Regulation of the Nigrostriatal Pathway by Metabotropic Glutamate Receptors during Development

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Dopamine neurons in the substantia nigra heavily innervate the striatum, making it the nucleus with the highest levels of dopamine in the adult brain. The present study analyzes the time course and the density of striatal innervation by nigral dopamine neurons and characterizes the role of the neurotransmitter glutamate during the development of the nigrostriatal pathway. For this purpose, organotypic cultures containing the cortex, the striatum, and the substantia nigra (triple cultures) were prepared from rat brains at postnatal day (PND) 0–2 and were cultured for up to 60 d *in vitro* (DIV). Dopamine fibers and neurons were labeled using tyrosine hydroxylase (TH) immunohistochemistry. Striatal TH-ir fiber density was quantitatively analyzed using confocal laser scanning microscopy (CLSM). In long-term triple cultures (44 ± 3 DIV), the striatal dopamine fiber density was high and was weakly correlated with the number of nigral dopamine neurons. The high striatal dopamine fiber density mainly resulted from an enhanced ingrowth and

ramification of dopamine fibers from nigral neurons during 8–17 DIV. The metabotropic glutamate receptor (mGluR) antagonist L-(+)-2-amino-3-phosphonopropionic acid (L-AP-3) selectively inhibited this dopaminergic innervation of the striatum, whereas ionotropic GluR antagonists had no effect. The L-AP-3-mediated inhibition was prevented by the mGluR agonist 1S,3R-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD). The inhibition of the striatal dopaminergic innervation by L-AP-3 was further confirmed by anterograde tracing of the nigrostriatal projection with *Phaseolus vulgaris* leucoagglutinin. These results indicate that glutamate, by acting on group I mGluRs, plays an important “trophic” role for the development of the nigrostriatal dopamine pathway.

Key words: development; substantia nigra; striatum; nigrostriatal projection; cortex; metabotropic glutamate receptor; dopamine; neurotrophic factor; organotypic culture

Dopamine neurons in the substantia nigra pars compacta (SNc) heavily innervate the striatum during development (Olson et al., 1972; Seiger and Olson, 1973; Voorn et al., 1988). This dopaminergic innervation is tissue-specific because dopamine neurons in organotypic mesencephalon cultures preferentially innervate the striatum but not the hippocampus or cerebellum (Østergaard et al., 1990; Holmes et al., 1995). Striatal neurons exert some growth-promoting effect on mesencephalic dopamine neurons. They increase the [^3H]dopamine uptake in dissociated mesencephalon cultures prepared from mouse or rat and also stimulate neurite outgrowth in dopamine neurons (Prochiantz et al., 1979; Hemmendinger et al., 1981; Tomozawa and Appel, 1986; Dal Toso et al., 1988; Zhou et al., 1994). The mechanisms involved in these interactions between dopaminergic nigral neurons and striatal neurons are not clear.

Glutamate is a highly abundant neurotransmitter in the striatum. The striatum receives major glutamatergic projections from most cortical areas (McGeorge and Faull, 1989) and also from the thalamus (e.g., Lapper and Bolam, 1992). The cortical projections have been shown to be important for proper maturation of striatal neurons (Plenz and Aertsen, 1996b). Furthermore, nigral dopa-

mine neurons themselves may contain glutamate (Shiroyama et al., 1996). Glutamate acts on both ionotropic receptors and G-protein-coupled metabotropic receptors (mGluR) (Seeburg, 1993; Nakanishi, 1994). mGluRs are divided into several groups based on agonist interactions and associated second messengers (Nakanishi, 1994). In the cortex, the striatum, and the substantia nigra, group I mGluRs (mGluR1, mGluR5) are particularly highly abundant (Shigemoto et al., 1992; Romano et al., 1995; Testa et al., 1995). Via these receptors, glutamate stimulates phosphoinositide (PI) hydrolysis in striatal and cortical neurons (Doble and Perrier, 1989; Schoepp et al., 1992; Bevilacqua et al., 1995; Lorezini et al., 1996; Manzoni et al., 1996; Thomsen et al., 1996).

Several lines of evidence suggest that mGluR group I activation and subsequent PI hydrolysis might play an important role in neuronal differentiation and synaptogenesis during development. First, morphological differentiation of pheochromocytoma 12 cells requires the activation of specific PI kinases (Jackson et al., 1996). Second, in the developing visual cortex, the stimulation of PI hydrolysis by excitatory amino acids only occurs during a critical postnatal period of synaptic modification (Bear and Dudek, 1991). This stimulation is inhibited by the mGluR group I antagonist AP-3. Third, growth factors that have important roles in the maturation and survival of neurons during development selectively upregulate PI-coupled mGluR5 in astrocytes (Miller et al., 1995). In the present study, we tested whether glutamate by acting via group I mGluRs might also be important for the development of the nigrostriatal pathway.

We have recently established that in cortex–striatum–substantia nigra organotypic cultures (triple cultures), striatal and cortical neuronal classes show an electrophysiology and morphology

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similar to that of corresponding classes in the *in vivo* and the acute slice preparation (Plenz and Kitai, 1996a,b, 1998). In the present study, this culture system was used to study the role of mGluRs during the development of the nigrostriatal pathway using tyrosine hydroxylase (TH) immunohistochemistry combined with confocal laser scanning microscopy (CLSM) and anterograde tracing with *Phaseolus vulgaris* leucoagglutinin (PHA-L).

