

Afferent Influences on Striatal Development in Organotypic Cocultures

ABIGAIL SNYDER-KELLER,^{1,2*} KUEI Y. TSENG,³ GREGORY D. LYNG,^{1,2}
DAVID J. GRABER,^{1,2} AND PATRICIO O'DONNELL³

¹Wadsworth Center, David Axelrod Institute, New York State Department of Health, Albany, New York

²Department of Biomedical Sciences, SUNY School of Public Health, Albany, New York

³Center for Neuropharmacology and Neuroscience, Albany Medical College, Albany, New York

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ABSTRACT Organotypic cocultures of striatum, cortex, and ventral mesencephalon were used to study the anatomical and physiological development of striatal neurons in the presence or absence of cortical and nigral (SN/VTA) inputs. Striatum and cortex were dissected from prenatal (E18–E22) or early postnatal (P0–P2) rats, and SN/VTA was dissected from E14–15 fetuses; pieces were maintained up to 3 weeks in static slice culture. Triple cocultures containing SN/VTA exhibited rapid and robust dopamine (DA) innervation of the striatum in a patchy pattern, and homogeneous distribution within the cortical piece, regardless of the orientations of the three pieces. DA fibers within the striatal piece overlapped striatal patch neurons, marked by DARPP-32 immunoreactivity, in striatal cultures prepared from all age rats, but development most analogous to that seen in vivo was observed with the use of late prenatal (E20–E22) striatum. The patch/matrix organization was maintained in cultures prepared from late prenatal striatum in the presence of cortical and nigrostriatal DA afferents. In addition, a more complete transition to a patchy organization was observed in E18/19 striatal cultures in the presence of cortical and DA innervation. Electrophysiological recording demonstrated the presence of both spontaneous and cortically evoked activity in striatal medium spiny neurons; this activity was greatly influenced by the presence of DA innervation. These findings demonstrate the importance of afferent innervation in the maturation of striatal neurons in organotypic cultures. **Synapse** 62:487–500, 2008. © 2008 Wiley-Liss, Inc.

INTRODUCTION

Organotypic coculture of brain regions is being increasingly used as an effective means of recreating brain circuitry in a simplified and easily manipulated model. These cultures have generally been used for either of two purposes: (1) to study cellular interactions in a controlled setting that morphologically approximates the in vivo situation, and (2) to study the anatomical development of patterns and circuits. In order to more effectively manipulate and control the factors that are thought to influence development of the striatum, we have used the static slice culture insert method of organotypic cultures originally developed by Stoppini et al. (1991). In contrast to the roller-tube culture procedure also commonly used (Gahwiler, 1988; Jaumotte and Zigmond, 2005; Ostergaard et al., 1990; Plenz and Kitai, 1998a,b), slices maintain a thickness of 100–200 μm during the culture period, and this makes them more suitable for the study of three-dimensional patterns within the

regions of interest. Our initial work demonstrated the feasibility of this approach for study of the development of the striatal patches after bromo-deoxyuridine birthdating (Snyder-Keller et al., 2001).

Several investigators have demonstrated that appropriate connectivity develops between brain

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Present address for Kuei Y. Tseng: Department of Cellular and Molecular Pharmacology, RFUMS/The Chicago Medical School, North Chicago, IL 60064.

Present address for David J. Graber: Department of Pathology, Dartmouth Medical School, Lebanon, NH 03765.

Present address for Patricio O'Donnell: Department of Anatomy and Neurobiology, University of Maryland School of Medicine, 20 Penn St., Baltimore, MD 21201.

*Correspondence to: Abigail Snyder-Keller, Wadsworth Center, David Axelrod Institute, New York State Department of Health, 120 New Scotland Ave, Albany, NY 12208, USA. E-mail: snykell@wadsworth.org

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regions grown in organotypic cocultures. Afferent innervation of forebrain regions demonstrates a specificity that relates to *in vivo* development: in cortex, thalamocortical connections are restricted to layer IV, and monoaminergic innervations demonstrate the appropriate densities (Bolz et al., 1990; Guthrie et al., 2005; Molnar and Blakemore, 1999; Palmer et al., 2001). Similarly, nigrostriatal dopaminergic (DA) innervation occurs readily *in vitro*, where DA afferents demonstrate the appropriate preference for striatal tissue over other target regions (Franke et al., 2003; Gomez-Urquijo et al., 1999; Holmes et al., 1995; Ostergaard et al., 1990). However, the pattern of innervation has varied greatly among studies, most of which have employed postnatal tissues. Our studies have concentrated on the use of striatal tissue that is derived from rat fetuses at various pre- and postnatal ages, thus allowing an assessment of the development of nigrostriatal connectivity that more closely approximates the *in vivo* situation. The use of embryonic day (E)14/15 ventral mesencephalon as the source of DA neurons is optimal in the organotypic situation, because the DA neurons are fully postmitotic and they express DA markers, but minimal axotomy is incurred upon dissection.

In this study, we compared prenatal striatal slices, either before (E18–19) or after (E21–22) striatal patch formation, with the more commonly used postnatal tissue. To investigate the relationship between DA innervation and the striatal patch compartment, we focused on dopamine and cAMP-regulated phosphoprotein-32 kDa (DARPP-32), a marker of striatal neurons that is expressed specifically by patch neurons during development (Foster et al., 1987). Finally, we used electrophysiological recording to demonstrate physiological activity in these cultures, and to assess the importance of cortical and nigral innervation in the anatomical and physiological development of striatal neurons.

MATERIALS AND METHODS

Preparation of organotypic cultures

Fetal tissue was obtained from timed-pregnant Sprague Dawley rats (day of sperm = E0). All animal procedures were approved by the Institutional Animal Care, and Use Committee of the Wadsworth Center. Laparotomies were performed under isoflurane anesthesia. The brains were rapidly stripped of meninges in Ham's F12 medium and were kept on ice for further dissection. The ventral mesencephalon (hereafter referred to as "SN/VTA" to indicate that both substantia nigra (SN) and ventral tegmental area (VTA) regions were included) was dissected out of E14–15 fetal brains. Forebrain slices were cut from the brains of rat pups ranging from E18 to P2 at 300- μ m thickness on a vibratome. The coronal sections were dis-

sected further, so as to separate striatum and cortex. Striatum, SN/VTA, and cortex were stored in separate small Petri dishes containing ice-cold Ham's F12 medium.

Six-well culture trays containing Costar clear membrane inserts (Costar, Corning, NY) were prepared by preincubation for 1 h with 1.25 ml/well of Neurobasal medium (Gibco) containing 20% horse serum. This base medium was supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), bicarbonate (1.2 mg/ml), and Hepes (4.5 mg/ml). Before placement, poly-lysine drops were added directly to some well membrane inserts with a sterile pipette, to restrict movement of the pieces during incubation. The sections were placed on the membranes by means of a sterile pipette with a cut and flame-polished tip. Pieces of cortex and/or SN/VTA were then placed about 1 mm from selected striatal pieces.

The cultures were incubated at 37°C under 5% CO₂ for a period ranging from 6 to 24 days, with the medium changed every 3–4 days. After 3 days *in vitro* (DIV), cultures were switched to serum-free Neurobasal medium containing B27 supplement (2%; Gibco). In cultures used for DARPP-32 immunostaining, brain-derived neurotrophic factor (BDNF; Amgen; 50 ng/ml) was administered to the medium underneath the cell culture inserts 20 h prior to fixation of the cultures.

Electrophysiology

All electrophysiological recordings were conducted following the same procedures as recently reported (Tseng et al., 2007). Cocultures were placed in a submersion chamber and perfused at 2 ml/min with artificial CSF containing (in mM) 125 NaCl, 25 NaHCO₃, 10 glucose, 3.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂ (pH 7.45, osmolarity 295 \pm 5 mOsm), oxygenated with 95% O₂/5% CO₂. Striatal neurons were identified under visual guidance using infrared-differential interference contrast (IR-DIC) video microscopy, with a 40 \times water-immersion objective mounted on an upright microscope (Olympus BX51). The image was detected with an IR-sensitive CCD camera (Dage-MTI) and displayed on a monitor. Patch pipettes (5–8 M Ω) were pulled from 1.5- μ m borosilicate glass capillary and were filled with (in mM) 115 K-gluconate, 10 HEPES, 2 MgCl₂, 20 KCl, 2 MgATP, 2 Na₂-ATP, 0.3 GTP internal solution (pH = 7.3, 280 \pm 5 mOsm), also containing Neurobiotin (0.125%). Whole-cell current-clamp recordings were performed with a computer-controlled amplifier (MultiClamp 700A; Axon Instruments, Sunnyvale, CA), and acquired with Axoscope 8.1 (Axon Instruments) at a sampling rate of 10 KHz.

