

Development of Inhibitory Synapses Among Striatal Neurons *in vitro*

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The development of excitatory and inhibitory synaptic connections has been studied in postnatal neurons from the caudate and putamen maintained in tissue culture. Excitatory postsynaptic potentials which were sensitive to the glutamate antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) appeared between 4 and 8 d *in vitro*. This is the first indication that glutamatergic excitatory neurons may be intrinsic to the striatum. Spontaneous inhibitory postsynaptic currents appeared at approximately the same time, several days after process outgrowth. Antibodies to glutamic acid decarboxylase (GAD), the synthetic enzyme for GABA, labeled neurons which produce bicuculline-sensitive, inhibitory postsynaptic currents. GAD immunoreactivity and immunoreactivity to synapsin I, a synaptic vesicle-associated protein, became localized to discrete sites along neurites 4–8 d after plating. It is concluded that the punctate GAD immunoreactivity identified possible sites of presynaptic transmitter release.

The synaptic organization of the striatum has been difficult to resolve using standard neuroanatomical techniques. The lack of a simple laminar architecture makes it difficult to predictably evoke and record synaptic potentials, as in the hippocampal slice. The presence of many histochemically identified GABAergic interneurons and projection neurons makes it likely that the majority of the intrinsic striatal neurons are inhibitory (Mugnaini and Oertel, 1985). However, physiologically this has been difficult to verify in both *in vivo* or *in vitro* preparations (Lighthall et al., 1981; Lighthall and Kitai, 1983). Because of this difficulty, synaptic transmission among striatal neurons has been studied in dissociated cultures from postnatal animals.

Since the majority of neurons are expected to use GABA as a transmitter, inhibitory responses attributable to GABA have been examined. Hippocampal inhibitory responses consist of both GABA_A and GABA_B hyperpolarizing potentials (Gahwiler and Brown, 1985; Newberry and Nicoll, 1985) and depolarizing GABA_A potentials localized to dendrites and present in immature tissue (Andersen et al., 1980; Alger and Nicoll, 1982; Mueller et al., 1984; Huguenard and Alger, 1986). Any or all

of these types of GABA conductances may be present in striatal tissue. These experiments examined the development of the inhibitory synaptic interactions using both electrophysiological and immunocytochemical techniques. Additionally, fast excitatory synaptic activity was studied electrophysiologically.

Materials and Methods

Cell culture. Primary cultures of neurons and glia from the anterior caudate and putamen of 1- or 2-d-old postnatal rat pups were prepared according to modifications of established procedures (Huettnner and Baughman, 1986; Nakajima et al., 1986). Caudate putamen (CP) was dissected out of coronal slices anterior to the appearance of the third ventricle as a roughly circular area posterior to the forceps minor of the corpus callosum, ventral and medial to the corpus callosum and lateral to the lateral ventricles. Acetylcholinesterase staining of sections taken midway through the dissected portion revealed that only caudate and putamen tissue were included (Fig. 1). Only a small amount of callosal tissue was apparent on the dorsal and lateral edges. Minced tissue was incubated in 1 μ g/ml papain (Sigma Type IV) in L-15 for 20 min before gentle trituration. The resulting cell suspension was centrifuged through L-15 containing 10 μ g/ml BSA and 10 μ g/ml trypsin inhibitor (Sigma). Cells were resuspended and plated (350,000/35 mm dish) onto existing glial feeder layers in MEM without glutamine containing 10% Nu Serum (this includes fibroblast growth factor, Collaborative Research), 20 units/ml penicillin, and 20 μ g/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Twenty-four hours after plating, each dish received an aliquot of fluorodeoxyuridine and uridine to final concentrations of 15 and 35 μ g/ml, respectively.

Glial cells were dissociated from 2-d-old rat pups from either cerebral cortex or caudate-putamen according to Nobel et al. (1984) and plated in T25 flasks in MEM supplemented as above. After 1 week the flasks were washed vigorously with cold L15 to remove surviving neurons and fed with supplemented MEM with 15 μ g/ml fluorodeoxyuridine and 35 μ g/ml uridine. Glial flasks were fed on a weekly basis for 1–7 weeks until passaged onto 35 mm polylysine-coated plastic petri dishes 4 d before neuronal plating. For fluorescence staining, cells were plated onto polylysine- and collagen-coated glass coverslips, glass insets in plastic petri dishes, or 8 chamber glass slides (Lab-Tek).

Electrophysiology. Individual CP neurons were voltage clamped with patch electrodes in whole-cell recording mode (Hamill et al., 1981) with an Axopatch 1B amplifier (Axon Instruments). Internal and external solutions are listed in Table 1. Data acquisition and voltage-step generation were controlled from the SPIKE program (Hillal Associates) through a Modular Instruments Inc. analog-digital converter connected to an IBM-AT computer. Because of the difficulty in maintaining adequate voltage control over the extensive processes of older cells, cells were voltage-clamped to -60 mV initially and only stepped briefly to voltages between -30 and -90 mV. Voltages reported here include a 6 mV correction for junction potentials developed by the low chloride internal solutions. Holding currents for cells less than 1 week *in vitro* were typically 10–20 pA, while for older cells, holding currents increased up to 100 pA. Cells with holding currents above 100 pA were discarded. Cell capacitance was calculated by integrating the current response to the offset of a -10 mV voltage step. Spontaneous activity was recorded onto a Gould chart recorder or Gould model 6504 FM tape recorder. GABA, bicucullin, and CNQX were applied by brief pressure pulses from a patch pipet positioned 10–20 μ m from a cell soma. Cells were allowed to recover for 40 sec between GABA applications to prevent

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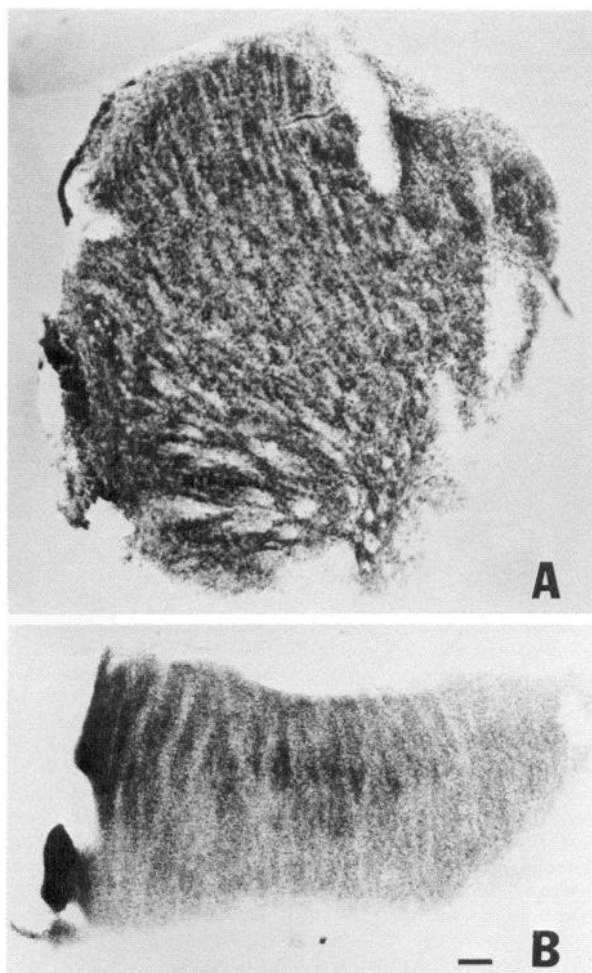


Figure 1. Acetylcholinesterase-stained sections (100 μm) through a portion of neonatal rat brain used in caudate-putamen dissections. "Striated" nature of the tissue is apparent in both coronal (*A*) and horizontal (*B*) sections (compare with Paxinos and Watson, 1986). Ventricular (medial) edge is at *left*, lateral at *right*. Dorsal is at the *top* in *A*, and anterior is at the *top* in *B*. Scale bar, 100 μm .

desensitization. Ten millimolar stock solutions of CNQX (Tocris Neuroamin, London) were made up in equimolar NaOH in water and diluted in external recording solution prior to use.