MATERIALS AND METHODS

Preparation of triple cultures

For the preparation of the cortex–striatum–substantia nigra organotypic cultures, coronal sections (350–400 μm) from rat brains (Harlan Sprague Dawley, Indianapolis, IN) at postnatal day (PND) 0–2 were cut on a microslicer (D.S.K., Ted Pella, CA). Slices containing the striatum and the cortex were used for dissection of dorsolateral cortical and striatal tissue (Fig. 1*A*). For the substantia nigra (including pars compacta and pars reticulata), ventrolateral sections from mesencephalic slices were selected, and medial tissue regions were avoided (Fig. 1*B*). The tissue was arranged in serial order (Fig. 1*C*) on a small rectangular piece of a Millicell-CM membrane (Millipore, Bedford, MA) with 20 μl of chicken plasma (Sigma, St. Louis, MO) on a coverslip. Then 20 μl of bovine thrombin (1000 National Institutes of Health units/0.75 ml; Sigma) was added. After plasma coagulation, the cultures were put into narrow culture tubes (Nunc, Naperville, IL), and medium was added (750 μl). The unbuffered standard medium consisted of 50% basal medium Eagle, 25% HBSS, and 25% horse serum with 0.5% glucose and 0.5 mM L-glutamine added (all Gibco, Grand Island, NY). After 3 and 27 d *in vitro* (DIV), 10 μl of mitosis inhibitor was added for 24 hr (4.4 mM cytosine-5- β -arabino-furanoside, 4.4 mM uridine, and 4.4 mM 5-fluorodeoxyuridine; calculated to final concentration; all Sigma). The medium was changed every 3–5 d [for further details, see Gähwiler (1981); Plenz and Aertsen (1996a); Plenz and Kitai (1996b)].

Pharmacological treatment

All drugs were added directly to the culture medium. Tetrodotoxin (TTX; Sigma), 6,7-dinitroquinoxaline-2,3-dione (DNQX; Research Biochemicals, Natick, MA), and ($\pm$)-2-amino-5-phosphonopentanoic acid (AP-5; Research Biochemicals) were dissolved directly in the culture medium. The mGluR antagonists L(+)-2-amino-3-phosphonopropionic acid (L-AP-3; Tocris Cookson, St. Louis, MO) and the mGluR agonist 1S,3R-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD; Tocris Cookson) were dissolved in 0.1N NaOH. Before application, the drug solution was neutralized by 0.1N HCl, and drugs were added directly to the culture tubes to the final concentrations. Normal groups were treated with the 0.1N NaOH/0.1N HCl mixture alone. Batches of cultures, consisting of 30–40 cultures, were prepared. One batch was always taken from one litter. After 8 DIV, each batch was divided into four to five groups containing six to eight triple cultures, and drugs were added. The pharmacological treatment was repeated at 12 DIV. The cultures were fixed at 16 DIV and were processed for immunohistochemistry.

Anterograde tracing of nigrostriatal fibers with PHA-L

The triple cultures were transferred to a chamber mounted on an inverted microscope (Nikon Diaphot 300; Nikon, Melville, NY). The glass bottom of the recording chamber allowed for visual selection of the substantia nigra within the triple cultures that were submerged in HBSS (Gibco) with 350 mg of NaHCO_3 added at $36.5 \pm 1^\circ\text{C}$. Glass micropipettes with a tip diameter of 5–10 μm were filled with PHA-L (2.5% in 10 mM phosphate; Vector Laboratories, Burlingame, CA) and positioned in the substantia nigra with the aid of a micromanipulator (MX-2; Narishige USA, Sea Cliff, NY). PHA-L was injected iontophoretically (5–10 μA ; duty cycle, 7 sec on–7 sec off; 20 min) (Gerfen and Sawchenko, 1984). Only one injection was made for each culture after 14 DIV. Because the injections were performed under nonsterile conditions, the days after the injections, cultures were treated with a culture medium with an antibiotic–antimycotic solution (0.125 ml/100 ml of medium; Gibco). After a survival time of 2 d, the cultures were double-immunostained for PHA-L and TH (see below).

Immunohistochemistry

Long-term cultures were fixed in 4% paraformaldehyde (PF) and 2% picric acid in 0.1 M phosphate buffer (PB), pH 7.4, overnight at 4°C and

Figure 1. Preparation of cortex–striatum–substantia nigra cultures (triple cultures). *A, B*, Cortical (cx), striatal (cp), and nigral (sn) tissue regions dissected from transversal rat brains at PND 0–2 indicated by black lines [E22; plate 7 and 19 taken from Altman and Bayer (1995)]. *C*, Arrangement of the dissected tissues during culturing. *D–H*, Quantitative analysis of striatal TH-ir fiber densities. *D*, A CLSM picture at high magnification taken 8 μm below the striatal surface. First, an estimation of the background intensity is achieved by placing a line onto regions with no TH-ir fibers present (*E*, background). Then, straight lines are placed at 0, 45, 90, and 135° (measure, only 0° shown). *E, G*, Typical pixel intensities over distance for a background and a measure line, respectively. *F*, The background distribution from which a threshold was chosen at mean + 4 $\times$ SD (filled arrows in *F, H*; broken lines in *E, G*). *H*, This threshold used to determine the number of pixels above threshold (PAT) in the pixel intensity distribution obtained from the measure (gray area).

then were incubated in 2% H₂O₂ in 0.1 M PBS and 0.3% Triton X-100 (30 min; Sigma). For developmental studies, triple cultures were washed (0.1 M PBS; three times for 10 min each; 4°C), fixed in 4% PF and 0.1 M PBS (30 min; 23°C), and washed again (0.1 M PBS; three times for 10 min each; 4°C). Subsequent washing was done in 0.1 M PBS (three times for 10 min each) if not otherwise indicated.

For TH immunohistochemistry, all triple cultures were incubated overnight in mouse anti-TH (1:500; Incstar, Stillwater, MN) in 0.1 M PBS containing 3% normal horse serum (Vector Laboratories) and 0.3% Triton X-100. They were washed and then incubated in fluorescein anti-mouse IgG (FITC; 1:150; Vector Laboratories) in PBS containing 0.3% Triton X-100 for 3 hr at room temperature and were covered in 2.5% 1,4-diazabicyclo-[2.2.2]-octane (50% glycerol in PBS; Sigma) or in Vectashield (Vector Laboratories). After washing in 0.05 M Tris-buffered saline (TBS; three times for 10 min each), the triple cultures were incubated in mouse monoclonal peroxidase-antiperoxidase (PAP; 1:500; Sigma) for 2 hr. They were washed (0.05 M TBS; three times for 10 min each) and reacted with 0.1% 3,3'-diaminobenzidine tetrahydrochloride (DAB; 0.1 M TBS; 0.002% H₂O₂). Cultures were then Nissl-stained and mounted.