All recordings were conducted at 33–35°C. The liquid junction potential was not corrected, and elec-

trode potentials were adjusted to zero before acquisition of the whole-cell configuration. Input resistance (calculated from the linear portion of the IV curve in the hyperpolarized direction), membrane potential, and cell excitability evoked by a 500-ms duration depolarizing current pulse were monitored periodically for each neuron. Spontaneous membrane potential fluctuations including plateau depolarizations and excitatory postsynaptic potentials (EPSPs) were also analyzed and compared among cells from different organotypic cocultures.

Cortico-striatal synaptic responses were evoked with a bipolar stimulating electrode placed in the cortical piece at around 2–3 mm from the recording site. The bipolar stimulating electrode was made with a pair of twisted Teflon-coated nichrome wires (75 μm) with the tips separated by 200–300 μm . Typically, the stimulation intensity was first adjusted so as to evoke a synaptic response of around 15–20 mV in amplitude (range from 0.3 to 0.5 mA) at the beginning of the recordings. The evoked response was induced by single pulses of 300- μs duration, delivered with a stimulus isolation unit (ISO-Flex, AMPI, Jerusalem, Israel), and was controlled by a pulse generator (Master-8; AMPI). To characterize the response, 2–4 series of 10–15 single pulses stimulation were delivered every 20 s, alternating with periods of minutes of spontaneous activity without stimulation.

Neurobiotin labeling

At the conclusion of recording, cultures were transferred to 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at room temperature. After fixation, cultures were rinsed in PBS and incubated in 0.5% Triton/PBS for 3 h, followed by overnight incubation in avidin–biotin–peroxidase complex (ABC Elite kit; Vector Laboratories) made in 0.2% Triton/PBS. After rinsing, cultures were reacted in 0.05% diaminobenzidine containing 0.0015% H_2O_2 for 5 min, to reveal brown neurobiotin-labeled cells.

Immunohistochemistry

The cultures were rinsed in PBS, and the inserts were then transferred to 4% paraformaldehyde/4% sucrose in PBS for 20 h at 4°C. During this time, cultures were excised by cutting the membrane of the insert with a scalpel. After fixation, the cultures were rinsed for 30 min and then washed in 0.2% Triton X-100 for 30 min. Nonspecific staining was blocked by pretreatment with 5% normal goat serum (NGS)/0.2% Triton/PBS for 2 h. The sections were incubated in primary antibodies diluted in 2.5% NGS for 40 h at 4°C: DARPP-32 (Cell Signaling; 1:75); tyrosine hydroxylase (TH; Chemicon; 1:600); and glutamate receptor1 (GluR1; Chemicon, 1:2500). The cultures

were then rinsed for 45 min, and washed for 10 min in 0.1% Triton, before a 2-h incubation at room temperature in biotinylated goat anti-rabbit secondary antibodies, followed by a 45-min rinse and a 2-h incubation in avidin–biotin complex linked to peroxidase (Elite ABC Kit, Vector Laboratories). Staining was revealed by incubation in 0.05% diaminobenzidine containing 0.0015% H_2O_2 in the presence (black stain) or absence (light brown stain) of 0.25% nickel ammonium sulfate. Double-labeling for DARPP-32 and TH was done with sequential immunostaining, with DARPP immunoreactivity developed first. The cultures were viewed and photographed with an Olympus Vanox Photomicroscope or Olympus BX-2 with Olympus 5050 digital camera.

RESULTS

After placement at 1 mm apart on the culture insert membranes, striatal, cortical, and nigral pieces all flattened and spread somewhat during the first few days in culture, but retained a thickness of about 200 μm . Pieces invariably fused within 3 days after placement. After this point, the size of the striatal piece was maintained consistently when both cortex and SN/VTA were present; however, in those cultures of striatum alone, a steady loss of volume was observed. This was particularly evident with the use of postnatal striatal tissue, which exhibited very poor survival when cultured alone. Fetal (E18–E22) or neonatal (P0–P1) striatal tissue, when cocultured with SN/VTA and/or cortex, appeared healthy for up to 3 weeks in vitro. Moreover, the subventricular zone exhibited significant expansion in size, particularly with the use of younger fetal striatal tissue, indicative of continued cell proliferation. Because the cultures remain attached to the transparent membrane after fixation, the spatial orientation of the pieces is preserved during the immunocytochemical processing, and connectivity between the pieces remains intact. Unless otherwise indicated, each finding described later is based on the observations of at least 10 cultures.

Influence of dopamine innervation on striatal development in organotypic cultures

Coculturing with E14/15 SN/VTA led to a substantial innervation of the striatal and cortical pieces with DA fibers (Fig. 1A). TH-immunoreactive fibers were observed to grow into the striatal piece by 4 DIV, the earliest time point examined, and they rapidly increased in density up to 10 DIV (Fig. 1D), beyond which no further changes in the density or pattern of innervation were observed. The striatal innervation at 10 DIV was characterized by patches of dense DA fibers on a background of lower innervation

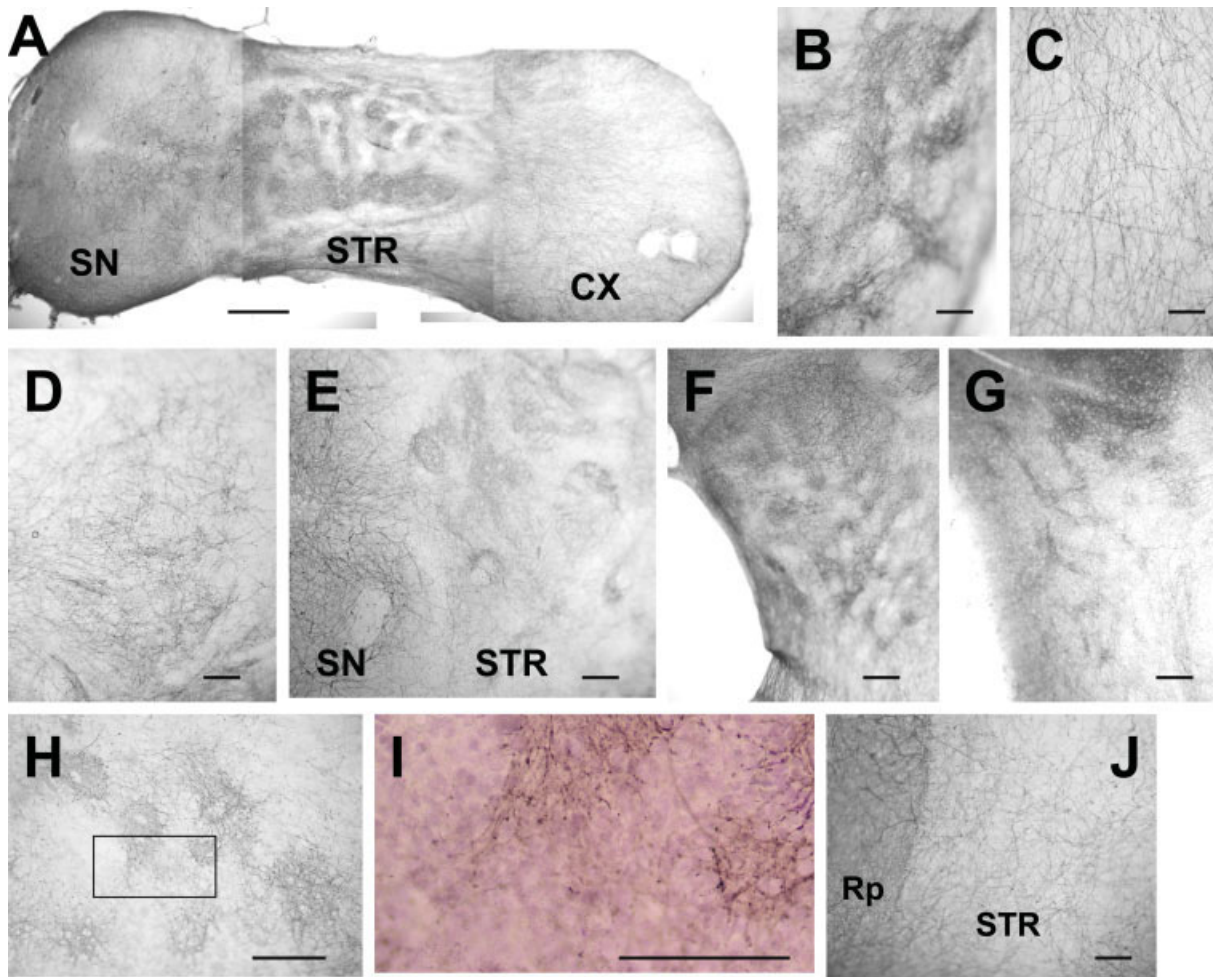


Fig. 1. Tyrosine hydroxylase (TH) immunostaining of DA innervation in organotypic cocultures. **A:** Low-magnification view of a triple coculture (E22 str + cx) exhibiting patchy DA innervation in the striatum, and less dense, homogeneous innervation in the cortex (15 DIV). **B:** High-power view of striatal DA innervation. **C:** High-power view of cortical DA innervation. **D:** Patches of DA fibers forming at 8 DIV (E20 str). **E:** Patches of DA fibers in an E19 striatum (15 DIV). **F:** Patches of DA fibers in a P0 striatum (15 DIV). **G:** DA