Synaptically coupled cell pairs were recorded using whole-cell patch clamping for the postsynaptic cell and extracellular stimulation of loosely sealed patch electrodes on the presynaptic cell (Dubinsky et al., 1988). The presynaptic patch electrode was connected to an A-M Systems Neuroprobe Amplifier model 1600 and contained internal solution in case intracellular contact was made. Pairs of phase-bright cells within 200 μm of each other with interposed neurites were chosen to test for synaptic interactions. All recordings were performed at room temperature. Synaptic connections were defined as monosynaptic if the latency from the peak of the presynaptically recorded action current to the onset of the postsynaptic current was <5.0 msec and varied by <0.7 msec between trials (sampling period 0.33 msec). Hirano et al. (1986) have measured the Q10 for monosynaptic connections among cultured cerebellar neurons and have shown that latencies <6 msec at 23°C correspond to 1 msec or less at 37°C. Many CP connections could follow high-frequency stimulation up to 20 Hz, but not all connections were tested in this manner since depression was frequently observed.

Immunocytochemistry. The staining procedures for the monoclonal antibody, GAD-6 (Chang and Gottlieb, 1988; kindly provided by Dr. David I. Gottlieb) and polyclonal antibody to synapsin I (De Camilli et al., 1983; kindly provided by Dr. Paul Greengard) were as follows. Initially, cells were fixed for 10 min in warm 4% paraformaldehyde and

Table 1. Electrophysiological solutions

Solute	A		B	
	External	Internal	External	Internal
NaCl	126.8	—	140.0	—
KCl	13.2	13.7	8.0	2.0
K-isethionate	—	126.3	—	138.0
CaCl ₂	2.0	—	2.0	—
MgCl ₂	1.0	—	1.0	—
Na-Hepes	10.0	10.0	10.0	10.0
Glucose	50.0	40.0	40.0	40.0
MgATP	—	2.0	—	2.0

Concentrations in mM.

0.1% glutaraldehyde in L-15. Following extensive rinsing in L-15, cells were incubated for 30 min in 20 mM Sorenson's phosphate buffer containing 0.1% glycine. Subsequently, primary antibody diluted (1:800 GAD-6, 1:400 anti-synapsin I) in 20 mM Sorenson's phosphate buffer with 0.45 M NaCl, 0.2% Triton X-100, 10% normal goat serum, and 0.1% Na azide was applied for 1 hr at room temperature. After rinsing in the buffer formulated above, cells were incubated in fluorescein-conjugated secondary antibodies for 1 hr (FITC-goat anti-mouse IgG and IgM, Cappel, for GAD-6 and FITC-goat anti-rabbit IgG, Boehringer-Mannheim, for synapsin I). Cells were rinsed for 30 min in buffer, followed by 3 rinses in 5 mM Sorenson's phosphate with 25 mM sucrose before mounting in a glycerol-PBS mixture that prevents bleaching (Citifluor). Substitution of normal mouse serum for GAD-6 or normal rabbit serum for anti-synapsin I for the primary antibodies or omission of the primary antibodies resulted in an absence of fluorescence staining in all experiments. Preabsorption of anti-synapsin I with 1.8 $\mu\text{g}/\text{ml}$ synapsin I also produced no detectable fluorescence signal. In double-labeling experiments, rhodamine-conjugated goat anti-mouse IgG (Jackson Immunocytochemicals) was used to visualize GAD-6. No cross-labeling was observed of either secondary antibody for the inappropriate primary antibody or normal control serum. No cell surface GAD-6 immunoreactivity was observed when live cultures were incubated with mAb GAD-6 (1:200, but see Docherty et al., 1987).

Immunostaining for the presence of GABA was performed with affinity-purified antibodies to GABA conjugated to BSA (GABA-5; Wenthold et al., 1986; kindly provided by John Zemple). After the above fixation, cells were incubated for 30 min in 0.1 M phosphate buffer with 5% normal goat serum and 0.2% Triton X-100. GABA-5 antibody was diluted 1:200 in this buffer and applied for 1 hr at room temperature. After rinsing, cells were incubated in fluorescein-conjugated goat anti-rabbit IgG (Boehringer-Mannheim) for 1 hr. Subsequently, cells were rinsed and mounted as above. No fluorescence signal was observed when normal rabbit serum was substituted for GABA-5, when the primary antibody was omitted, or when GABA-5 was preabsorbed with GABA-conjugated to ovalbumin.

While these antibodies are well characterized (GAD-6 and anti-synapsin I both recognize single bands on gels: Chang and Gottlieb, 1988; De Camilli et al., 1983), the possibility remains that they may be recognizing some similar, as yet unidentified cellular antigen. Therefore, although the terms GAD-6, GABA-5, or synapsin I immunoreactivity are used throughout this paper, they should be interpreted to mean GAD-like, GABA-like, and synapsin I-like immunoreactivity.

Cultures were initially stained with antibodies directed against neuron-specific enolase (NSE, ICN Immunobiologicals), phosphorylated and nonphosphorylated neurofilaments (SMI 31 and SMI 32, Sternberger-Meyer Immunocytochemicals, Inc.), and glial-fibrillary acidic protein (GFAP; Bignami et al., 1972) to differentially identify neurons and astrocytes. All phase-bright ovoid or pyramidal shaped cells with 2–5 long, thin, relatively straight processes and eccentric nuclei stained positively for NSE, SMI 31, or SMI 32 and were identified as neurons. All large, flat, spread cells stained positively for GFAP and were classified as astrocytes. A small population of mostly flat, phase-dark cells with multiple, thick, short, crooked processes, possibly oligodendroglia, remain to be positively identified. These cells were excluded from electrophysiological and immunocytochemical experiments. SMI 31 and SMI 32 staining of the glial feeder layers was performed to confirm the