For double labeling of PHA-L and TH, triple cultures were incubated in mouse anti-TH (1:1000) and biotinylated goat anti-PHA-L (1:1000; Vector Laboratories) in 0.1 M PBS with 3% normal goat serum, 3% normal horse serum (Vector Laboratories), and 0.3% Triton X-100 (48 hr; 4°C). After washing, they were incubated in fluorescein avidin D (FITC; 1:150; Vector Laboratories) and Texas Red anti-mouse IgG (1:150; Vector Laboratories; 0.1 M PBS; 0.3% Triton X-100; 3 hr; 23°C).

The fluorescent stains were analyzed using CLSM (Bio-Rad MRC 1000; Olympus Immunochemicals, Lake Success, NY). Optical sections (0.5–5 μ m) were taken throughout the entire depth of the tissue. For each section, a Kalman filter ($n = 3$) and background subtraction ($n = -1$) were used to increase the signal-to-noise ratio.

Quantitative analysis of morphological parameters

Number of nigral TH-ir neurons. The number of nigral TH-ir neurons was estimated using a fluorescence light microscope (BX50; Olympus America, Melville, NY) with a CCD camera attached to a computer image analysis system (IPLab Spectrum; Signal Analysis Corporation, Vienna, VA). During early stages of the project, the TH-ir neuron numbers obtained from the fluorescent pictures were counterchecked after conversion to a permanent stain using PAP. Counting differences between both methods were <5%, and for most cultures the number of TH-ir neurons was obtained from fluorescent pictures. In cases with very high TH-ir numbers (>300), the neuronal density of TH-ir neurons was calculated from five small areas under 40 $\times$ magnification and averaged. Then, the total number of neurons was estimated from the total area covered by TH-ir neurons.

Somatic cross-sectional area of nigral TH-ir neurons. The somatic cross-sectional area of TH-ir neurons was obtained from 100 $\times$ fluorescent pictures. A square grid of fixed size (240 $\times$ 310 μ m) was arbitrarily placed over an area with TH-ir neurons, and all neurons within that area or intersecting two borders were measured digitally by outlining the cell bodies. On average, 10 cultures per group with 40 cell bodies per culture were analyzed.

Striatal TH-ir fiber densities. The striatal density of TH-ir fibers was obtained from optical sections taken at 100 $\times$ magnification using CLSM. The sections had an optical thickness of 0.8 μ m and were positioned 6–8 μ m below the striatal surface. First, a line was placed on tissue regions with no TH-ir fibers present, and the pixel intensities along this line were measured (Fig. 1*D,E*, background). Then, four straight lines were placed at 0, 45, 90, and 135°, and the pixel intensities along each line were obtained (Fig. 1*D,G*, measure). A threshold value was chosen at mean plus 4 $\times$ SD from the background pixel intensity distribution (Fig. 1*F*) and was used to determine the number of pixels above threshold (PAT) in the measures (Fig. 1*H*). The PAT values were normalized to 100 μ m and averaged (nPAT). This measuring was repeated for seven arbitrarily chosen locations within the striatum for each culture. The results from the seven locations were then averaged. The calculations were done in Mathematica (Wolfram Research, Champaign, IL) on a Sun SPARCstation (Sun Microsystems, Mountain View, CA).

Anterograde tracing with PHA-L. The location of the PHA-L injection was based on the presence of labeled neurons and glia cells in the nigral culture and, in some cases, on the tissue damage caused by the injection electrode. At the striatal level, seven locations were arbitrarily chosen, and each striatal location was screened for the presence of PHA-L-ir

fibers at 100 $\times$ magnification using CLSM. At each location, optical sections were taken from the surface to the bottom of the tissue in steps of 2 μ m. The number of PHA-L-ir fibers per optical section was counted and averaged over all sections and all locations for each culture. To test for TH and PHA-L double labeling, we scanned sections with PHA-L-ir and TH-ir fibers singly under high resolution.

Striatal culture thickness as revealed by the depth distribution of TH-ir fibers. The striatal culture thickness was measured using vertical scans at 100 $\times$ magnification (step size, 0.8 μ m), thereby analyzing the distribution of TH-ir fibers. For each culture, seven arbitrarily chosen locations were examined, and the results were averaged. In general, the striatum flattened twice as much as the cortical or nigral tissue during culturing. Judging from the penetration depth of the TH stain in those thicker areas, we always achieved a complete penetration of the striatum with the antibodies.

Neuronal density estimations. The density of striatal neurons was analyzed from Nissl-stained cultures using a light microscope with a drawing tube attached. For each culture, a square grid of fixed size (200 $\times$ 320 μ m) was arbitrarily placed over a striatal area, and all neurons within that area or intersecting two borders were counted. Ten areas were analyzed and averaged per culture.

Data are expressed as mean $\pm$ SEM if not otherwise stated. For the statistical analysis, the one-way ANOVA with a *post hoc* Student–Newman–Keuls test (significance level, $p < 0.05$) has been used if not otherwise stated. Correlation was estimated by regression analysis combined with F and t statistics (Zar, 1984).

RESULTS

Nigral TH-ir neurons and striatal TH-ir fiber density in long-term triple cultures

In long-term triple cultures grown for 44 ± 3 DIV ($n = 23$), intensively labeled TH-ir neurons were found exclusively in the nigral culture. These neurons were normally located within a subregion of the substantia nigra with an average of 135 ± 28 TH-ir neurons per culture. TH-ir neurons were characterized by a relatively large somatic cross-sectional area of $305 \pm 4 \mu\text{m}^2$ ($n = 372$) and three to five primary dendrites that extended for several hundred micrometers. Primary and higher order dendrites were generally smooth and sparsely branched (Fig. 2*A,B*). The axon of TH-ir neurons heavily arborized in the striatum (Fig. 2*C,D*) that showed an average TH-ir fiber density of 70.0 ± 6.4 nPAT. In the long-term cultures, this density was only weakly correlated with the number of nigral TH-ir neurons (see Figs. 2, 4*H*; $F = 3.56$; $r = 0.38$; $df = 1, 21$; $p = 0.073$; $y = a + bx$ with $a = 62 \pm 7$ and $b = 0.070 \pm 0.038$; mean $\pm$ SEM). The thickness of the striatal culture was $39.1 \pm 4.5 \mu\text{m}$ ($n = 23$) as measured by the average depth distribution of TH-ir fibers.