innervation in a P2 striatal slice is most dense in the medial aspect (left side; 14 DIV). **H:** DA patches in an E20 striatal coculture, counterstained with hematoxylin (purple profiles in I, high magnification of box in H). **J:** Serotonin-immunoreactive fibers in striatal piece of a coculture of E20 striatum with E14 raphe. SN, SN/VTA; STR, striatum; CX, cortex; Rp, raphe. Scale bar = 500 μ m in (A); 100 μ m in (B), (C), (I), and (J); 200 μ m in (D–H).

(Fig. 1B). TH-immunoreactive fibers also grew through the striatal piece to enter the cortical piece (both somatosensory and prelimbic), where they exhibited a homogeneous, and less dense, distribution (Fig. 1C). At longer times in vitro, the DA innervation was still clearly patchy in the striatum and homogeneous in cortex.

This patch pattern of DA innervation was even observed in cocultures of SN/VTA with E18/19 striatum (Fig. 1E), in which the compartmentalization of striatal neurons was not yet established at the time of culturing. A similar pattern was observed in late fetal (E20–22; Fig. 1A) and early postnatal (P0–P1; Fig. 1F) striatal slices, in which the compartmentalization of medium spiny neurons (MSN) into patch and matrix is already established (Snyder-Keller, 1991). Surprisingly, however, in P2 or older striatal

slices, patches of DA fibers were most notable within the medial aspect, with the rest of the striatum receiving less innervation (Fig. 1G).

Two experiments were performed to verify that the patchiness of TH-immunoreactive fibers within the striatum was not due to inconsistencies in either the thickness of the striatal pieces or the distribution of striatal neurons. First, counterstaining with hematoxylin revealed a homogeneous distribution of cell bodies both inside and outside of patches of TH-immunoreactive fibers ($n = 5$; Fig. 1H,I). Moreover, coculturing with fetal (E15) metencephalon containing the raphe nucleus led to a homogeneous innervation of the striatal piece with serotonergic fibers, even though the overall density was comparable with that seen with TH-immunoreactive fibers (Fig. 1J).

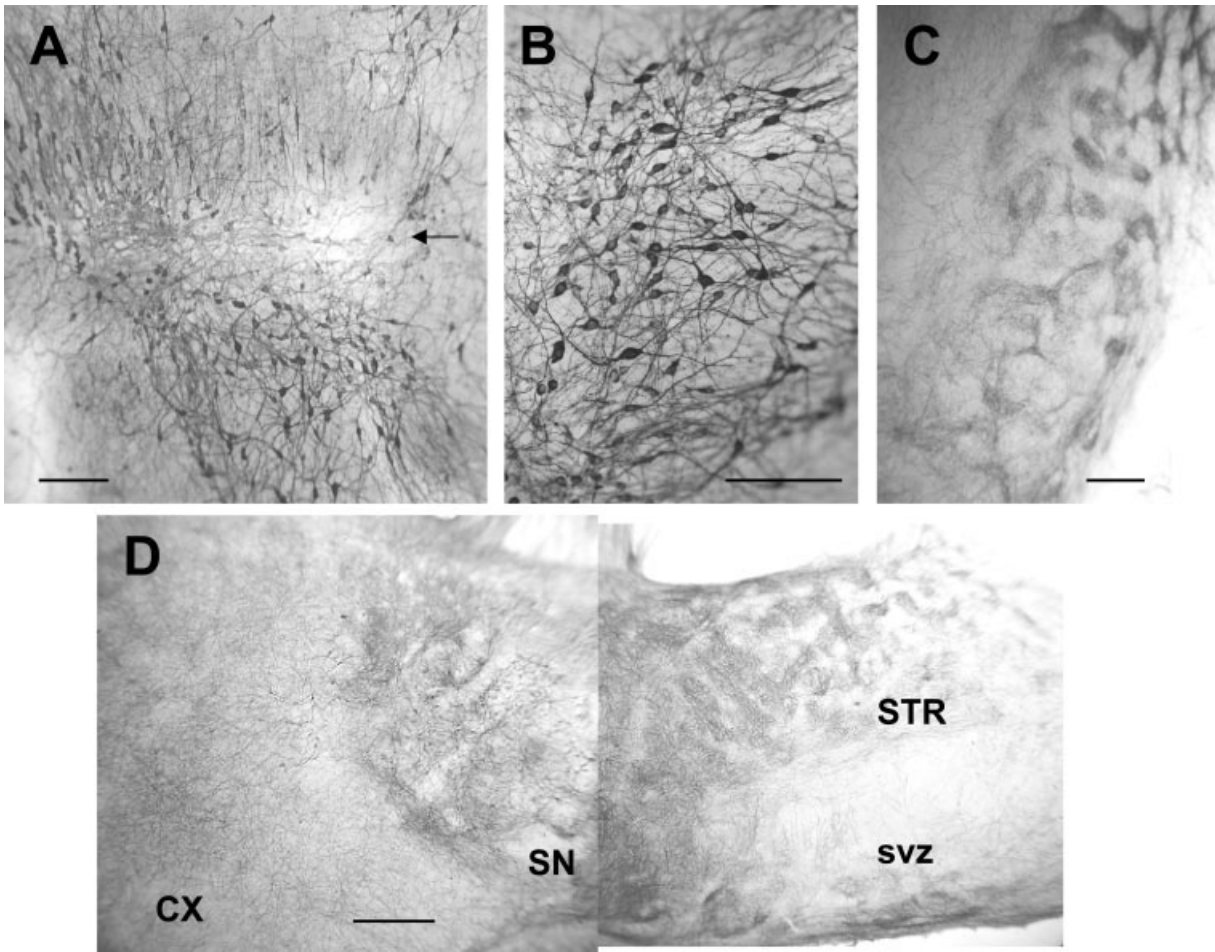


Fig. 2. DA neurons in the SN/VTA of organotypic cocultures and pattern of ingrowth. **A:** Low power view of TH-immunoreactive neurons in a SN/VTA piece; arrow points to the midline. **B:** High-power view of TH-immunoreactive neurons in the SN/VTA piece. **C:** Pattern of DA innervation in the striatal piece (E21) cocultured

with medial portion (within 1 mm of the midline) of the SN/VTA (15 DIV). **D:** Triple coculture of cortex-SN/VTA-striatum, in that order (12 DIV). Striatum and cortex are from E21 fetuses. svz, subventricular zone. Scale bar = 200 μ m in (A–C); 500 μ m in (D).

Within the SN/VTA piece, TH-immunoreactive neurons were densely distributed on either side of the midline (Fig. 2A). Although separation of DA neurons into SN and VTA may not have occurred by E14/15, we examined the innervation arising from medial and lateral regions of the E14/15 ventral mesencephalon pieces. In pieces that were limited to tissue less than 1 mm from the midline, numerous TH-immunoreactive neurons were present that conformed in size and shape to the majority of DA neurons present in the whole SN/VTA pieces (Fig. 2B). Consistent with the idea that lateral migration has not yet occurred at this age, fewer TH-immunoreactive neurons were present in the lateral pieces (data not shown). Interestingly, DA innervation from the medial SN/VTA pieces exhibited a patchy pattern that was even more striking than that observed with the standard SN/VTA dissection (Fig. 2C).