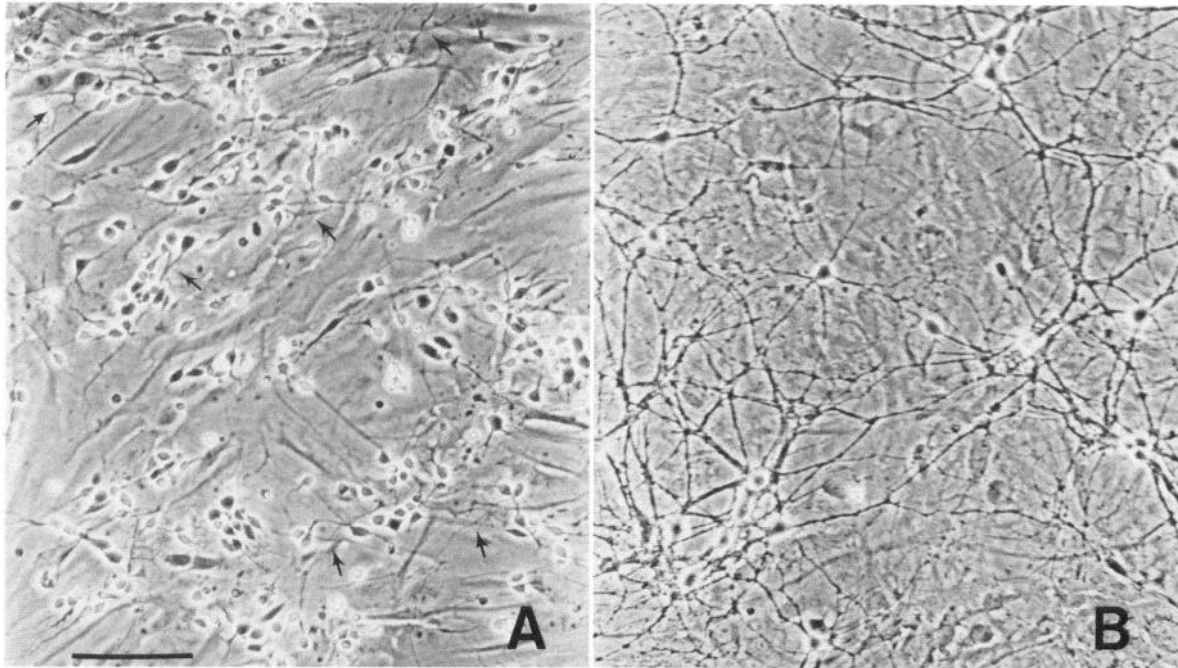


Figure 2. Phase-contrast micrographs of neonatal striatal neurons maintained *in vitro*. *A*, Neuritic process extension (arrows) 24 hr after plating. *B*, Appearance of mature cultures with fasciculated processes and large phase-bright neurons, in this case 7 weeks *in vitro*. Scale bar, 100 μ m.

removal of all cortical neurons. Only 9 poorly developed cells with possible neuronal profiles were immunoreactive on 14 dishes from 2 separate glial platings. Cells with such an unhealthy appearance were not used for electrophysiology.

Results

Survival of striatal neurons in culture

The photomicrographs in Figure 2 show dissociated CP neurons maintained in culture for 24 hr and 7 weeks. Within 4 hr after dissociation, CP neurons attached to the glial feeder layer and began to extend processes. By 24 hr, these processes contacted neurites and somas of other neurons. Over the next week, process extension continued, forming a meshwork of interconnecting fascicles over the glial substrate. During this time, culture appearance changed sufficiently that positive identification of the same neuron was not possible with daily photographic observation. Clumps of neuronal cell bodies often formed in the denser portions of some cultures. After a week, the cultures appeared to stabilize and relocation of identified neurons on subsequent days was possible. Cultures maintained this healthy appearance for 4–7 weeks *in vitro*. The appearance of grainy neurites and abruptly truncated processes indicated that cultures were dying. Once this occurred, no phase-bright neurons remained after another week.

Neuronal survival was quantified by counting 10 representative fields per dish on successive days for several different platings (Fig. 3). Over 50% of the plated neurons died between days 4 and 8 *in vitro*. After 2 weeks, the number of surviving neurons stabilized.

Spontaneous activity

By electrophysiological criteria, CP neurons appeared healthy at all ages *in vitro*. Stable recordings could be obtained from several hours up to 4 weeks after plating. The parallel combination of input resistance and electrode seal resistance was 1.2

± 1.3 G Ω ($N = 72$). The average resting potential in solution A was 54.0 ± 8.0 mV (mean \pm SD, $N = 61$). This decreased from -58.0 ± 10.0 mV at 1 d *in vitro* (div) to -49.5 ± 3.6 mV (13) in the third week after plating. Fast overshooting action potentials, sometimes accompanied by afterhyperpolarizations, were observed in 77% of striatal neurons after 1 div, increasing to 100% of neurons after day 8.

Synaptic activity was classified as excitatory or inhibitory according to the following criteria. Postsynaptic currents which were outward above -60 mV, reversed at -60.9 ± 2.1 mV (solutions B), and were blocked by 100 μ M bicuculline were considered to be inhibitory postsynaptic currents (IPSCs). In-

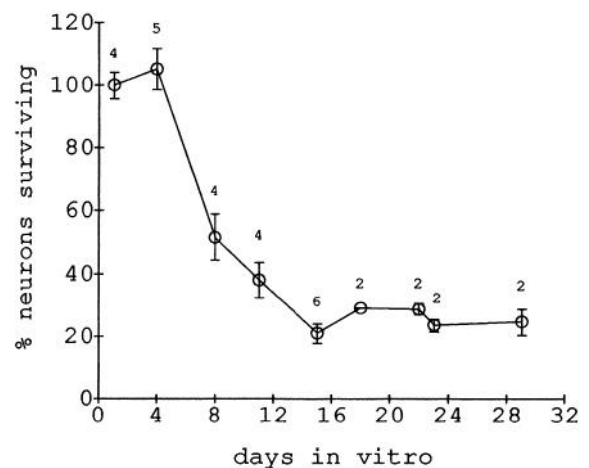


Figure 3. Survival of neonatal CP neurons in long-term cultures. Number of platings counted at each time point are indicated. Numbers expressed as percentage of neurons present on day 1 (means \pm SEM). Initially, a 350×250 μ m field contained an average of 450 neurons.

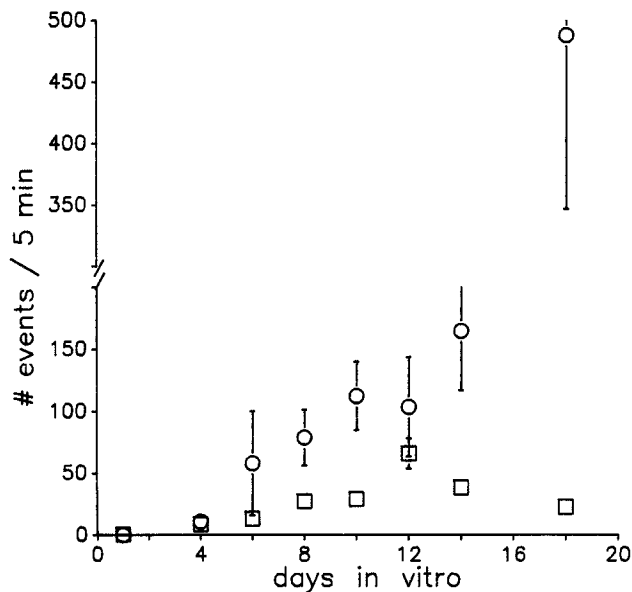


Figure 4. Development of spontaneous synaptic activity in CP cultures. The number of spontaneously occurring excitatory (circles) and inhibitory (squares) postsynaptic currents were recorded during 5 min at -60 and -40 mV, respectively. Numbers are mean \pm SEM, in solution B.

ward postsynaptic currents which could not be reversed at negative holding potentials and were blocked by $200 \mu\text{M}$ CNQX (Yamada et al., 1989) were considered to be excitatory postsynaptic potentials (EPSCs).

A low incidence of spontaneous synaptic activity could be observed in 17% of cells 1 d after plating. By day 4, 67% of all cells had EPSCs and 74% exhibited IPSCs. The number of spontaneous EPSCs increased steadily with age *in vitro* (Fig. 4), while

the number of IPSCs increased up to 12 div and subsequently declined. Spontaneous synaptic activity persisted unchanged in the presence of $1 \mu\text{g/ml}$ TTX (sufficient to block all sodium action potentials) through day 8. After day 10, TTX reduced the frequency of spontaneous events.

Evoked synaptic activity recorded from pairs of cultured neurons was encountered reliably during the third week *in vitro*. Fourteen percent (4 out of 29) of all pairs of neurons impaled on day 8 were synaptically coupled. This incidence increased to 61% (16/26) by day 14.