Development of the nigrostriatal pathway

In the developmental study, we attempted to ascertain the time of the main ingrowth of TH-ir fibers into the striatum. Triple cultures from one single batch were analyzed at 5, 8, 11, 14, and 17 DIV for the number of nigral TH-ir neurons and the striatal density of TH-ir fibers (Fig. 3). The number of TH-ir neurons per culture did not differ between the different groups ($F = 0.89$; $df = 4, 26$; $p = 0.48$; 308 ± 32 neurons/culture/group).

At 5 DIV, no TH-ir fibers were visible in the striatum. However, many TH-ir elements were randomly dotted throughout the striatum (Fig. 3*A*). At 8 DIV, some TH-ir fibers with varicosities were seen in the striatum. At 11 DIV, the striatum was intensively and homogeneously innervated by TH-ir fibers. During the following 6 d, the density of striatal TH-ir fibers increased further (Fig. 3*A,B*). The striatal TH-ir fiber density at 17 DIV was significant higher when compared with that at 5 DIV and 8 DIV (Fig. 3*B*). At 17 DIV, the striatal TH-ir density did not differ from those densities measured in long-term cultures (67.9 ± 12.2 nPAT

Figure 2. TH-ir neurons in the substantia nigra and corresponding striatal TH-ir fibers in long-term cortex–striatum–substantia nigra organotypic cultures. *A, B*, Nigral TH-ir neurons in a long-term triple culture with a low ($n = 22$) and a high ($n = 360$) total number of TH-ir nigral neurons, respectively. *C, D*, Corresponding striatal TH-ir fibers for the cultures shown in *A* and *B*, respectively. Note the slightly increased number of striatal TH-ir fibers for the culture with high total numbers of TH-ir neurons. Pictures are projections of a series of optical sections that covered the total depth of the nigral and striatal tissue. *cp*, Striatum; *sn*, substantia nigra. Scale bar: *A–D*, 100 μ m.

at 17 DIV vs 71.4 ± 5.1 nPAT at 44 DIV; two-tailed t test; compare Figs. 3*B*, 4*H*).

Analysis of the branching pattern of individual TH-ir axons revealed that during the first week in culture, TH-ir fibers radiated from the nigral tissue and grew several millimeters in the surrounding plasma clot. No indication of directional outgrowth was visible; however there was a tendency for TH-ir fibers not to traverse the tissue that most likely corresponded to the substantia nigra pars reticulata (see also Fig. 5). A significant difference in growth pattern was present depending on whether TH-ir axons came close to the striatal border or not. Whereas fibers growing away from the striatum only rarely gave off collaterals (Fig. 3*C*), fibers growing into or passed the border of the striatum showed a high tendency of branching by 8 DIV (Fig. 3*D*).

Glutamate transmission and the ingrowth of TH-ir fibers to the striatum

We tested for pharmacological effects during the period of strongest innervation (from 8 to 16 DIV) of the striatum by TH-ir

fibers. In a first set of experiments, the effect of TTX that is known to block sodium spike activity was measured. TTX (1 μ M) strongly reduced the striatal density of TH-ir fibers to 14.5% of the normal level ($n = 5$ per group; data not shown).

Then, the effect of GluR blockade was tested. In a previous study, the ionotropic GluR antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) at 10 μ M was shown to block completely striatal activity in the triple cultures (Plenz and Kitai, 1998). For the developmental studies, we used the closely related ionotropic GluR antagonist DNQX at 50 μ M; DNQX is more water-soluble than CNQX with an inhibition of binding (IC_{50}) to cortical membranes twice that of CNQX (Honoré et al., 1988). Furthermore, in the striatum, the NMDA GluR antagonist AP-5 at 30 μ M was shown to abolish completely NMDA-GluR-mediated responses (Jiang and North, 1991). Similar dose–response relationships also hold for cortical neurons (Jones and Baughman, 1988; Bear et al., 1996) and nigral dopamine neurons (Chergui et al., 1993; Futami et al., 1995; Mercuri et al., 1996). L-AP-3 was chosen for the mGluR antagonist because it was reported to inhibit mGluR-mediated phosphoinositide hydrolysis (Schoepp et al., 1990) and to affect striatal synaptic plasticity (Calabresi et al., 1992).

DNQX (50 μ M), AP-5 (50 μ M), and L-AP-3 (100 μ M) applied together significantly reduced the striatal density of TH-ir fibers during the development of the triple cultures (Fig. 4*A*). This effect was because of the action of L-AP-3. DNQX and AP-5 applied alone had no effect. The number of TH-ir neurons per culture did not differ between groups ($F = 0.15$; $df = 4, 27$; $p = 0.96$; 218 ± 12 neurons/culture/group).

The effect of the mGluR antagonist L-AP-3 on the development of the nigrostriatal pathway

At the light microscopic level, the effect of the mGluR antagonist L-AP-3 on the development of the nigrostriatal system was apparent (Figs. 4*B–E*, 5*C*). Only very few striatal TH-ir fibers were present in the L-AP-3 compared with the normal group. However, individual TH-ir fibers when present in the L-AP-3 group were as intensely labeled for TH as were those under normal conditions (Fig. 4*D,E*). Similarly, under normal conditions, as well as in the L-AP-3 group, the cell body and the dendrites of TH-ir nigral neurons were intensively stained for TH (Figs. 5*A,C*, 6*D,F*).

