.1\%$)	$16.0 \pm 1.0^*$ ($28.2 \pm 1.5\%$)

Results represent means $\pm$ SEM of grain area and percentage of positively labeled medium-sized cells from at least 600 neurons analyzed per treatment group, either in the graft patches or in the host caudate-putamen (i.e., 100–150 cells per graft or caudate-putamen per animal, $n = 5-7$). Statistical analyses were not performed for either the PPE or the PPT probes in the 6-OHDA-denervated host caudate-putamen, as spared host caudate-putamen was available for analysis in only three or four of the grafted animals (450–600 neurons examined). Grafted groups were compared using a one-way ANOVA followed by Scheffé's *F* test and control and haloperidol-treated host caudate-putamen were compared using Student's unpaired *t* test: ** , $P < 0.001$; * , $P < 0.01$. † , $P < 0.05$, different from control caudate-putamen using the PPT probe; Student's paired *t* test.

Effects of 6-OHDA Lesions. Four weeks after the animals had been subjected to the DA-depleting 6-OHDA lesions ipsilateral to the grafts, a 22% increase in the hybridization signal for PPE in the graft ($P < 0.01$, $n = 7$) and a 40% increase in the spared host striatum (Fig. 1 *A* and *B*) were detected by film analysis. These changes were restricted to the patches of expression, and, hence, the area of PPE mRNA expression did not change in the denervated grafts. The hybridization signal for PPT mRNA after the lesion was reduced from control values by 31% in the grafts ($P < 0.01$, $n = 7$) and by >50% in the spared portions of the host caudate-putamen (Fig. 1 *D* and *E*). Again, the area of PPT mRNA expression in the denervated grafts was not significantly different from the control grafts. These observations indicate that the denervation-induced changes in graft PPE and PPT mRNA levels were largely confined to the patches of expression. Microscopic examination of grain densities, therefore, was performed in the patch regions.

In the microscopic analysis, the grain density per PPE-labeled cell increased as a result of the DA-depleting lesion, by 84% in the grafts and by 125% in the spared host caudate-putamen (Table 1 and Fig. 2 *A–D*), whereas the percentage of labeled cells remained relatively unchanged both in the patches of PPE expression in the transplants and in the spared host caudate-putamen (Table 1). Analysis of grain density for PPT mRNA showed a reduction of 53% in the graft patches compared to the nontreated control grafts and of 51% in the spared portions of the host caudate-putamen (Table 1 and Fig. 3 *A–D*). Although the percentage of neurons positively hybridizing with the PPT probe dropped from 50.8% in the control grafts to 38.2% in the denervated grafts, this reduction did not reach statistical significance (Table 1). The 6-OHDA lesion did not affect either PPE or PPT mRNA expression in the contralateral intact caudate-putamen.

Effects of Chronic Haloperidol Treatment. Chronic haloperidol treatment (2 mg/kg, i.p., for 10 days) resulted in increases in the PPE hybridization signal from control values in the grafts of 42% ($P < 0.01$, $n = 6$) and in the contralateral host caudate-putamen of 67% ($P < 0.01$, $n = 6$) (Fig. 1 *A* and *C*) as determined by film analysis. The PPT hybridization signal was reduced by 21% in the grafts ($P < 0.01$, $n = 6$) and by 50% in the contralateral host caudate-putamen ($P < 0.01$, $n = 6$). As after the 6-OHDA lesion, the changes were confined to the graft patches. Thus, the area of expression was not significantly altered by haloperidol treatment (Fig. 1 *D* and *F*).

Analysis of the grain density overlying the PPE-labeled neurons in the graft patches and the contralateral host caudate-putamen showed considerable increase in grain area per cell after haloperidol treatment—146% in the graft and 175% in the normal caudate-putamen when compared to control animals (Table 1 and Fig. 2 *A*, *B*, *E*, and *F*). Again, the percentage of neurons positively labeled with the PPE probe did not differ significantly from the untreated graft or caudate-putamen (Table 1). The PPT hybridization signal as determined by grain density measurement was reduced by 52% in the graft patches and by 51% in the host caudate-putamen after haloperidol treatment (Table 1 and Fig. 3 *A*, *B*, *E*, and *F*). The percentage of PPT-labeled cells in the graft patches was reduced from an average of 50.8% to an average of 32.4%, but this difference was not statistically significant. Also in the contralateral intact caudate-putamen, there was a trend toward fewer positively labeled PPT expressing neurons in the haloperidol-treated animals, from 40.4% in the untreated caudate-putamen to 28.2% in the treated caudate-putamen; again, this difference did not reach statistical significance.

DISCUSSION

These experiments show that the levels of PPE and PPT mRNA expression in striatal grafts change in response to surgical removal of the host nigrostriatal DA afferents or

chronic DA D₂ receptor blockade. The pattern of change within the grafts resulting from these DA-specific manipulations paralleled that previously observed in the normal caudate-putamen, with an increase in PPE mRNA and a decrease in PPT mRNA (12, 14–18). The effect was observed as a change in the level of mRNA per cell rather than in the number of positive labeled cells, although there was a (non-significant) trend toward fewer PPT-labeled cells in the lesioned and haloperidol-treated animals, both in the graft and in the host caudate-putamen. The slight reduction in number of PPT-labeled cells most likely reflects the facts that the grain density for PPT mRNA was generally decreased after these manipulations and that this decrease was sufficient to reduce the number of grain counts in a population of weakly labeled cells to below the current cut-off level (3 times the background density; ref. 13). The magnitude of changes in mRNA levels in the host caudate-putamen, produced by either the 6-OHDA lesion or the haloperidol treatment, was similar to that reported earlier for both PPE and PPT (12, 14–18). In the striatal grafts, the magnitude of induced changes was similar to that found in the host caudate-putamen for the PPT message but 20–30% lower for the PPE message. Effects similar to those reported here concerning the regulation of enkephalin in grafted striatal neurons have recently been observed by Liu *et al.* (19).