Postsynaptic response

CP neurons responded to GABA at all ages studied. Postsynaptic responses to pressure application of $100 \mu\text{M}$ GABA were recorded for membrane holding potentials between -90 and -30 mV in solutions A. Current-voltage plots of these responses were generally linear, although some exhibited rectification at potentials below -70 mV (Fig. 5C, filled circles). Responses to GABA reversed at the same membrane potential as synaptically driven inhibitory currents (Fig. 5). In order to evaluate postsynaptic sensitivity to GABA, the slope conductance was calculated from the current-voltage plot between -70 and -30 mV. The slope conductance increased over time (Fig. 6A). However, when these values were normalized to cell capacitance to correct for neuronal size, no developmental increase was detected (Fig. 6B). The GABA_B receptor agonist baclofen ($100 \mu\text{M}$) did not alter membrane current in this same voltage range ($N = 8$). Application of GABA to proximal processes produced smaller-amplitude, slower-rising responses that reversed at the same potential as somal application.

Presynaptic maturation

In order to study the development of presynaptic structures, a reliable marker for presynaptic inhibitory neurons was needed.

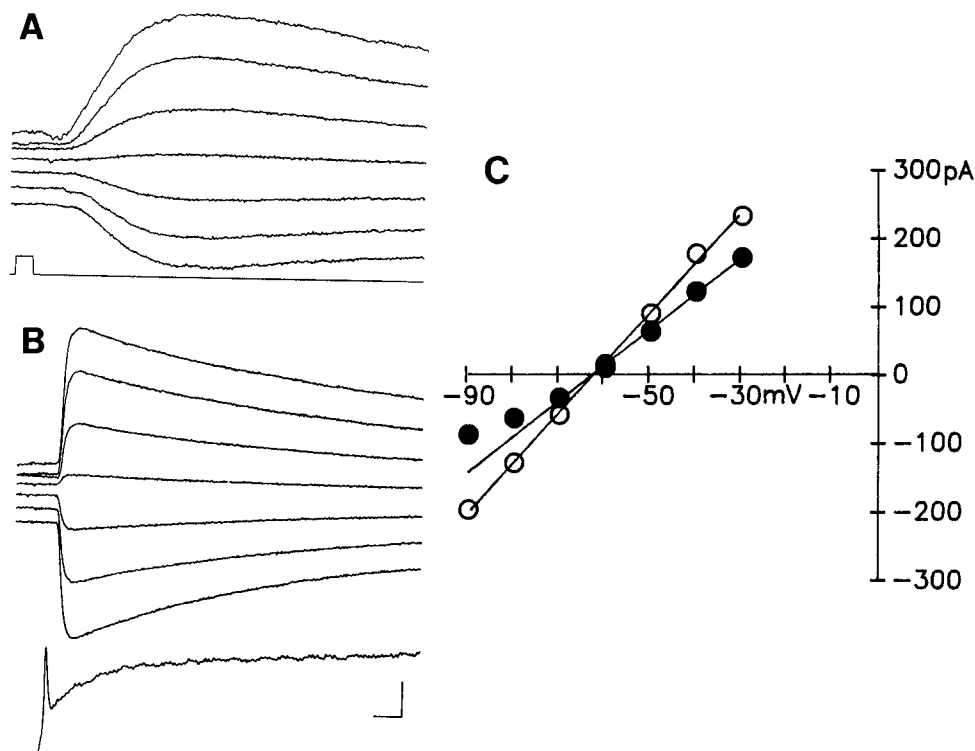


Figure 5. Inhibitory postsynaptic currents (B, open circles in C) reversed at the same membrane potential as pressure-applied $100 \mu\text{M}$ GABA (A, filled circles in C). A and B are from the same cell recorded in solution B. Lower trace in A indicates GABA application; lower trace in B is extracellularly recorded action current. Scale bar, 25 msec, 60 pA in A; 10 msec, 60 pA, 10 pA in B.

GAD, the synthetic enzyme for GABA, has typically been employed to locate putative inhibitory neurons. However, it is not known if GAD is an exclusive marker or if it is also present in other non-GABA-releasing neurons. To test this possibility, electrophysiologically identified, monosynaptically coupled pairs of neurons were subsequently stained for GAD with a monoclonal antibody (GAD-6; Chang and Gottlieb, 1988). GAD-6 immunofluorescence was scored by 2 observers, including one who had no knowledge of the electrophysiological results.

As expected, in 92% ($N = 12$) of monosynaptic (latency < 5.0 msec) inhibitory connections, the presynaptic neuronal soma was immunoreactive for GAD-6 (Fig. 7A). In presynaptic excitatory neurons, GAD-6 immunoreactivity was absent 77% ($N = 13$) of the time (Fig. 7B). In 3 instances, excitatory presynaptic neurons (synaptic latencies 0.9, 2.0, and 3.1 msec) contained immunoreactivity for GAD-6 (Fig. 7C). Overall, 79% (11/14) of GAD-6-positive presynaptic neurons were indeed inhibitory. Only 9% (1/11) of unstained presynaptic neurons produced bicuculline-sensitive inhibitory postsynaptic currents.¹

Presynaptic maturation was therefore examined using the GAD-6 antibody. GAD-6 immunoreactivity was also correlated with immunoreactivity to antibodies directed against both synapsin I, a synaptic vesicle-associated protein (De Camilli et al., 1983) and GABA conjugated to glutaraldehyde (GABA-5; Wenthold et al., 1986). GAD-6 stained neurites and cytoplasmic portions of the majority of CP somas (Fig. 8). The intensity of somal staining was increased by overnight pretreatment with 1 μ g/ml colchicine in the growing medium (Fig. 8B). The proportion of somas exhibiting GAD-6 immunofluorescence remained constant at all ages examined (Fig. 8C). Colchicine pretreatment did not alter the number of GAD-6-positive neurons detected.

In mature cultures, synapsin I immunoreactivity has been localized to discrete areas along neuritic processes (Weiss et al., 1986). When cultures were stained simultaneously for synapsin I and GAD-6, the punctate GAD-6 immunoreactivity exactly colocalized with the punctate synapsin I immunofluorescence (Fig. 9). As expected, some processes staining positively for synapsin I did not contain any GAD-6 immunoreactivity.

Developmentally, however, the patterns of GAD-6 and synapsin I immunoreactivity differed slightly (Fig. 10). Initially, GAD-6 immunoreactivity was observed only in neuronal somas in asymmetric patterns resembling cytoplasmic (non-nuclear) areas. At this time, synapsin I staining was found uniformly distributed in somas and along processes. In the next few days, synapsin I became increasingly localized to small discrete areas along neurites and the somal staining became less prominent but did not disappear. In some cells during the first week, GAD-6 immunoreactivity appeared uniform throughout the soma and processes. More often, GAD-6 immunofluorescence became progressively more localized to neuritic puncta. Colocalization experiments suggested that synapsin I puncta developed before the GAD-6 puncta, but quantitative intensity measurements were not attempted. In contrast to GAD-6 immunofluorescence,