To verify that the differences in pattern of innervation between striatum and cortex were not a function

of distance from the SN/VTA after placement on the membrane, we prepared some cultures in which cortex was placed between the striatum and SN/VTA. Cortical DA innervation was more dense in these cultures, but still homogeneous in distribution (data not shown). TH-immunoreactive fibers grew past the cortex to innervate the striatum, where they terminated in a patchy distribution. Additional experiments were done using a choice paradigm, in which SN/VTA was placed between the striatum and the cortex. TH-immunoreactive fibers grew in both directions, and again established the appropriate pattern of innervation: patches in the striatum, and homogeneous in the cortex ($n = 6$; Fig. 2D). No significant differences in innervation density were observed between cocultures containing somatosensory cortex and those containing prefrontal cortex. In addition, DA innervation of cortex was always homogeneous, regardless of the age of cortex used (E18–P2; data not shown).

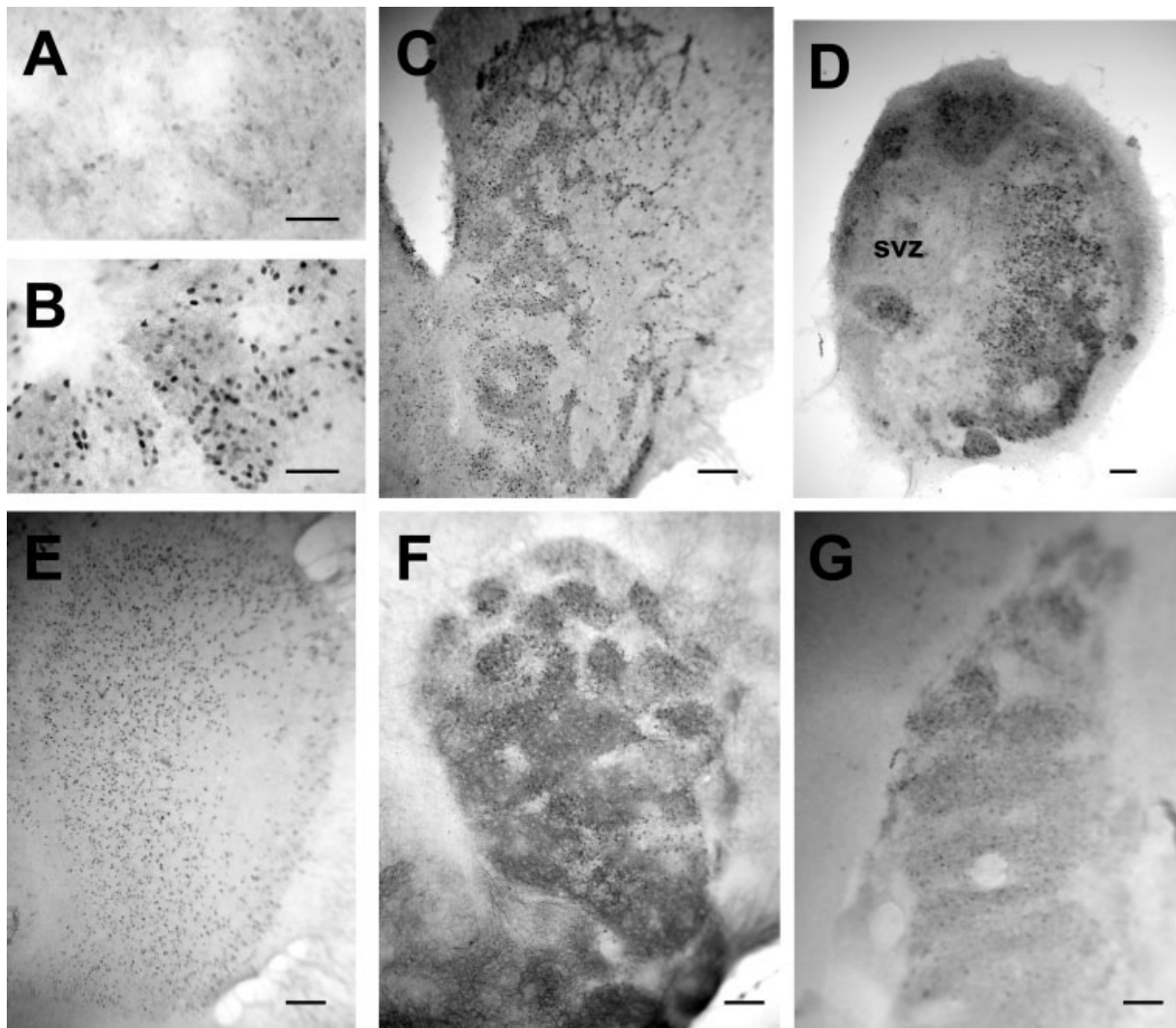


Fig. 3. DARPP-32 expression in organotypic cocultures. **A:** High-power view of DARPP-32 expression in striatal neurons in an E20 coculture in the absence of exogenous BDNF. **B:** 20 h after addition of BDNF (50 ng/ml) to the medium, DARPP-32 expression in striatal neurons (E20) is more robust. **C:** Patches of DARPP-32-immunoreactive neurons in E22 striatum cocultured with SN/VTA and E22 cortex (11 DIV). **D:** DARPP-32-immunoreactive neurons are more homogeneously distributed in an E21 striatum cultured

alone (14 DIV). **E:** Homogeneous distribution of DARPP-32-immunoreactive neurons in E19 striatum at 5 DIV. **F:** Transition to patches of DARPP-immunoreactive neurons in an E19 striatum cocultured with E21 cortex and SN/VTA (14 DIV). Culture also stained for TH, but patches of black DARPP-immunoreactive cells are still visible. **G:** E19 striatum cocultured with E21 cortex only (14 DIV). Scale bar = 100 μ m in (A) and (B); 200 μ m in (C–G).

Phenotypic development of striatal neurons in cocultures of striatum with SN and cortex

To determine whether DA fibers were innervating striatal neurons that populate the patches (or “striosomes”), we used an antibody to dopamine and cAMP-regulated phosphoprotein (DARPP-32), which has been shown to selectively stain the patch neurons during the late prenatal/early postnatal period (Foster et al., 1987). DARPP-32 expression in striatal cultures was generally weak (Fig. 3A) unless BDNF (50 ng/ml) was added to the medium for at least 12 h (Fig. 3B), in agreement with findings of previous studies indicating the dependence of DARPP-32

expression in vitro on the addition of BDNF to the medium (Ivkovic and Ehrlich, 1999; Ivkovic et al., 1997; Nakao et al., 1995). In addition, the intensity of expression of DARPP-32, as well as the distribution of DARPP-32-immunoreactive neurons, was clearly dependent on the age of the striatal tissue and coculture conditions. Cultures prepared from E21–22 fetuses and immunostained at early times in vitro exhibited a patchy distribution of DARPP-32-immunoreactive neurons (Fig. 3C), which approximated the distribution observed in vivo. However, this patchy distribution was only maintained for the entire culture period if striatal tissue was cocultured with