A summary from eight experiments with 100 μ M L-AP-3 allowed us to examine the correlation between the number of nigral TH-ir neurons and the resulting striatal densities of TH-ir fibers in more detail. Under normal conditions at 16 DIV, a clear correlation between the striatal TH-ir fiber density and the number of nigral TH-ir neurons was found (Fig. 4*F*; $F = 15.89$; $df = 1, 44$; $p < 0.001$) with the striatal TH-ir fiber density increasing on average by 8.5 nPAT per 100 TH-ir nigral neurons ($r = 0.55$; $a = 27 \pm 8$; $b = 0.085 \pm 0.022$). This slope dependency was not significantly different from the regression found in the long-term triple cultures (Fig. 4*H*; $t = 0.238$; $p > 0.5$). In the presence of L-AP-3, this correlation was completely absent (Fig. 4*G*; $F = 0.55$; $df = 1, 33$; $p = 0.46$; $r = 0.13$; $a = 22 \pm 2.3$; $b = 0.004 \pm 0.006$) and significantly different from the correlation found at 16 DIV under normal conditions ($t = 3.47$; $p < 0.001$). L-AP-3 did not affect the survival of TH-ir neurons because the average number of nigral TH-ir neurons was similar for both groups [326 ± 39 for normal ($n = 45$) vs 323 ± 61 for L-AP-3 ($n = 34$); two-tailed t test]. Also, no effect was found on the striatal culture thickness by L-AP-3 (Table 1). The distributions for the somatic cross-sectional area revealed that a considerable portion of TH-ir

Figure 3. Development of the striatal TH-ir fiber density and growth characteristics of TH-ir nigral fibers in triple cultures. *A*, Development of the striatal TH-ir fiber density (*upper row*) at 5, 8, 11, 14, and 17 DIV. The earliest ingrowth of new TH-ir fibers occurs between 5 and 8 DIV (*arrowhead*). The cytoplasmic area of the cell body in TH-ir neurons is relatively small during the first week but increases during the second week (*lower row*). Note the constant size of the nucleus during this postnatal period. All pictures are single optical sections. *B*, Statistical analysis of TH-ir fiber ingrowth into the striatum. The striatal TH-ir fiber density is low at 5 and 8 DIV and increases during the following 9 d (mean $\pm$ SEM). At 17 DIV, the striatal TH-ir fiber density has reached a significantly higher level (*) when compared with that at 5 and 8 DIV. *C*, TH-ir fibers (DAB converted) with an axonal growth cone (*arrowhead*) several millimeters outside the nigral tissue within the plasma clot (8 DIV). Note the low tendency to branch (*arrow*). *D*, Single TH-ir fiber that grows at the border of the striatal area (8 DIV). Note the early branching point (*arrow*) and the multiple growth cones (*arrowheads*) that result from this and subsequent branching points. *cp*, Striatum; *sn*, substantia nigra. Scale bar: *A*, *C*, *D*, 100 μ m.

neurons grown in the presence of L-AP-3 had a smaller somatic cross-sectional area when compared with normal (Fig. 4*I*; $305 \pm 3.7 \mu\text{m}^2$ for normal vs $266 \pm 3.3 \mu\text{m}^2$ for L-AP-3; $n = 372/\text{group}$; two-tailed t test). Furthermore, the striatal neuronal density was slightly lower in the presence of L-AP-3 (Table 2).

Manipulation of the nigrostriatal system by mGluR agonists and antagonists

The putative role of mGluRs during the development of the nigrostriatal pathway was further supported by experiments using the mGluR agonist 1S,3R-ACPD and the mGluR antagonist L-AP-3 together (Figs. 5, 6). A comparison of whole mount pictures from triple cultures indicated that 1S,3R-ACPD had an overall beneficial effect on the neuronal growth in the culture system (Fig. 5*A,B*).

Dose–response relationships (Fig. 6*A*) showed that there was a tendency for higher concentrations of 1S,3R-ACPD to result in slightly higher striatal TH-ir fiber densities. L-AP-3 had no effect

on the striatal density of TH-ir fibers at 10 μM but significantly reduced this density at 100 μM . L-AP-3 at 1000 μM resulted in a complete degeneration of striatal and cortical tissue but not nigral tissue (data not shown), and no measurement on striatal TH-ir fiber densities was done for this group. When given both the agonist and the antagonist together, 1S,3R-ACPD at 100 μM did not reverse the inhibitory action of 100 μM L-AP-3 on the striatal TH-ir fiber density (Figs. 5, 6*B*) but at 1000 μM did (Fig. 6*B–G*). No significant differences were found for the number of TH-ir neurons and the striatal culture thickness in these combined agonist and antagonist experiments (Table 1).

Tracing the nigrostriatal projection with PHA-L

To test whether L-AP-3 decreases the enzyme TH within fibers or whether L-AP-3 reduces the number of nigrostriatal fibers, we traced the nigrostriatal projection anterogradely with PHA-L. One single PHA-L injection was placed iontophoretically at 14 DIV into each nigral part of cultures grown under normal con-

Figure 4. Effect of glutamate receptor blockade on striatal TH-ir fiber density during development. **A**, Effect on TH-ir fiber density of drugs applied alone or in combination (50 μ M DNQX; 50 μ M AP-5; 100 μ M L-AP-3). Values are mean $\pm$ SEM. Numbers in parentheses indicate the number of cultures per group. The asterisk indicates significantly different from normal ($p < 0.05$). **B**, **C**, Striatal TH-ir fibers from the normal and the L-AP-3 groups. Pictures are projections of a series of optical sections that covered the total striatal depth. **D**, **E**, Corresponding striatal TH-ir fibers at higher magnification (single optical section; 7 μ m below the

conditions (normal; $n = 12$) and cultures grown in the presence of 100 μ M L-AP-3 from 8–16 DIV (L-AP-3; $n = 15$). After 2 d of survival, the cultures were fixed and analyzed for TH and PHA-L. If the injection site was within an area with high numbers of TH-ir neurons present ($400 \times 400 \mu$ m; >20), the injection was classified SNc⁺; otherwise, it was SNc[−]. In normal and SNc⁺ cultures ($n = 5$, Fig. 7A), PHA-L-ir fibers were always found in the striatum in addition to a high density of striatal TH-ir fibers (Fig. 7B). The average number of PHA-L fiber segments per section in those cultures was 12 ± 2 . PHA-L-ir fibers were also positive for TH (three sections examined for each culture, Fig. 7B). In normal and SNc[−] cultures ($n = 7$), PHA-L-ir fibers were never found in the striatum despite a high striatal density of TH-ir fibers (data not shown). In L-AP-3 and SNc⁺ cultures ($n = 7$, Fig. 7C), a significantly lower number of PHA-L-ir fiber segments was found in the striatum (0.1 ± 0.1) compared with the number found in normal and SNc⁺ cultures (two-tailed Student's t test). Only few striatal TH-ir fibers were present per section (Fig. 7D). In L-AP-3 and SNc[−] cultures ($n = 8$), as in the normal and SNc[−] group, no striatal PHA-L-ir fibers were found.