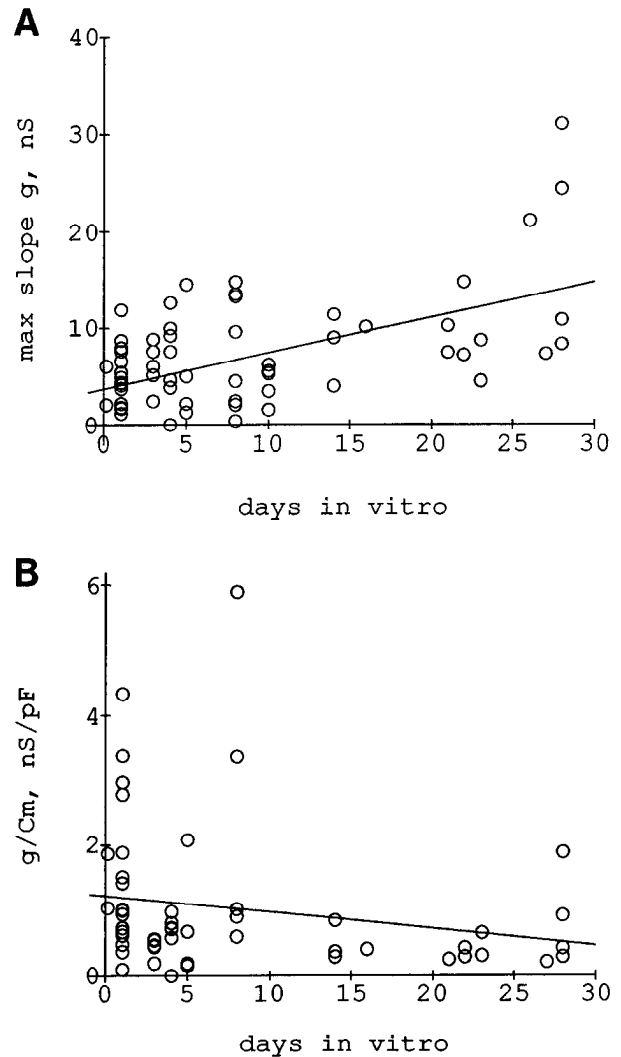


Figure 6. Sensitivity of CP neurons to pressure-applied GABA. *A*, Maximum slope conductance for 66 neurons appears to increase with age. *B*, Same data as *A*, normalized for cell capacitance. Slope of linear-regression line does not significantly differ from 0 (t test, $p = 0.15$, solution A).

GABA-5 staining was uniform throughout the soma and processes of CP neurons. The percentage of GABA-5-positive neurons increased from 40% at 1 div to 90% by the end of the first week.

The appearance of discrete puncta of GAD-6 immunoreactivity around neuronal somas is presented in Figure 11. The time course of their appearance resembles the time course of onset of spontaneous synaptic activity (Fig. 4). To test this correlation, GAD-6 immunostaining was performed after electrophysiological recording of spontaneous activity on identified cells. In 29 neurons with spontaneous IPSCs, punctate GAD-6 immunostaining was always present in processes adjacent to the soma or proximal neurites. Ten out of 14 younger neurons with no spontaneous IPSCs (3 min of continuous recording at -40 mV) lacked GAD-6 puncta adjacent to their somas or proximal processes. In the remaining 4 neurons without spontaneous IPSCs, only 1 or 2 appropriately placed, dim puncta could be observed. In these cases, it is conceivable that the observation period should have been extended in order to observe IPSCs.

¹ mAb GAD-6 labeled 66.7% ($n = 33$) of postsynaptic somas, a proportion comparable to the number of GAD-6-labeled cells overall (see Fig. 8C). Since the whole-cell recording technique was invariably employed for postsynaptic recording, internal dialysis of cellular contents did not compromise the GAD-6 immunostaining. Every attempt was made to stimulate presynaptic cells extracellularly to insure intact cytoplasmic contents. However, in the few instances where intracellular recording was unavoidable, the immunocytochemical results were accepted as valid because of the clear presence of GAD-6 stain in postsynaptic cells.

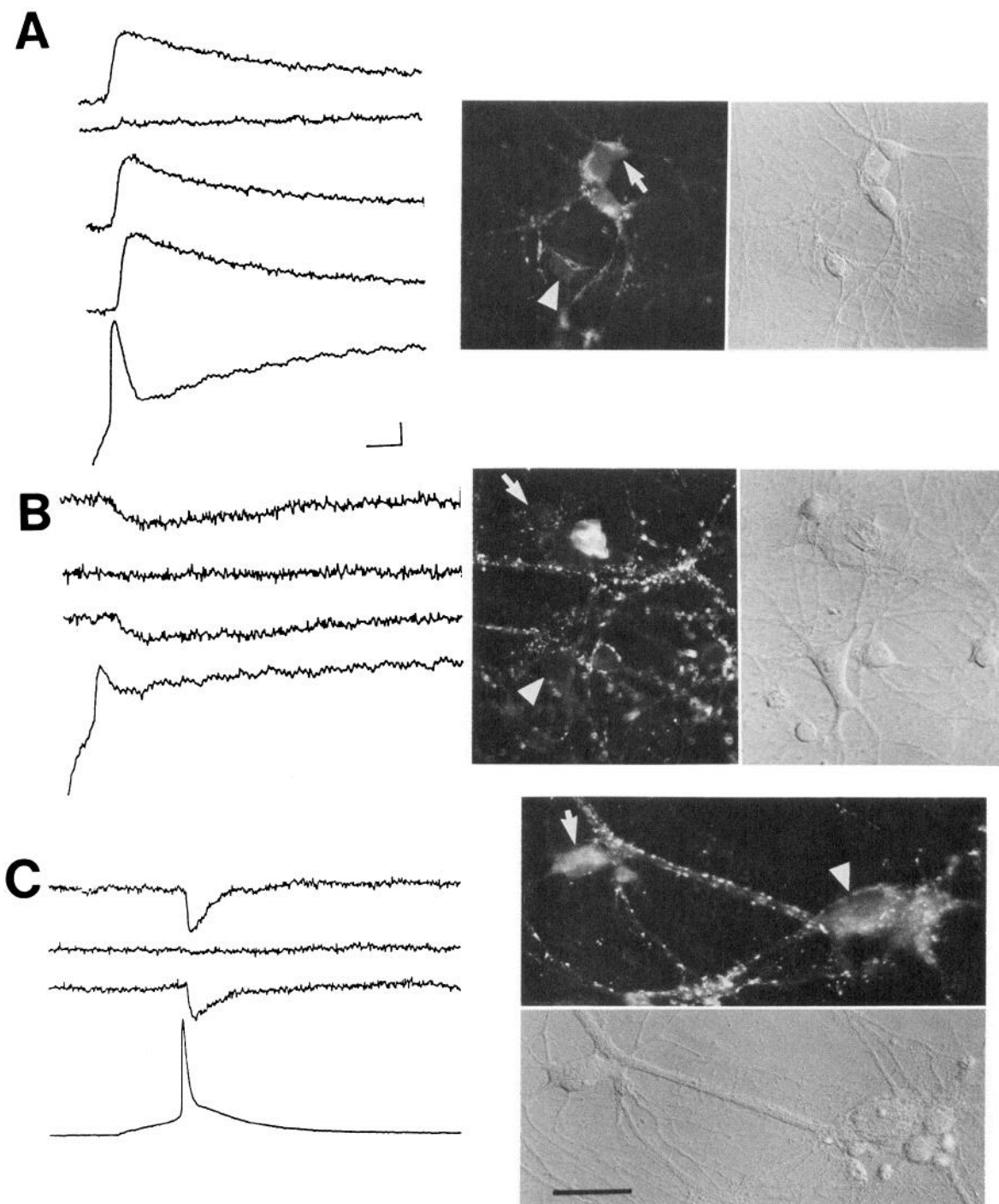
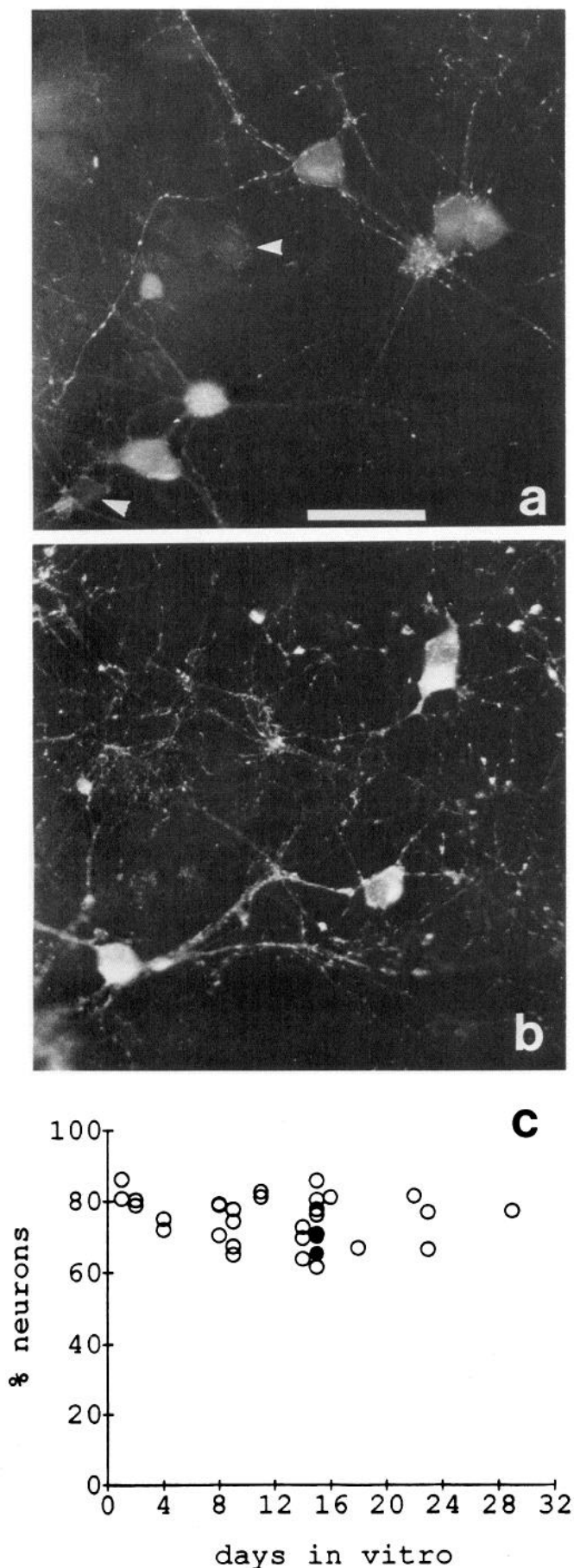