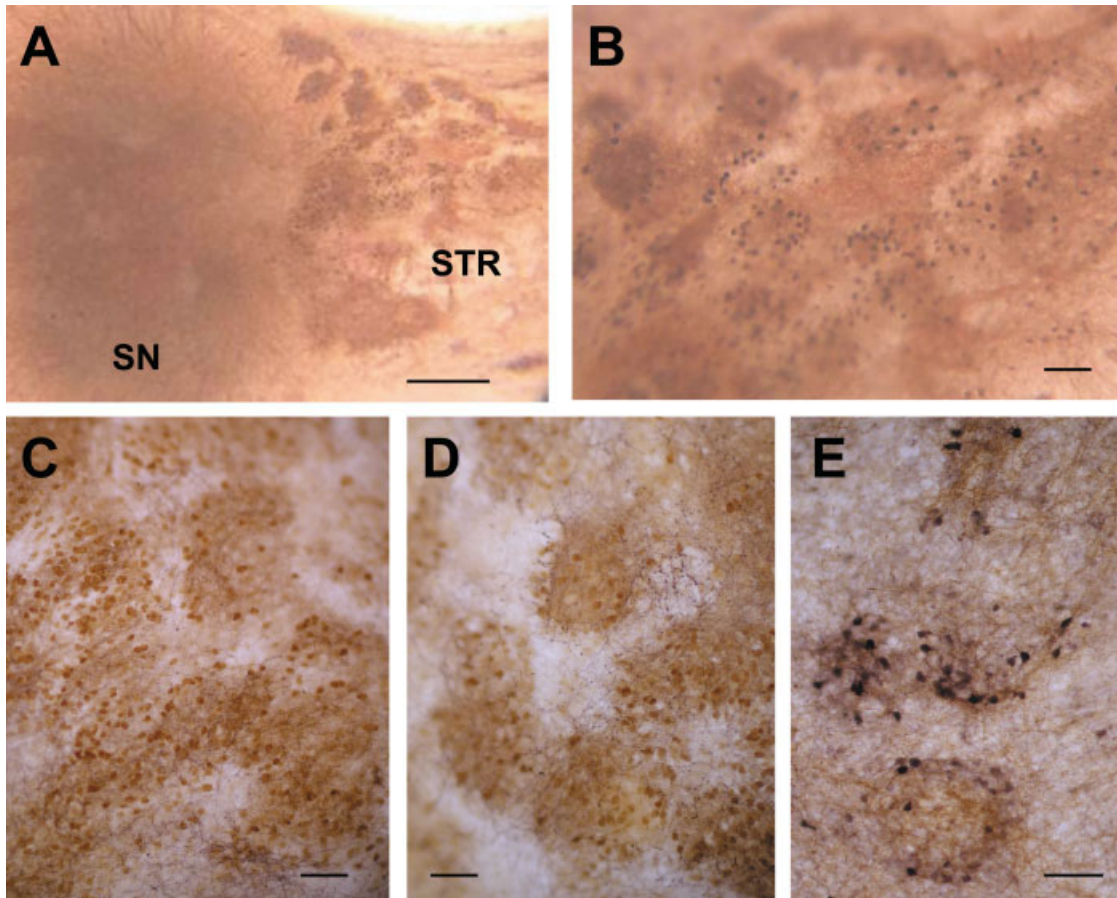


Fig. 4. Correspondence between DA innervation and developing striatal patches. Low-power (A) and high-power (B–E) views of E21 (A, B, E), P0 (C), and E19 (D, with E21 cx) cocultures double-labeled for DARPP-32-immunoreactive striatal neurons and TH-immunore-

active DA fibers. In (A), (B), and (E), DARPP-immunoreactive neurons are black, and DA fibers are brown; in (C) and (D) the reverse is true. Scale bar = 500 μ m in (A); 100 μ m in (B–E).

SN/VTA and cortex. In E21 striatum cultured alone, the DARPP-32-immunoreactive neurons were observed to merge into one large cluster within the slice (Fig. 3D). A strict analysis to compare the degree of patchiness of striatal DARPP-immunoreactive neurons between striatal cultures and cocultures was precluded by the fact that striatal tissue cultured alone invariably appeared to decrease in size with time *in vitro*, indicating poorer survival in the absence of afferents.

In cultures prepared from E18/19 striatal tissue, DARPP-32-immunoreactive neurons were distributed homogeneously at short times *in vitro* (see Fig. 3E), consistent with the fact that E19 is prior to *in vivo* patch formation (Snyder-Keller, 1991). Again, the addition of BDNF to the medium was necessary to elevate DARPP-32 expression sufficiently to reveal DARPP-32-immunoreactive neurons. Coculturing with either source of afferent innervation known to contain BDNF (cortex or substantia nigra) also increased DARPP-32 expression, although not to the extent observed with exogenous BDNF (data not

shown). Most importantly, with increasing time in culture, the distribution of DARPP-32-immunoreactive cells in E18/19 striatal cocultures transitioned to a patchy pattern, especially if both SN/VTA and cortex were present in the cocultures (compare Figs. 3F and 3G). In agreement with our previous findings (Snyder-Keller, 2004), coculturing with E21 cortex (rather than E19 cortex) was more effective at promoting patch formation when only cortex was included. Moreover, the additional presence of SN/VTA resulted in patch formation in 88% (with E19 cortex) to 100% (with E21 cortex) of cocultures, compared to 17 and 60%, respectively, in the absence of SN/VTA.

Correspondence between DA innervation and developing striatal patches

Double-labeling was performed to determine whether patches of DA innervation, revealed by TH immunocytochemistry, overlapped the patches of DARPP-32-immunoreactive striatal neurons. In cocultures

containing SN/VTA, clusters of DARPP-32-immunoreactive neurons were observed to lie within the patches of TH-immunoreactive fibers (Fig. 4). Substantial, albeit not complete, overlap was observed in both late prenatal (E21–22; Figs. 4A, 4B, and 4E) and postnatal (P0; Fig. 4C) striatal slices. In cultures prepared from E18/19 fetuses, a partial correspondence between DA fibers and developing striatal patches was also observed (Fig. 4D).

Electrophysiological analysis of functional connections in organotypic cocultures

To verify the physiological nature of the afferent innervation revealed using anatomical means, we subjected cocultures of perinatal striatum (E18–19 and E20–22) with cortex and/or SN/VTA to electrophysiological recording. After 10–24 DIV, cultures were transferred to the recording chamber of an Olympus BX50WI fixed-stage upright microscope, and perfused in oxygenated (95% O₂/5% CO₂) artificial CSF. Using IR-DIC video microscopy to visualize putative medium spiny neurons (MSN), we performed whole-cell patch clamp recordings in individual neurons from the striatal piece. MSNs were identified electrophysiologically by their characteristic ramp depolarization and delayed action potential firing in response to depolarizing pulses, as well as by the inward rectification with negative current injection (Figs. 5A and 5B).

Striatal neurons recorded from corticostriatal cocultures containing an E18–19 ($n = 8$) or E20–22 ($n = 18$) striatum showed similar resting membrane potential (E18–19: -69.8 ± 2.2 mV; E20–22: -69.4 ± 2.3 mV; mean \pm SD) and input resistance (E18–19: 208.9 ± 54.2 M Ω ; E20–22: 261.3 ± 81.7 M Ω), and they all exhibited spontaneous EPSPs lasting from 30 to 80 ms (Fig. 5C). Spontaneous EPSPs could be blocked with bath application of the non-NMDA antagonist CNQX, confirming that these events were driven by glutamatergic excitatory inputs. Similarly, electrical stimulation of the cortical piece evoked short (\sim 100-ms) EPSPs in both preparations (\pm SN/VTA) and was blocked with CNQX ($n = 6$), indicating the presence of functional glutamatergic excitatory corticostriatal connections (Fig. 5D).

The presence of a SN/VTA piece in corticostriatal cocultures significantly enhanced the responses of MSNs to cortical stimulation, resulting in prolonged plateau depolarizations lasting hundreds of ms (measured as decay to half-amplitude) (Fig. 6A). Moreover, MSNs recorded from corticostriatal-SN/VTA cocultures with an E20–22 striatum ($n = 26$) exhibited significantly longer plateaus than those with an E18–19 ($n = 7$) striatum (Fig. 6A). A two-way ANOVA analysis revealed a significant interaction effect of $F(1,52) = 14.35$ ($P < 0.0004$) between striatal ages and the

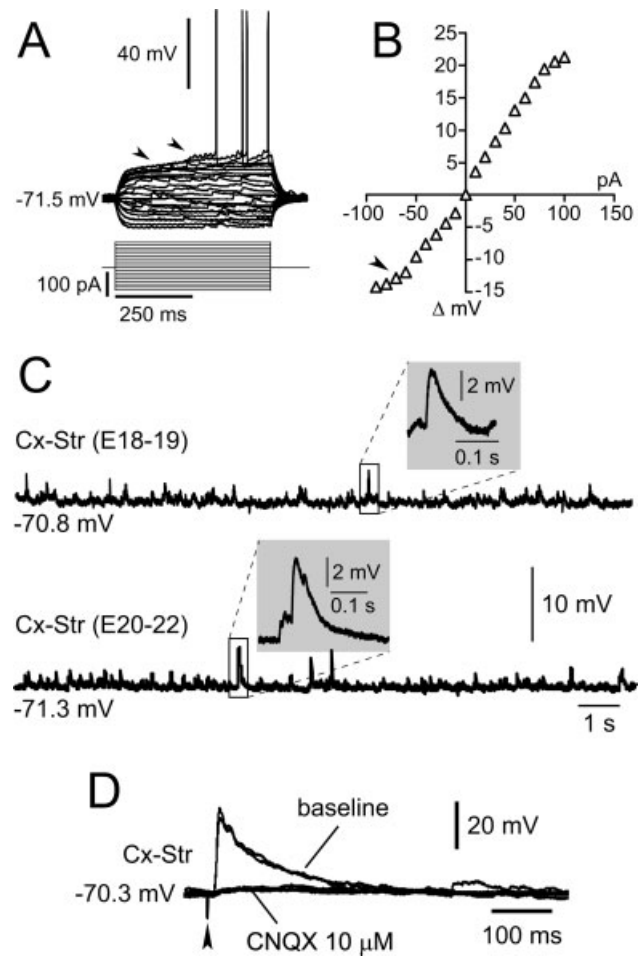


Fig. 5. Electrophysiological characteristics of striatal MSNs in corticostriatal organotypic cocultures. **A**: Typical voltage responses to somatic current steps (500 ms, from -100 to $+100$ pA) in a striatal MSN. Note the characteristic ramp depolarization and delayed action potential firing responses (arrowheads) to suprathreshold current steps. **B**: Current–voltage plot obtained from the traces shown in (A), illustrating the typical inward rectification with negative current injection (arrowhead). **C**: Examples of traces obtained from cortico–striatal (Cx–Str) cocultures containing either an E18–19 or an E20–22 striatum. All MSNs recorded from Cx–Str E18–19 and E20–22 cocultures exhibited similar spontaneous excitatory postsynaptic potentials (insets). **D**: Traces of cortical evoked responses recorded in MSNs before and after bath application of a non-NMDA antagonist CNQX. Electrical stimulation of the cortical piece (indicated with an arrowhead) typically induces a short (\sim 100-ms) depolarizing postsynaptic potential in MSN of Cx–Str cocultures. This response could be completely eliminated with a non-NMDA receptor antagonist (CNQX 10 mM), indicating that the response was mediated by glutamate.

presence of SN/VTA on the duration of the evoked response (Fig. 6B). These results indicate that the impact of DA innervation on corticostriatal cocultures differs between E18–19 and E20–22 striatal ages.