DISCUSSION

Development of the nigrostriatal pathway *in vivo* and in triple cultures

In the rat *in vivo*, TH-ir and dopamine-ir neurons are present at embryonic day 13–13.5, and the innervation of the ganglionic eminence takes place 2 d later (Tennyson et al., 1975; Specht et al., 1981; Voorn et al., 1988). At PND 1, the morphology of dopamine neurons is almost mature (Tepper et al., 1994). At PND 0–2, patches with dense dopamine fibers are distributed throughout the striatum. From PND 8–20, dopamine fibers become more diffuse in the striatum, and the number of varicose dopamine fibers increases dramatically, reaching almost adult levels at the end of the third week after birth (Voorn et al., 1988). In the triple cultures, nigral TH-ir neurons showed the typical morphology and distribution described during *in vivo* development (Tepper et al., 1994), and the period of strongest innervation of the striatum occurred during 8–17 DIV that correspond to a postnatal period of PND 10–19. Also during this period, the striatal culture becomes diffusely and very homogeneously innervated by TH-ir fibers. Thus, in the triple cultures, the time course and spatial characteristics of the postnatal development of the nigrostriatal pathway corresponds well with those *in vivo*. The diffuse distribution of highly varicose TH-ir fibers after 17 DIV very closely matches the appearance of dopamine fibers in the rat striatum *in vivo* at a corresponding age [Voorn et al. (1988), their

striatal surface). **F**, Correlation between striatal TH-ir fiber density and the number of nigral TH-ir neurons under normal conditions after 16 DIV (linear regression, $r = 0.55$; $n = 45$; $p < 0.001$; slope, 8.5/100 nigral TH-ir neurons; $n = 8$ experiments combined). **G**, Correlation between striatal TH-ir fiber density and the number of nigral TH-ir neurons grown in the presence of 100 μ M L-AP-3 after 16 DIV (linear regression, $r = 0.13$; $n = 34$; $p = 0.46$; slope, 0.4/100 nigral TH-ir neurons; $n = 8$ experiments combined). The effect of L-AP-3 is independent of the total number of TH-ir neurons present in the substantia nigra. **H**, Correlation between striatal TH-ir fiber density and the number of nigral TH-ir neurons for long-term cultures (44 $\pm$ 3 DIV; linear regression, $r = 0.38$; $n = 23$; $p = 0.073$; slope, 7.0/100 nigral TH-ir neurons). **I**, Distribution of the somatic cross-sectional area of nigral TH-ir neurons grown under normal conditions and in the presence of 100 μ M L-AP-3. L-AP-3 results in a slightly but consistently smaller cell body area of TH-ir neurons ($n = 372$ per group). Scale bar: **B**, **C**, 200 μ m; **D**, **E**, 50 μ m. **cp**, Striatum.

Figure 5. Effect of metabotropic glutamate receptor treatment on TH-ir fiber ingrowth into the striatum during development. Cortex–striatum–substantia nigra organotypic cultures were grown for 16 DIV and stained for TH using FITC as a fluorochrome (*light*). Drugs were added from 8 to 16 DIV. *A*, Normal. *B*, mGluR agonist 1S,3R-ACPD at 100 μ M. *C*, mGluR antagonist L-AP-3 at 100 μ M. *D*, mGluR agonist 1S,3R-ACPD at 100 μ M and mGluR antagonist L-AP-3 at 100 μ M. In all conditions, the number of TH-ir neurons in the substantia nigra is high, and numerous dendritic processes are present. Pictures are a montage of confocal pictures covering the total depth for each culture. *cp*, Striatum; *cx*, cortex; *sn*, substantia nigra. Scale bar: *A–D*, 500 μ m.

Fig. 25*G,I*). The dot-like TH-ir elements seen during the first week of culturing in the striatum most likely represent the degenerating early dopamine fibers that were severed during the preparation of the triple cultures.

In long-term cultures, TH-ir fibers were also found in a few cases in the cortex (Plenz and Kitai, 1996b). In those cases, the fibers were thin, sinuous, and smooth with irregular swellings and showed a layered distribution similar to that described *in vivo*

(Berger et al., 1974; Lindvall et al., 1974). These cortical TH-ir fibers differed in their appearance from striatal TH-ir fibers and were only very few compared with the many striatal dopamine fibers.

The results from the PHA-L experiments furthermore demonstrated that mainly dopamine neurons from the nigral tissue innervated the striatal culture. When injections were placed outside the nigral TH-ir region, no PHA-L-ir fibers were detected in

Figure 6. *A*, Dose–response relationship of the mGluR antagonist L-AP-3 and the mGluR agonist 1S,3R-ACPD is shown. L-AP-3 (100 μ M) significantly decreased the striatal TH-ir fiber density (*). At a concentration of 1000 μ M L-AP-3, the striatal tissue degenerated, and no measurements could be done. The mGluR agonist 1S,3R-ACPD did not result in any significant increase in the striatal density of TH-ir fibers. However, a tendency toward higher densities with increased concentrations was present. The broken line and dotted lines indicate the mean $\pm$ SEM for the normal group ($n = 11$). *B*, 1S,3R-ACPD applied at 100 μ M has no significant effect on the L-AP-3-induced reduction of striatal TH-ir fibers. ($F = 10.02$; $p = 0.0002$; $df = 3, 66$; $p > 0.05$). *C*, 1S,3R-ACPD applied at 1000 μ M significantly prevents the L-AP-3-induced reduction of striatal TH-ir fibers ($F = 4.59$; $p = 0.0062$; $df = 3, 55$; * $p < 0.05$). Numbers in parentheses indicate cultures per group. Values are mean $\pm$ SEM. Data are pooled from three (*B*) and two (*C*) experiments. *D–G*, Corresponding confocal pictures for the experiments in *C* are shown. *cp*, Striatum; *sn*, substantia nigra. Scale bar: *D–G*, 100 μ m.

the striatum. Thus, neurons from regions outside the SNc (e.g., substantia nigra reticulata), despite the lack of target tissue, did not innervate the striatum in the triple cultures.