Figure 7. GAD-6 immunofluorescence of identified presynaptic (arrows) and postsynaptic (arrowhead) neurons. Evoked postsynaptic currents recorded at -60 mV (solution B) are offset to demonstrate block by appropriate antagonist. In fluorescence and differential interference contrast micrographs, scale bar, $50 \mu\text{m}$. *A*, Presynaptic neuron producing a bicuculline-sensitive IPSC was GAD-6 positive. Traces are (top to bottom) control IPSC, IPSC after $100 \mu\text{M}$ bicuculline, recovery, IPSC after $200 \mu\text{M}$ CNQX, extracellular presynaptic action current stimulus. Scale bar, 10 msec, 10 pA. *B*, Presynaptic neurons producing a CNQX-sensitive synaptic response had no GAD-6 immunoreactivity. Traces are control EPSC, EPSC after $200 \mu\text{M}$ CNQX, recovery, and extracellular action current stimulus. Scale bar, 10 msec, 5 pA. *C*, Presynaptic neuron with GAD-6 immunofluorescence produced a CNQX-sensitive postsynaptic response. Traces are control EPSC, EPSC after $200 \mu\text{M}$ CNQX, recovery, and intracellular action potential stimulus. Scale bar, 15 msec, 7.5 pA, 10 mV.

No correlation was observed between the number of presynaptic puncta and the frequency of spontaneous IPSCs. The absence of such a relationship suggests that synaptic efficacy varies greatly among these putative presynaptic sites.

Discussion

These experiments demonstrate that *in vitro* striatal neurons exhibit glutamatergic excitatory activity and GABAergic inhib-



itory activity. Immunoreactivity to the GAD-6 antibody serves as a marker for inhibitory GABAergic neurons. The appearance of GAD-6 immunoreactivity in putative presynaptic sites corresponds to the onset of spontaneous inhibitory activity in these cultures.

Survival of striatal neurons in vitro

The death of a subpopulation of striatal neurons (not necessarily GABAergic) between days 4 and 8 *in vitro* may be attributable to several causes. *In vivo*, 25% of striatal neurons die during a period of naturally occurring cell death at 4–8 d postnatally (Fentress et al., 1981). Striatal cultures prepared from embryonic day 17 rat pups also show abrupt reductions in cell number immediately after plating but not at similar conceptual ages (Surmeier et al., 1988). While known growth factors have been supplied in the culture media (Morrison et al., 1987), unidentified growth factors may still be required for cell survival. Since 70% of striatal neurons project to other CNS structures (Bolam et al., 1981; Loopuijt and Van der Kooy, 1985), the absence of appropriate targets may adversely affect the survival of projecting neurons. Because the period of active synaptogenesis occurs simultaneously, the surviving population may be determined by their ability to form functional synaptic contacts with other intrinsic striatal neurons.

GAD-6 immunoreactivity as marker for GABAergic cells

Since the developmental observations reported in this paper depend heavily upon immunocytochemical labeling, it was necessary to clearly establish that the antigen recognized by the GAD-6 monoclonal antibody was a marker for inhibitory neurons. On Western blots of unfractionated rat whole brain, GAD-6 recognizes a single 59 kDa protein that has been purified, sequenced, and shown to be homologous to the protein coded for by a cDNA for feline GAD (Chang and Gottlieb, 1988). Relocation of electrophysiologically identified synaptic pairs clearly demonstrated that mAb GAD-6 overwhelmingly labeled inhibitory neurons. This is the first confirmation of any immunocytochemical marker for GABAergic neurons. The absence of label in one 17-d-old inhibitory neuron may be attributable to the developmental decrease in somal labeling observed during the third week *in vitro*.

Two populations of GABAergic neurons have been reported in the striatum; those that stain lightly or intensely with probes for GAD (Oertel and Mugnaini, 1984; Chesselet et al., 1987; Kita and Kitai, 1988). Both of these populations are observed in culture and together account for 76% of all neurons. GAD-like immunoreactivity in intact caudate and putamen is reported present in from 40–90% of all neurons (Penny et al., 1986; Mugnaini and Oertel, 1985), the most recent estimate being 85% (Kita and Kitai, 1988). Colchicine pretreatment, routinely used

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Figure 8. GAD-6 immunofluorescence in CP neurons. *A*, The majority of neurons in this 15-d-old culture contained cytoplasmic GAD immunoreactivity. Unstained neurons indicated by arrowheads. *B*, Pretreatment of sister culture overnight with 1 μ g/ml colchicine-enhanced cytoplasmic staining. Scale bar, 50 μ m for *A* and *B*. *C*, Percentage of GAD-6 positive CP neurons remained constant over 4 weeks *in vitro*. Each symbol represents the percentage of GAD-6-positive neurons observed in 10 microscopic fields (350 \times 250 μ m). An average of 167 ± 24 ($N = 34$; range, 40–651) neurons was counted per point. Filled circles are from cultures pretreated with 1 μ g/ml colchicine; open circles, untreated cultures.