In the normal caudate-putamen, enkephalin and substance P are localized largely in separate populations of medium-sized densely spiny neurons, most of which are projection neurons innervating the globus pallidus and the pars reticulata of the substantia nigra, respectively (8, 9). Cells expressing PPE mRNA and PPT mRNA in the rat are of approximately equal abundance, each type amounting to a little more than 50% of the total medium-sized neuron population in the caudate-putamen (17). The medium-sized densely spiny neurons represent the principal synaptic target for the nigrostriatal DA afferents (20), and there is evidence from electron microscopic immunohistochemical studies that both the enkephalin- and substance P-containing neuronal subtypes are directly innervated by the DA terminals (21, 22). Thus, the response of the striatal target neurons to DA denervation or chronic receptor blockade indicates that the nigrostriatal DA pathway normally exerts a tonic regulatory, but opposite, effect on neuropeptide synthesis in these two subclasses of striatal neurons (8, 9).

Previous immunohistochemical studies (4, 5, 23) indicate that the striatal grafts are composed of a mixture of striatal and nonstriatal tissue, and that the DARPP-32-positive patches, which are also acetylcholinesterase and calbindin positive, represent the striatal compartment of the grafts, embedded in tissue with essentially nonstriatal features. The striatal graft patches are densely and selectively innervated by host DA fibers (4, 5) and express both D₁ and D₂ receptor binding (23, 24), as well as the D₁-receptor related phosphoprotein DARPP-32 (5). In agreement with previous studies (4, 23, 25, 26) the present results show that enkephalin- and substance P-synthesizing neurons are confined primarily to the DARPP-32-positive (i.e., striatum-like) patches of the transplants. Although the overall abundance of neurons containing PPE mRNA and PPT mRNA was much lower than that in the intact caudate-putamen, the density of PPE and PPT mRNA-expressing neurons in the patches was similar to that in the contralateral control caudate-putamen. Cells expressing the two peptide mRNA transcripts thus comprised 51–54% of all medium-sized cells in the graft patches (as defined by cresyl violet stain), compared to 40–55% in the contralateral host striatum.

The levels of neuropeptide mRNAs within single neurons are likely to reflect the synthetic activity of the cell. Indeed, the alterations in neuropeptide mRNA levels in response to a 6-OHDA lesion or to DA receptor antagonism do translate

into parallel changes in peptide levels (8). Furthermore, similar DA-specific manipulations have been shown to induce changes in glucose utilization (27, 28) and electrophysiological activity (29–31) complementary to those found here in the enkephalinergic (striatopallidal) and substance P-containing (striatonigral) neuronal populations. The present results provide evidence, therefore, that the host DA afferents to the grafts are functional and that they exert a tonic regulatory control over the activity of grafted striatal neurons. In support of this hypothesis, previous studies (32–34) using induction of the Fos protein as a cellular marker for DA-mediated postsynaptic effects have shown that pharmacological activation of the DA afferents induces functional postsynaptic responses in grafted neurons, comparable to those found in the intact host caudate-putamen and that these changes occur selectively in the patch regions. Furthermore, consistent with the observations that striatal grafts are rich in GABAergic (GABA, γ -aminobutyric acid) neurons (25, 26, 35), we have recently observed that K^+ -evoked extracellular GABA overflow in intrastriatal striatal grafts is potentiated after removal of the DA afferents, similar to what is found in the DA-denervated caudate-putamen (unpublished data).

Although the current findings suggest that the tonic stimulatory influence of the host DA afferents on the substance P-containing graft neurons is close to normal, the inhibitory influence on the enkephalin-containing graft neurons appears to be less effective, since the response to DA blockade was 20–30% lower than that in the host caudate-putamen. The fact that PPE and PPT mRNA expression in the graft showed opposite responses to DA blockade, as in the normal caudate-putamen, suggests that these peptides are likely to be present, for the most part, in separate populations of grafted striatal neurons each under a differential DA regulatory control. Since the PPE- and PPT-expressing neurons each accounted for more than 50% of the medium-sized cells in the graft patches, these two neuronal populations (i.e., enkephalin- and substance P-containing cells) may comprise the vast majority of the medium-sized neurons in the striatal compartment of the grafts. This implies that the host DA afferents may have considerable impact on the functional regulation of the graft's striatal compartment, the neurons of which give rise to the efferent connections with the host brain (5).

In conclusion, the present results using ISHH show that removal or blockade of the host DA afferents to striatal grafts invokes long-lasting differential functional changes in the two principal classes of medium-sized graft neurons and that these changes are confined to the patch areas of the grafts that are known to be densely innervated by the host nigrostriatal DA pathway. Together with previous behavioral findings (6, 7), these data provide direct evidence that neurons in the striatal compartment of intrastriatal striatal grafts are under functional dopaminergic regulation from the host nigrostriatal pathway. In the intact animal, DA afferents are known to exert a profound regulatory influence over striatal functions and striatum-related behaviors. It seems highly probable, therefore, that the ability of the striatal grafts to interact with the host DA system may be important for mediation of the graft-induced recovery of sensorimotor behaviors previously observed with the types of grafts studied here (36, 37).

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