In summary, the developmental features of the nigrostriatal

pathway *in vivo* are also primarily expressed in the triple cultures with respect to the time course of striatal innervation, spatial distribution, the morphology of dopamine fibers, and the selectivity of tissue innervated.

Table 1. Striatal culture thickness as measured by the depth distribution of striatal TH-ir fibers and number of nigral TH-ir neurons per group for the combined mGluR agonist/antagonist experiments

	Normal	1S,3R-ACPD (100 μ M)	L-AP3 (100 μ M)	1S,3R-ACPD (100 μ M) + L-AP3 (100 μ M)	Normal	1S,3R-ACPD (1000 μ M)	L-AP3 (100 μ M)	1S,3R-ACPD (1000 μ M) + L-AP3 (100 μ M)
Number of cultures	16	18	18	18	15	14	17	13
Striatal culture thickness (μ m) ¹	26.8 $\pm$ 2.3*	27.7 $\pm$ 2.2*	22.9 $\pm$ 3.1*	19.6 $\pm$ 1.7*	22.8 $\pm$ 3.0 [†]	25.0 $\pm$ 2.5 [†]	23.8 $\pm$ 3.5 [†]	19.6 $\pm$ 2.5 [†]
Number of TH-ir nigral neurons ²	305 $\pm$ 87**	270 $\pm$ 64**	247 $\pm$ 65**	252 $\pm$ 63**	367 $\pm$ 56 ^{††}	488 $\pm$ 52 ^{††}	402 $\pm$ 103 ^{††}	409 $\pm$ 76 ^{††}

Not significantly different: * $F = 2.29$; $p = 0.09$; $df = 3, 66$; ** $F = 0.14$; $p = 0.94$; $df = 3, 68$; [†] $F = 0.53$; $p = 0.66$; $df = 3, 55$; ^{††} $F = 0.42$; $p = 0.74$, $df = 3, 55$.

Table 2. Neuronal densities of the striatal tissue under the various pharmacological treatments

Group	<i>n</i>	Striatal density (n/mm ²)
Normal	8	6184 ± 363
100 μ M 1S,3R-ACPD	7	6007 ± 276
1000 μ M 1S,3R-ACPD	4	6351 ± 151
100 μ M L-AP3	10	5099 ± 249*
100 μ M 1S,3R-ACPD + 100 μ M L-AP3	5	5486 ± 319
1000 μ M 1S,3R-ACPD + 100 μ M L-AP3	4	5930 ± 319

Groups are significantly different ($F = 2.74$; $p = 0.036$; $df = 5,32$). The striatal neuronal density is slightly but significantly reduced in the presence of 100 μ M L-AP3(*). Results are combined from two experiments shown in Figure 6B and C, respectively.

Target specificity of nigral dopamine axons

In the triple cultures, dopamine axons from the SNc not only preferentially innervated the striatum, but they, to a very high level, exclusively ramified within the striatum. The sequence of radiated outgrowth and increased branching within striatal territory ultimately led to the intense innervation of the striatal area that resulted in the typical macroscopic innervation patterns shown in Figure 5. These results indicate that the arborization of nigral dopamine axons is facilitated by the striatum.

A growth-promoting effect of striatal neuronal tissue has been described by several *in vitro* studies. In mouse dissociated dopamine neurons, [³H]dopamine uptake and dopamine synthesis are significantly increased in the presence of striatal target cells (Prochiantz et al., 1979). This effect is specific to striatal neurons, does not involve striatal glia (Di Porzio et al., 1980), and can be mimicked by striatal neuronal membrane fractions (Prochiantz et al., 1981). The strongest effect is obtained from striatal neuronal membrane fractions taken during the second and third week after birth. This period corresponds with the period during which the main increase in striatal dopamine fibers takes place in the triple cultures. In rat dissociated dopamine neurons, in addition to enhancing [³H]dopamine uptake three- to fourfold, extracts from striatal tissue have also been reported to stimulate neurite outgrowth (Tomozawa and Appel, 1986; Dal Toso et al., 1988; Zhou et al., 1994). Studies using organotypic mesencephalic slices cocultured with either the striatum or nontarget-specific brain areas (Østergaard et al., 1990; Holmes et al., 1995) further demonstrated that mesencephalic dopamine axons preferentially innervate the striatum. Taken together, these results strongly indicate that striatal neurons promote neurite outgrowth from dopamine neurons. Our study extends these findings both at a more quantitative and morphological level, in an *in vitro* system in which neurons develop into a mature state with electrophysiological and morphological properties similar to those described *in vivo* and in the acute slice (Plenz and Aertsen, 1996a,b; Plenz and Kitai, 1996a,b, 1998).

The correlation between the striatal TH-ir density at 44 DIV was similar to the correlation obtained at 16 DIV, although the range of TH-ir neuron numbers covered was smaller in the long-term cultures. In general, the striatal TH-ir fiber density increased on average by 7.0 ± 3.8 nPAT per 100 nigral TH-ir neurons in the long-term cultures as well as at 16 DIV. Long-term cultures were also used for electrophysiological analysis (Plenz and Kitai, 1998), and during the preparation of these cultures, we

Figure 7. Reconstruction of the nigrostriatal projection using PHA-L injections into the substantia nigra. *A*, Nigral region with TH-ir neurons (*A1*) and the corresponding PHA-L injection site (*A2*) in a triple culture grown for 16 DIV under normal conditions. The location of the PHA-L injection (*x*) is revealed by the radiation of PHA-L-ir fibers (*A2*, arrow) from a relatively dark area. *B*, PHA-L-ir fibers that are also positive for TH-ir neurons (*B1*, optical section (depth, 0.5 μ m; 100 $\times$) at a depth of 4 μ m from the surface of the striatal tissue. *B2*, Corresponding striatal region showing the presence of PHA-L-ir fibers. Note that every fiber in that region is also TH-ir as indicated by the arrows in *B1* and *B2*. *C*, Nigral region with TH-ir neurons (*C1*) and the corresponding PHA-L injection site (*C2*, *x*) in a triple culture grown for 16 DIV with the presence of L-AP-3 from 8 to 16 DIV. *D1*, Confocal projection of a striatal region covering a total depth of 5 μ m at 40 $\times$ magnification showing the presence of few striatal TH-ir fibers in the L-AP-3 group. The density of TH-ir fibers at the striatal level is much reduced in the L-AP-3 group compared with that in the normal group (*B1*). *D2*, Corresponding region demonstrating the lack of PHA-L-ir fibers in the striatum. *cp*, Striatum; *sn*, substantia nigra. Scale bar: *A*, 100 μ m; *B*, 20 μ m; *C*, *D*, 200 μ m.