After intracellular Neurobiotin injection, cultures were fixed and reacted to reveal the location of the recorded cells. Nearly all (95%) of the Neurobiotin-labeled cells exhibited the MSN phenotype (Fig. 7). Full dendritic labeling permitted the visualization of

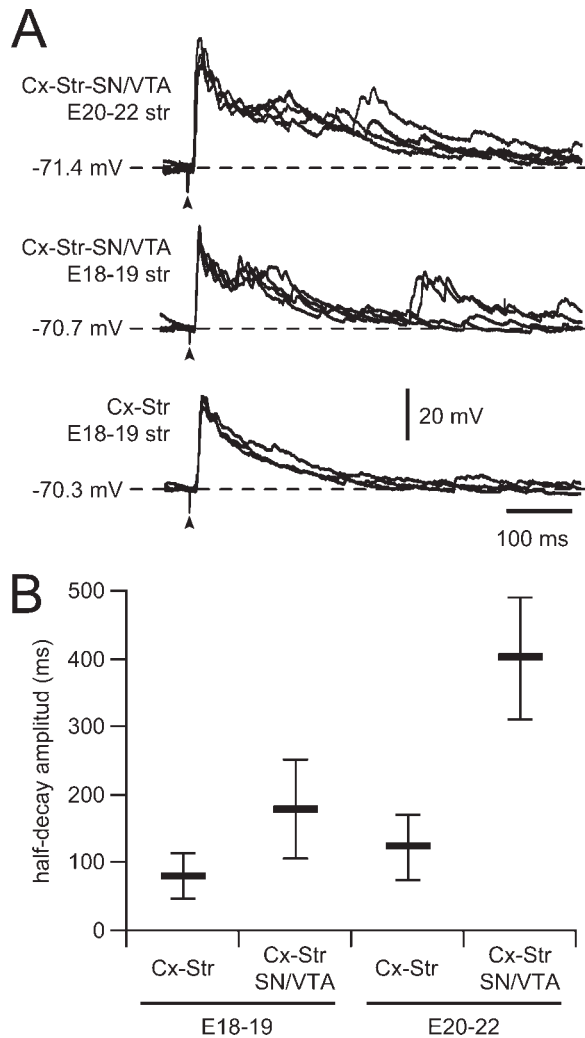


Fig. 6. Effect of cortical stimulation on MSN responses recorded from corticostriatal cocultures containing a substantia nigra (SN/VTA) piece. **A:** Traces of cortically evoked responses in MSNs recorded from corticostriatal E20–22 and E18–19 cocultures containing a SN/VTA piece. Cortical stimulation resulted in prolonged plateau depolarizations only in cocultures with SN/VTA; in corticostriatal cultures, a short EPSP was observed instead. The onset of the cortical stimulation is indicated with arrowheads. **B:** Bar graphs summarizing the duration of cortically induced MSN responses recorded from corticostriatal cocultures containing either an E18–19 or an E20–22 striatum with or without a SN/VTA piece. The duration of the evoked responses were measured from the half-decay amplitude. Plateau depolarizations could be observed only in corticostriatal cocultures containing a SN/VTA piece. However, the duration of these events was significantly longer in cocultures containing an E20–22 striatum.

spines on most labeled neurons. No apparent differences in MSNs were observed in young (E19) and older (E22) striatal tissues (Figs. 7A and 7B). Double labeling of cocultures containing neurobiotin-filled neurons revealed these neurons' location in striatal patches. For example, when cocultures containing SN/VTA were additionally immunostained to reveal the distribution of nigrostriatal DA innervation, recorded cells

were always found to lie within a patch of TH-immunoreactive fibers (Fig. 7C). Neurobiotin-labeled neurons were also found to lie within clusters of DARPP-32-immunoreactive neurons (Fig. 7D). Because cultures used for electrophysiological recording were not exposed to BDNF, the DARPP labeling was weak. Thus, we also double-labeled with GluR1, another marker of patch neurons during development (Snyder-Keller and Costantini, 1996); as expected, we found that the recorded neurons lay within GluR1-immunoreactive patches (Fig. 7E).

DISCUSSION

These findings underscore the utility of the static-insert type of organotypic cultures, originally developed by Stoppini et al. (1991), for both anatomical and physiological studies of striatal development. Particularly with the use of prenatal tissue, cocultures of striatum with SN/VTA and/or cortex can be maintained up to 21 DIV. Key features of the developing striatum, such as patchy DA innervation and clustering of DARPP-32-immunoreactive patch neurons, are present as early as 1 week in vitro. Furthermore, because development proceeds at a rapid rate in these cultures—with complete innervation with DA fibers achieved by 10 DIV—cultures do not need to be maintained for periods beyond a few weeks. Although roller-tube cultures can be maintained for longer periods (Jaumotte and Zigmond, 2005; Ostergaard et al., 1996; Plenz and Kitai, 1998a,b), maturation of the DA innervation takes 3–4 weeks using this technique (Jaumotte and Zigmond, 2005; Ostergaard, et al., 1996). Moreover, the excessive thinning that occurs with the roller-tube technique renders it less suitable for the study of the three-dimensional cellular organization of the cultured brain regions. This study builds upon our previous studies (Snyder-Keller, 2004; Snyder-Keller et al., 2001), in demonstrating that the extensive connections between SN/VTA, cortex, and striatum that develop in this model, not only exhibit the appropriate pattern of distribution, but are also important for the physiological development of the striatal medium spiny neurons.

Dopamine innervation in organotypic cocultures

In cocultures containing E14/15 SN/VTA, dense innervation with TH-immunoreactive fibers was observed within the first week in vitro. Furthermore, the DA innervation exhibited a regionally appropriate pattern of ingrowth, with patchy innervation of the striatum, as occurs during the perinatal period in vivo (Voorn et al., 1988; Snyder-Keller, 1991), and homogeneous and less dense innervation of the cortex. These differences were seen regardless of the order and orientation in which the pieces had been