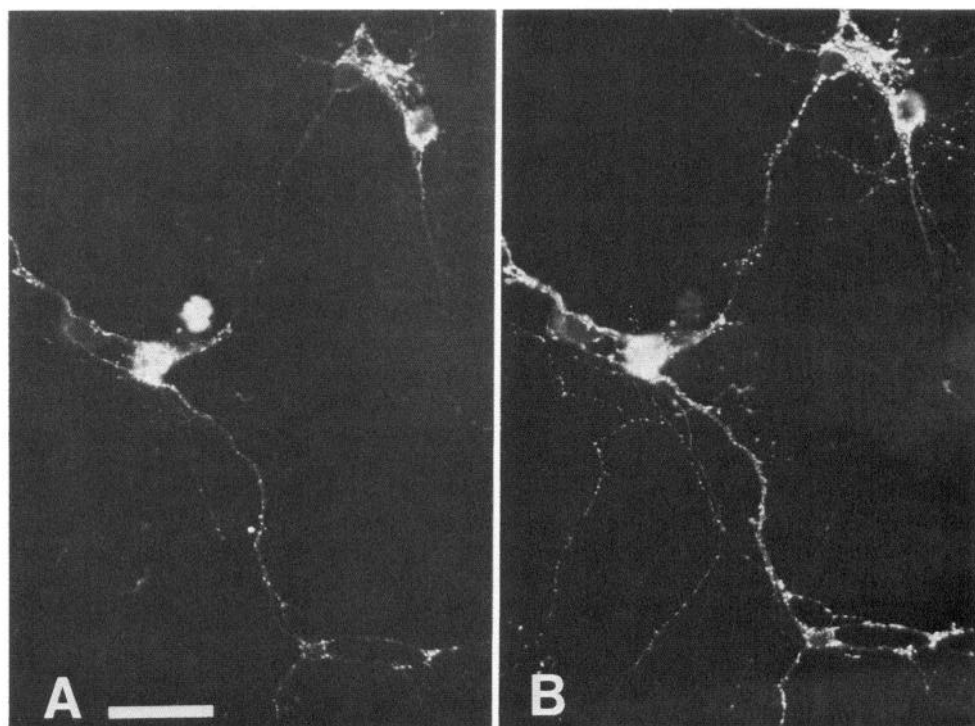


Figure 9. Colocalization of GAD-6 immunoreactivity with anti-synapsin I immunofluorescence in a 22-d-old culture. *A*, mAb GAD-6 visualized with a rhodamine-conjugated goat anti-mouse IgG. *B*, Synapsin I immunofluorescence visualized with a fluorescein-conjugated goat anti-rabbit IgG. Scale bar, 50 μ m.

in vivo to enhance GAD staining (Oertel and Mugnaini, 1984), clearly enhanced the intensity of immunofluorescence *in vitro* but did not alter the percentage of GAD-6-containing neurons. In postnatal cultures, the proportion of neurons with immunoreactivity to GABA-5 is 90%, compared with previously reported values of 46% from embryonic cultures (Surmeier et al., 1988). Since GABA levels increase from 20% of adult levels at embryonic day 15 to 60% at birth (Coyle and Enna, 1976), the postnatal cultures probably reflect a more accurate estimate of GABA-like immunoreactivity. The higher incidence of GABA-5 immunoreactivity (90%) compared with GAD-6 immunoreactivity (76%) has been observed previously (Nishimura et al., 1985; Mosinger and Yazulla, 1987). This may reflect a low level of cross-reactivity of the GABA-5 antibody (Wenthold et al., 1986), the early development of GABA uptake processes, or the fact that, developmentally, GAD lags behind GABA in reaching adult levels (Coyle and Enna, 1976). Indeed, the developmental changes in the pattern of GAD-6 immunoreactivity occur more slowly than those observed for GABA-5 (Fig. 10).

The appearance of synapsin I immunoreactivity in nerve cell bodies has been observed previously both *in vivo* (De Camilli and Greengard, 1986; Goldenring et al., 1986) and at early times *in vitro* (Weiss et al., 1986). In rat cerebellum, synapsin I is coded for by a 4.5 kb transcript that is expressed prior to postnatal day 7 and, subsequently, by a 3.4 kb transcript that peaks in its expression during synaptogenesis (Haas and DeGennaro, 1988). It is possible that the developmental changes observed in the pattern of synapsin I immunoreactivity correspond to this shift in mRNA expression.

Inhibitory synaptic activity

Striatal neurons maintained in tissue culture exhibit inhibitory synaptic currents sensitive to bicuculline which reverse at the GABA reversal potential. These "hyperpolarizing" synaptic re-

sponses are produced by activation of GABA_A type conductances. No "depolarizing" type GABA responses were observed. GABA_B responses, activated by baclofen, were undetectable, as might be expected from the paucity of GABA_B type receptors in striatal tissue *in vivo* (Wamsley et al., 1986).

These studies revealed striking parallel time courses of several aspects of the development of inhibitory synaptic function. During an apparent critical period between days 4 and 8 *in vitro*, neurons stabilize their position, both GAD and synapsin I immunoreactivity become colocalized to discrete sites along processes, inhibitory spontaneous synaptic activity becomes readily apparent, and GAD-containing processes appear encircling somata. Since the sensitivity to GABA remained constant throughout the culture lifetime, maturation of postsynaptic receptors is not considered to influence this developmental pattern. These experiments suggest that the development of functional inhibitory synaptic activity may, in part, be limited by the localization of the inhibitory transmitter synthetic enzyme, GAD, to presynaptic sites. Indeed, when neurons devoid of spontaneous IPSCs were subsequently stained with GAD-6, punctate immunoreactivity was not identifiable presynaptically along the soma or proximal processes.

In previous studies of cultured embryonic spinal cord, the onset of inhibitory synaptic transmission lags behind that of excitatory synaptic activity (Jackson et al., 1982; O'Brien and Fischbach, 1986). In cultures of neonatal striatum, both excitatory and inhibitory activity developed roughly in parallel. The more rapid onset of inhibitory activity in this study may reflect the higher levels of GAD present at birth than at embryonic ages (Coyle and Enna, 1976). Another difference in these preparations is the much higher percentage of inhibitory neurons in striatal cultures (76%) compared with the estimated 15% in spinal cord tissue (Mugnaini and Oertel, 1985).