selected smaller and more lateral tissue regions to avoid the ventral tegmental area. This difference in nigral tissue size most likely explains the on-average smaller neuron numbers and consequently the weaker correlation found in those cultures.

In summary, our results suggest that during development, dopamine neurons preferentially ramify within the striatum. The correlation between the striatal dopamine fiber density and the nigral dopamine neuron numbers indicates that axons of nigral dopamine neurons ramify independently from the presence of other dopamine fibers in the striatum.

Localization of group I mGluRs in the cortex, striatum, and substantia nigra

The most striking finding of the present study is that mGluRs play an important role during the development of the nigrostriatal pathway. The ingrowth of dopamine fibers into the striatum and subsequent ramification within the striatum were strongly inhibited by the mGluR antagonist L-AP-3. This inhibition was prevented by the mGluR agonist 1S,3R-ACPD. L-AP-3 and 1S,3R-ACPD are acting preferentially on mGluR group I receptors (mGluR1, mGluR5) that, via their PI hydrolysis-linked second messenger functions, provide vitally important support for synaptogenesis during development (Bear and Dudek, 1991; Miller et al., 1995; Jackson et al., 1996). Because both drugs were added to the medium, no conclusions can be drawn as to the site of drug action. However, several hypothesis can be presented. First, L-AP-3 could act directly on nigral dopamine neurons preventing their growth. If so, L-AP-3 seemed to arrest the growth of nigral dopamine neurons because it did not change the overall morphology, the survival rate, or the intensity of the TH stain of nigral dopamine neurons but mainly prevented their axonal growth. This growth-arresting effect of L-AP-3 is also supported by the smaller somatic cross-sectional cell body area of TH-ir neurons in the presence of L-AP-3 (compare Fig. 3A). Second, L-AP-3 could interrupt a signaling cascade between nigral axons and striatal tissue that normally leads to the intensive ramification of dopamine fibers within the striatum. Third, L-AP-3 could act at the cortical level, which in turn affects the maturation of the striatal and nigral system.

A cortical and/or striatal action of both drugs is supported by the intensive expression of mGluR5 mRNA (Testa et al., 1995) and the mGluR5 receptor (Romano et al., 1995) in the adult cortex and striatum. This expression is even stronger in the developing cortex and striatum (Testa et al., 1994; Romano et al., 1996). Furthermore, a developmental peak of mGluR-stimulated PI hydrolysis occurs during the early postnatal weeks in the rat at a period of intense synaptogenesis (Palmer et al., 1990). In the developing substantia nigra of the rat, by PND 0, the mGluR1 is highly abundant (Shigemoto et al., 1992). In addition, nigrostriatal axon terminals seem to carry mGluR receptors because 6-OHDA lesion irreversible reduces the binding sites for mGluR at the striatal level (Wullner et al., 1994).

In summary, group I mGluR are particularly highly expressed in the cortex, the striatum, and the substantia nigra, and this expression is enhanced during early development. Thus, L-AP-3 and 1S,3R-ACPD by primarily acting via these receptors could exert the growth effects described for the nigrostriatal pathway in this study.

Second messenger pathways involved in the regulation of the nigrostriatal pathway by group I mGluRs

In the developing and adult striatum, *trans*-ACPD (Manzoni et al., 1996; Thomsen et al., 1996) and 1S,3R-ACPD (Schoepp et al., 1992) strongly increase striatal PI turnover, and this increase is inhibited by L-AP-3 (Lorezini et al., 1996). Thus, the inhibition of PI hydrolysis via L-AP-3 could be one major pathway through which the development of the nigrostriatal pathway is severely affected. In striatal neurons and transfected *Xenopus* oocytes, L-AP-3 was shown to act as a competitive inhibitor on PI formation (Manzoni et al., 1991) and for group I mGluRs (Saugstad et al., 1995; but see Schoepp et al., 1990). In our study, the reversal of the L-AP-3-mediated inhibition of the nigrostriatal pathway was achieved with relatively high doses of 1S,3R-ACPD. Even with those high doses, this effect is probably mediated via mGluRs because it was shown that ACPD as high as 1 mM is not active on ionotropic glutamate receptors in the striatum (Manzoni et al., 1996).

In the developing and adult cortex, *trans*-ACPD increases PI turnover (Mortensen et al., 1995; Bevilacqua et al., 1995) and intracellular Ca^{2+} concentration (Koh et al., 1991b) in cortical neurons. The increase in PI turnover is reduced by L-AP-3 (Mortensen et al., 1995; Mistry et al., 1996). It is also known that 1S,3R-ACPD up to 1 mM is not neurotoxic for cortical neurons (Koh et al., 1991a) and even protects cortical neurons from NMDA-induced neurotoxicity (Koh et al., 1991b). In triple cultures grown in the presence of 1S,3R-ACPD, no signs of neurotoxicity could be detected as evidenced by the macroscopic appearance (Fig. 5B) and the striatal neuronal density estimate (Table 2). On the contrary, as judged from the robustness of the tissue during handling and the autofluorescence, these cultures appeared even “healthier” than normal, indicating an overall beneficial effect of 1S,3R-ACPD during development.

In conclusion, our results demonstrate that glutamate, by acting on group I mGluR subtypes, plays an important role in the development of the nigrostriatal pathway. Thus, mGluR agonists may be useful for restoring developmental deficits and/or preventing neurodegeneration of the dopaminergic pathways in the basal ganglia.

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