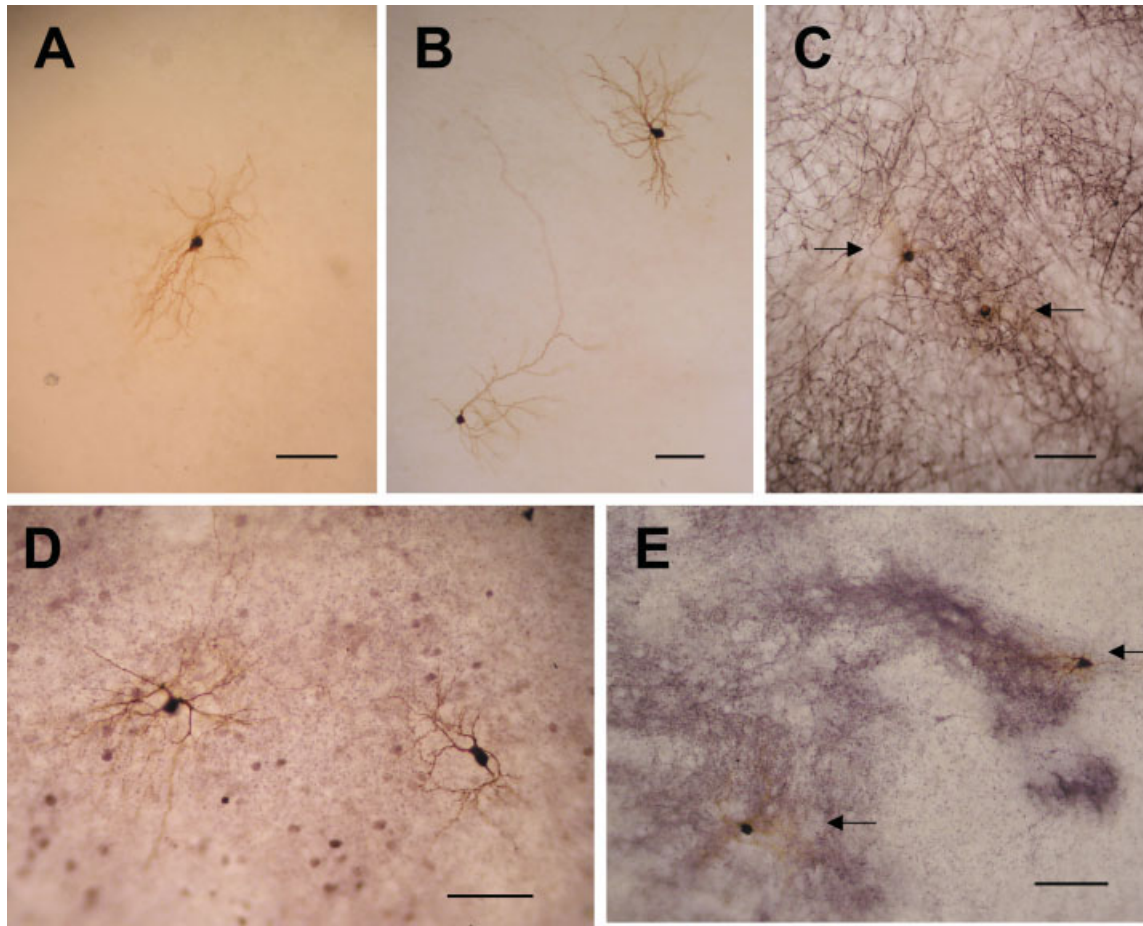


Fig. 7. Neurobiotin labeling of recorded striatal neurons. **A:** Neurobiotin-labeled MSN in E21 striatum. **B:** Two neurobiotin-labeled MSNs in E19 striatum. **C:** Two neurobiotin-labeled MSNs (arrows) within a patch of TH-immunoreactive fibers (P0 striatum).

D: Two neurobiotin-labeled MSNs in regions of DARPP-32 immunoreactivity (E21 striatum). **E:** Two neurobiotin-labeled MSNs lying within GluR1-immunoreactive patches in E21 striatum. Scale bar = 100 μ m for all.

placed. When the SN/VTA was restricted to 1 mm on either side of the midline, a distinctively patchy distribution of DA afferents was still revealed throughout the striatum. This is not surprising, given that full lateral migration of DA neurons, to differentiate between lateral SN and medial VTA, may not have occurred by E14/15 in the rat; this inference is extrapolated from the mouse system, in which SN and VTA DA neurons still lie in one cluster near the midline at E15 (Nishikawa et al., 2003). Thus, differentiation between innervation arising specifically from SN and that from VTA, as can be done with the use of postnatal tissue in culture (Franke et al., 2003; Jau-motte and Zigmond, 2005; Plenz and Kitai, 1998b), is not possible with the use of younger ventral mesencephalic tissue. Immunocytochemical discrimination between VTA and SN neurons of the pars compacta, as has been done with transplanted neurons (Thompson et al., 2005), will be necessary if we are to determine whether different subsets of DA neurons give rise to striatal and cortical innervation.

Synapse

Dopamine fibers exhibited a much more robust ingrowth into prenatal striatum than into postnatal striatum. Although researchers employing the roller-tube technique have observed DA innervation from older SN/VTA into postnatal striatum (Ostergaard et al., 1990, 1996; Plenz and Kitai, 1998b), it is difficult to compare the densities achieved with the two approaches. Using an approach similar to ours, Gates et al. (2004) demonstrated robust innervation from E12 SN/VTA into both E19 and P4 striatum, but not into either younger (E15) or older (adult) striatal tissue. Becq et al. (1999) reported homogeneous DA innervation into P4 striatum. However, late prenatal (E19–21) striatal tissue is likely to possess more growth-promoting factors than postnatal tissue, which has already developed substantial DA innervation. It should be noted that because of the existence of DA fibers, excision of postnatal striatal tissue results in substantial terminal degeneration, discernible as dotted TH immunoreactivity within the first few days of culturing, predominantly within the

lateral half of the striatal slice (Snyder-Keller et al., 2001). The preexisting DA innervation *in vivo* is most dense laterally (Snyder-Keller, 1991), and the presence of degenerating DA terminals in this region might serve to inhibit new DA ingrowth. Although fairly extensive DA ingrowth was observed in P0–P1 striatal slices, a dramatic difference was observed by P2, with the new DA ingrowth restricted to the medial aspect of the slice. The most robust innervation occurred in E21–E22 striatum (removed just prior to birth), which exhibited dense patchy distribution of DA afferents throughout the slice.

Because neurons of the E18/19 striatum have not yet established the patchy organization characteristic of older striatal tissue, the presence of patches of DA fibers in organotypic cultures using E18/19 striatum cannot be considered to be a growth of DA fibers in response to cues elaborated by preexisting clusters of striatal patch neurons. It is also unlikely that DA fibers possess an intrinsic cue for patchy arborization, given that they project in a homogeneous pattern into cortical pieces. An alternative scenario is that DA fibers project to and arborize in the vicinity of corticostriatal terminals that release BDNF. This possibility might be assessed through comparison of DA innervation in E18/19 striatum in the presence or absence of cocultured cortex. Although the patches were most obvious in triple cocultures containing cortex, direct comparison to SN/VTA-striatal cultures was difficult, because in the latter cultures, the striatal tissue tends to “shrink” up toward the SN/VTA, thus losing some of the surface area that is important for visualization of these spatial features. Nevertheless, these observations demonstrate that the appropriate cues for establishment of the nigrostriatal DA pathway, as well as the appropriate distribution within the striatum, are maintained *in vitro*. Although the exact nature of these cues has not yet been determined (Riddle and Pollock, 2003), factors such as ephrins (Janis et al., 1999; Yue et al., 1999), netrins (Hamasaki et al., 2001), growth factors (Lopez-Martin et al., 1999; Oo et al., 2003), and extracellular matrix molecules (Charvet et al., 1998), in addition to afferent influences (see below), are likely to have roles.

Compared to the more commonly used postnatal tissues (Franke et al., 2003; Holmes et al., 1995; Jau-motte and Zigmond, 2005; Ostergaard et al., 1990, 1996; Plenz and Kitai, 1998a,b), the fetal SN/VTA used in our system provides a source of DA neurons that is closer in developmental stage to the end of neurogenesis and initiation of outgrowth, processes which occur on E14 in the rat (Gates et al., 2004; Voorn et al., 1988). In our cultures, the use of postnatal SN/VTA resulted in a very small number of surviving DA neurons (data not shown). Even dissection on E16 resulted in lower survival, consistent with the idea that extraction of the ventral mesencephalon

after the initial outgrowth of DA neurons has occurred results in significant axotomy with consequent cell death. It should be noted, however, that these dramatic age-dependent differences in survival may be specific to the static slice culture method.

Patch development in organotypic cocultures

A prominent feature of the perinatal striatum is the presence of clusters of striatal neurons that possess a common phenotype and connections (Jain et al., 2001; Snyder-Keller, 1991). These patches (or “striosomes”) are maintained into adulthood; at that stage they represent a subset of striatonigral neurons that project back to the nigral DA neurons (Gerfen et al., 1987). Correspondence between the initial DA afferents and these patch neurons has been shown in the developing striatum *in vivo* (Snyder-Keller, 1991). The present findings demonstrate that a similar overlap develops in these cocultures, although not as complete as the overlap observed *in vivo*.