The frequency of spontaneous synaptic events did not become

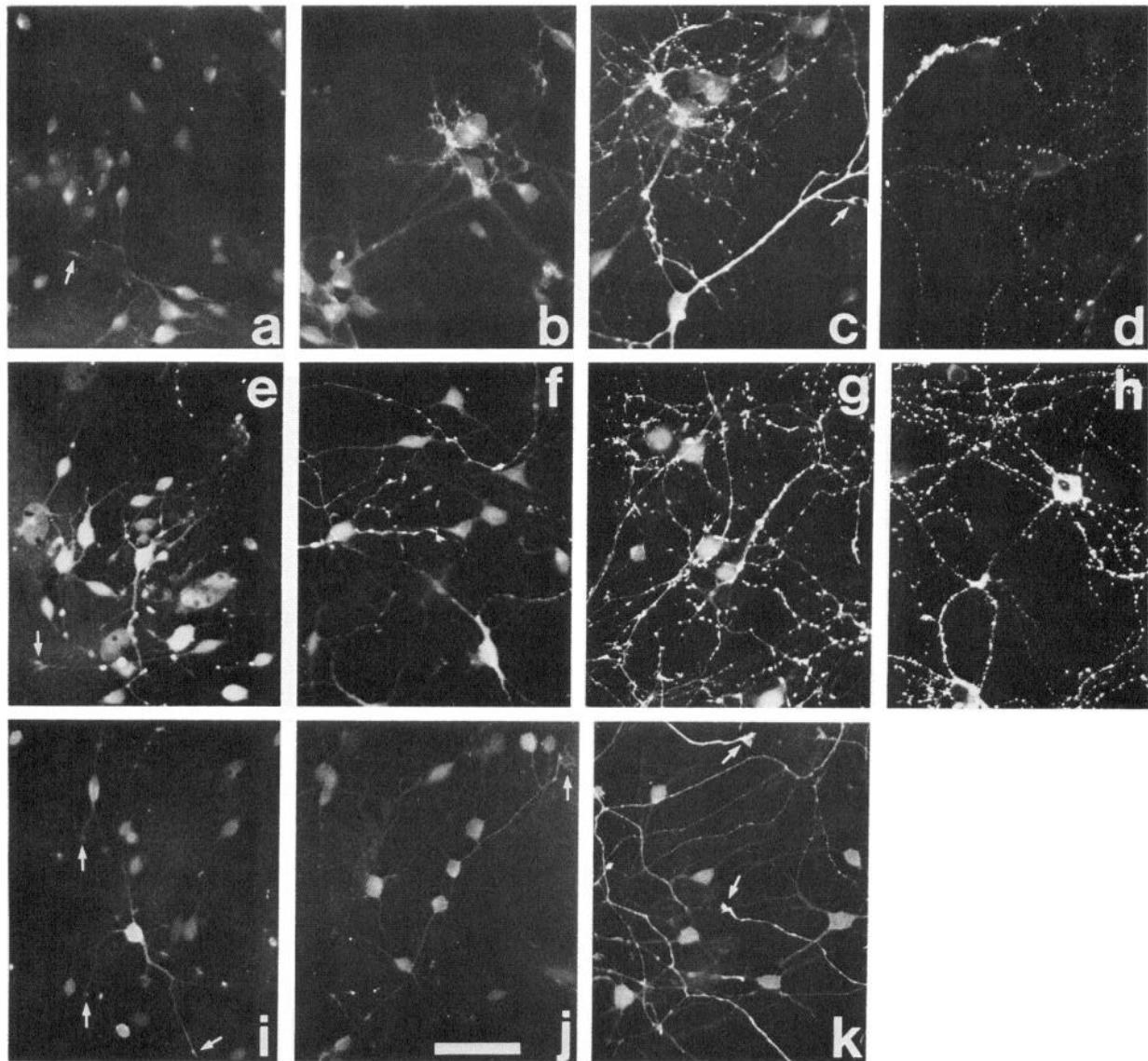


Figure 10. Developmental changes in the staining patterns of GAD-6 (*a–d*), synapsin I (*e–h*), and GABA-5 (*i–k*). Sister cultures were stained separately for each antibody on the following days: *a*, *e*, and *i*, 1 div; *b*, *f*, and *j*, 4 div; *c*, *g*, and *k*, 11 div; and *d* and *h*, 23 div. Arrows indicate the presence of immunoreactivity in growth cones. All staining was visualized with fluorescein-conjugated secondary antibodies. Intensity of immunostaining increased with age for GAD-6 and GABA-5. Photographic exposures were slightly longer in *a* and *i* to capture the weaker initial immunoreactivity. Scale bar, 50 μm .

TTX sensitive until after 10 div. However, the effects of calcium channel blockers or varying calcium and magnesium concentrations have not been examined. Therefore, it is uncertain whether these TTX-insensitive events are true “miniature” IPSCs. Developmental increases of sodium current density have been reported in cultured spinal cord and cortical neurons (MacDermott and Westbrook, 1986; Huguenard et al., 1988). Similarly, overshooting action potentials are often difficult to evoke in younger striatal cultures. Calcium-dependent action potentials have been reported in striatal neurons in the presence of potassium channel blockers (Kita et al., 1985; Cherubini and Lanfumey, 1987), and the presence of local dendritic calcium spiking cannot be ruled out (Llinás et al., 1984). Thus, an apparent dissociation may exist between the onset of spontaneous synaptic release and the onset of sodium action potential driven synaptic events.

Of particular interest are the very earliest synaptic events observed as early as 24 hr *in vitro*. These rare events may originate from the few neurons with punctate GAD-6 staining at this time period (Fig. 10). Since these occur when neurons are still quite mobile, it is possible that they occur at transient presynaptic sites or by some mechanism that does not require fully assembled presynaptic machinery. Motoneuron growth cones are capable of both evoked and spontaneous release of transmitter (Hume et al., 1983; Young and Poo, 1983; Sun and Poo, 1987). Since both GAD-6 and GABA-5 immunostaining appear in growth cones (Fig. 10), it is possible that growth cones from central neurons also release transmitter.

Excitatory synaptic activity

The presence of spontaneous glutamatergic excitatory activity is noteworthy among neurons from the caudate and putamen.

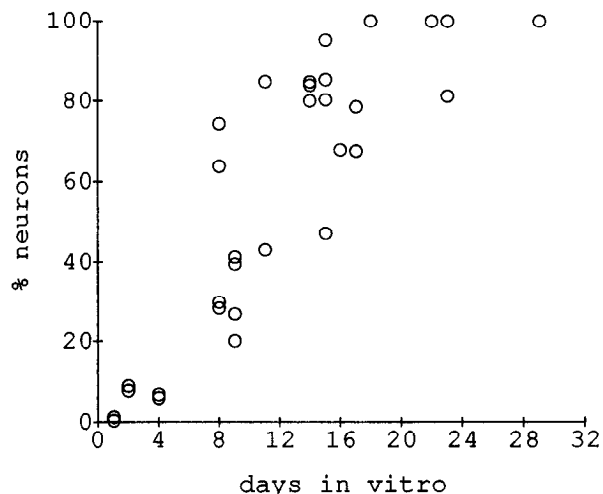


Figure 11. Percentage of CP neurons with punctate GAD-6 immunoreactivity in processes adjacent to their somas. Each data point represents the percentage of somas with punctate staining encountered in 10 microscope fields of one culture.

Glutamatergic afferents from cortex have been assumed to be the primary source of striatal excitatory activity (Hassler et al., 1982; Heimer et al., 1985). Experiments in striatal slices report excitatory events sensitive to the NMDA antagonist amino-phosphonoheptanoic acid resulting from local field stimulation, presumably of endogenous striatal neurons (Lighthall and Kitai, 1983; Cherubini et al., 1988), but these may have resulted from fibers of passage. The observations in dissociated cultures indicate that striatal neurons are capable of using glutamate as a transmitter. It is unlikely that these are cortical neurons since great care was taken during dissections to exclude all extrastriatal tissue (Fig. 1). Although this expression could be an artifact of the culture environment, it is more likely an indication of the presence of glutamatergic neurons intrinsic to the striatum. Since <1% of the cultured striatal neurons expressed choline acetyltransferase-like immunoreactivity or stained histochemically for acetylcholinesterase (unpublished observations), cholinergic excitatory activity was expected to be rare and was not studied.

The presence of GAD-6 immunoreactivity in 3 excitatory presynaptic neurons may represent a certain degree of false-positivity since a low level of nonspecific background staining is always present with this technique. However, other neuronal somas in these cultures were clearly unstained. The most intriguing possibility is that these neurons represent a class of central neurons which are either pleuripotent or plastic with respect to their transmitter phenotype (Landis, 1984; Docherty et al., 1987). Cortical and striatal GABAergic neurons contain markers for neuropeptides (Hendry et al., 1984; Penny et al., 1986; Alho et al., 1988), and retinal amacrine cells can express both cholinergic and GABAergic traits (Brecha et al., 1988; Kosaka et al., 1988; Vaney and Young, 1988). An overlap of GABAergic and aspartate-containing neuronal populations has previously been suggested (Storm-Mathisen and Ottersen, 1986). Considering the prevalence of excitatory activity in cultures with only 24% of the neurons lacking GAD-6 immunoreactivity, it is plausible that a population of GABAergic neurons may also express excitatory properties. Alternatively, a small number of excitatory neurons may innervate a large number of cells. The

possibility that a single striatal neuron expresses both excitatory and inhibitory properties is currently being explored.

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