Striatal patch neurons in these cultures can be most readily revealed with antibodies to DARPP-32, a dopamine and cyclic AMP-regulated phosphoprotein linked to DA D1 receptors that is highly expressed by patch neurons during the late prenatal (E20–22) and early postnatal periods *in vivo* (Foster et al., 1987). We concentrated on the use of this marker to demonstrate the phenotypic development of striatal MSNs in the presence or absence of afferent innervation. As reported previously (Ivkovic and Ehrlich, 1999; Ivkovic et al., 1997; Nakao et al., 1995), acute exposure to BDNF was necessary to enhance expression of DARPP-32 *in vitro*. Therefore, BDNF was added to the medium for the final 18 h prior to fixation. Although BDNF is known to affect striatal cell survival and differentiation (Mizuno et al., 1994; Ventimiglia et al., 1995), its presence during the final hours of culturing is not expected to alter the distribution of DARPP-32-immunoreactive neurons. However, because the known source of BDNF in the striatum is the corticostriatal and nigrostriatal fibers (Altar et al., 1997; Conner et al., 1997; Seroogy et al., 1994), the presence of cocultured tissue providing these afferent sources is likely to increase the expression of DARPP-32 in the absence of exogenous BDNF. Consistent with this hypothesis, we observed stronger DARPP-32 expression in cocultures that contained both nigral and cortical inputs, suggesting that endogenous BDNF released from these afferents had a similar effect *in vitro*.

One issue that we wished to address was whether the patch/matrix organization forms in organotypic striatal cultures that were dissected out prior to *in vivo* patch formation. Using E18/19 striatal tissue, which had not yet developed patches *in vivo*, we observed a homogeneous distribution of DARPP-

32-immunoreactive neurons initially, which transitioned to a more patchy distribution when cocultured with SN/VTA and cortex. Cultures consisting of striatum alone exhibited a single region of DARPP-32-immunoreactive neurons, and coculturing with cortex alone (no SN/VTA) produced an intermediate phenotype. The presence of patches in cocultures is unlikely due to selective cell death, because in pilot studies we found that cocultures had considerably less propidium iodide staining, indicative of cell death. Moreover, older striatal tissue that exhibited patches of DARPP-32-immunoreactive neurons prior to culturing was found to lose its clear patchiness when cultured in the absence of corticostriatal and nigrostriatal afferents. However, striatal tissue cultured in the absence of afferent inputs exhibited poorer growth and survival in general, in addition to displaying fewer DARPP-32-immunoreactive neurons. Thus, although afferent innervation from substantia nigra or cortex cocultured with striatum appears to influence the formation or maintenance of patches, quantification of this effect is difficult under the present conditions. In addition, the exact nature of such an influence remains to be elucidated.

Afferent influences on striatal development

Previous studies from our lab and others have demonstrated an interdependence of striatal neurons and nigral DA neurons. In dissociated cultures, the presence of DA neurons increased the survival of striatal patch neurons, and vice versa (Aronica et al., 1996). Similar results have been observed in transplanted tissue (Costantini et al., 1999), where the presence of fetal striatal tissue enhanced the survival and outgrowth of DA neurons into DA-depleted mature striatum. In vivo studies fall into two categories: lesion studies and knockout studies. Snyder-Keller (1991) demonstrated, through the use of in utero (E17) 6-hydroxydopamine injections, that the patch/matrix organization of striatal neurons still developed in the absence of DA inputs. Comparable prenatal lesion studies produced similar findings (van der Kooy and Fishell, 1992), suggesting that DA inputs are not necessary for the striatal patch/matrix formation. In mice homozygous null for DA, the striatal patch/matrix organization also appears to be intact (Kim et al., 2002). Thus, it would appear that dopamine afferents cannot be the sole driving force for this organization. Nevertheless, the possibility that cortical glutamatergic afferents act in concert with nigral DA afferents to guide patch formation still needs to be considered. Our findings of selective expression of glutamate receptors (Snyder-Keller and Costantini, 1996) and *trkB* receptors (Costantini et al., 1999) on striatal patch neurons early in development support this hypothesis.

Corticostriatal and nigrostriatal afferents are sources of BDNF in the striatum (Altar et al., 1997; Baquet et al., 2005; Conner et al., 1997; Seroogy et al., 1994), in addition to the respective neurotransmitters glutamate and dopamine. BDNF has been shown to increase neuronal survival (Ventimiglia et al., 1995) and promote differentiation (Mizuno et al., 1994) of striatal MSNs. Expression of DARPP-32 is particularly dependent upon BDNF (Ivkovic and Ehrlich, 1999; Ivkovic et al., 1997; Nakao et al., 1995) and occurs first in striatal patch neurons (Foster et al., 1987). Because the earliest corticostriatal and nigrostriatal fibers target the patches both in vivo (Snyder-Keller et al., 2003) and in vitro (Snyder-Keller, 2004; Snyder-Keller et al., 2001; this study), the presence of cocultured tissue providing these afferent sources is likely to increase the expression of DARPP-32 within striatal patch neurons. Further studies are necessary to determine whether BDNF released from these afferents actually induces the initial clustering of striatal patch neurons. In other regions of the brain, BDNF has been shown to play a role in pattern formation (Cabelli et al., 1997; Schwartz et al., 1997); thus, this neurotrophic factor may also participate in striatal patch/matrix formation.

Morphological development of the striatal MSNs, which make up 95% of the striatal neuron population, has been shown to be affected by afferent inputs. Full dendritic development, including the elaboration of spines, is thought to require afferent inputs; Segal et al. (2003) demonstrated the necessity of coculturing with afferent sources to elicit spines on striatal neurons. Using conditional knockout mice that lack cortical BDNF expression, Baquet et al. (2004) demonstrated a dramatic loss of spines on striatal neurons. Examination in our study of neurobiotin-filled neurons revealed extensive dendritic branching and the presence of spines. Although MSNs were most readily obtained in cocultures, examples of fully developed MSNs could also be found in striatal tissue cultured alone. It should be noted that the process of identifying cells for recording is biased in favor of the best-developed ones; the presence of these recorded neurons in patches (based on TH and GluR1 immunoreactivity) would suggest that the more mature patch neurons, given their earlier birthdate (van der Kooy and Fishell, 1987), are preferentially selected. Indeed, the striatal MSNs in these striatal slices have already been contacted by nigrostriatal DA afferents prior to their removal from the fetal brain; however, the absence of axospinous DA synapses at birth (Antonopoulos et al., 2002) suggests that DA afferents do not induce spine formation. Although most evidence to date suggests that the elaboration of spines, as well as the ingrowth of corticostriatal afferents, is a postnatal event (Tepper et al., 1998), our findings reveal an earlier development of striatal MSNs (or,

alternatively, a more rapid development *in vitro*). Previously we demonstrated the capacity of prenatal cortex (as early as E19) to robustly innervate perinatal striatal tissue in organotypic cocultures (Snyder-Keller, 2004). In addition, cortex is known to send projections to the brainstem as early as E19 (Schreyer and Jones, 1982), and these same neurons are known to send collaterals into the striatum (Levesque et al., 1996). Although technical limitations preclude the demonstration of the collaterals prenatally *in vivo*, the possibility that striatal neurons have already been influenced by cortical collaterals as early as E19, must be considered. Such inputs, releasing BDNF as well as glutamate, are likely to be the primary influence on spine development.

Physiological maturation of striatal neurons in organotypic cocultures

The physiological nature of the afferent innervation observed anatomically was verified using electrophysiological recording. In cortico-striatal cocultures, MSNs were shown to possess spontaneous activity, regardless of the age of the striatal tissue used for the cocultures. No significant differences in the resting membrane potential and input resistance were obtained between striatal MSNs from E18–19 and those from E20–22. Moreover, cortical stimulation elicited EPSPs in striatal MSNs from both age groups, demonstrating the existence of functional excitatory corticostriatal connections. Furthermore, both spontaneous activity and cortically evoked activity were blocked with CNQX, a non-NMDA antagonist. These observations support our prior anatomical demonstration of robust cortical ingrowth into striatal slices using biocytin tracing (Snyder-Keller, 2004), and provide evidence that the corticostriatal projections are functional.

The presence of DA innervation was found to significantly enhance the responses of striatal MSNs to cortical stimulation, resulting in prolonged plateau depolarizations (see also Tseng et al., 2007). Furthermore, this effect was more dramatic in cocultures prepared from E20–22 striatal tissue than in preparations from E18/19 striatal tissue. Thus, despite the fact that apparently mature (as judged on the basis of anatomical features) MSNs were observed in the cocultures prepared with the younger (E18/19) striatal tissue, striatal neurons may remain physiologically immature in this organotypic coculture preparation. Given the superior anatomical and physiological features of cocultures prepared from late prenatal (E20–22) striatum, as compared with either younger prenatal (E18–19) and or postnatal (P0–2) striatum, cocultures prepared from late prenatal tissue appear most useful for further investigation of factors influencing striatal development and function. Nevertheless, data

obtained from all age cultures in this study support the premise that the corticostriatal and nigrostriatal afferents can influence the development of striatal neurons, and perhaps patch/matrix compartmentalization, in an activity-dependent manner.

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