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# Synaptogenesis of cultured striatal neurons in serum-free medium: A morphological and biochemical study

**(neuronal differentiation/synapse formation/synapsin I/signal transduction/γ-aminobutyric acid release)**

SAMUEL WEISS*‡, JEAN-PHILIPPE PIN*, MICHÈLE SEBBEN*, DOROTHY E. KEMP*, FRITZ SLADECZEK*, JACQUELINE GABRION†, AND JOËL BOCKAERT*

*Centre National de la Recherche Scientifique–Institut de la Santé et de la Recherche Médicale, Centre de Pharmacologie-Endocrinologie, B. P. 5055, 34033 Montpellier Cedex, France; and †Laboratoire de Neuroendocrinologie (UA Centre National de la Recherche Scientifique 639), Université des Sciences et Techniques du Languedoc, Place Eugene Bataillon, Montpellier, France

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ABSTRACT **Striatal neurons were cultured from the fetal mouse brain and maintained in serum-free medium for 14–21 days *in vitro* (DIV). Pretreatment of the culture dishes successively with a polycation followed by fetal calf serum resulted in rapid neuron attachment and neurite proliferation. After 9–10 DIV, electron microscope observations revealed the presence of vesicles in axon terminals forming mature synapses with axons and perikarya of adjacent neurons and in varicosities along extended axons. Synapsin I, a synaptic vesicle-specific protein, was present only in neuronal perikarya after 3 DIV, in perikarya and in varicosites along extended axons after 6 DIV, and in varicosities and contact points between axon terminals and adjacent axons or perikarya after 11–14 DIV. Neurotransmitter-stimulated intracellular formation of cAMP decreased markedly during neuronal differentiation. Inositol phosphate formation in response to neurotransmitters, however, increased significantly throughout the period of striatal neuronal development. $K^+$ (56 mM) depolarization resulted in a 2-fold increase in endogenous γ-aminobutyric acid (GABA) release from striatal neurons, 50% of which was $Ca^{2+}$-dependent, between 3 and 11 DIV. Between 11 and 14 DIV, subsequent to synapse formation (as revealed by electron microscope observations), GABA release evoked by 56 mM $K^+$ increased up to 5-fold, 75% of which was $Ca^{2+}$-dependent. It appears that the complete differentiation of striatal neurons in serum-free medium may provide a suitable model for the study of the physiological and regulatory mechanisms involved in nerve cell development.**

In the last few years, the generation of primary cultures of central nervous system (CNS) neurons has been carried out to examine their functional maturation, in the absence of glial cells and in a defined molecular environment (1, 2). Several advances that have resulted in improved neuronal survival and differentiation include the embryonic age of the nervous tissue to be cultured (3), the nature of the adhesive substrate (4), and particular elements in the culture medium (5). However, evidence for functional differentiation of specific structures in the mammalian CNS, after dissociation and generation in primary culture, has been limited (6, 7).

The neostriatum has been demonstrated to be involved in the pathophysiological aspects of extrapyramidal disorders, such as parkinsonism (8). Though a great deal is known regarding the biochemical anatomy of the striatum (9), few attempts have been made to examine the functional development of striatal neurons. In fact, recent findings indicating the involvement of neuronal degeneration in the pathology of numerous CNS disorders (10, 11) obviate the importance of elucidating the molecular mechanisms involved in the development and maintenance of neurons.

Significant findings as to the mechanisms involved in neuron development in CNS regions such as the mesencephalon (12) and hypothalamus (13) have permitted us to begin a multidimensional approach to the study of striatal neurons in primary culture, devoid of nonneuronal cell types. In the present study, we report the anatomical, biochemical, and physiological properties of striatal neurons, differentiated for up to 3 weeks *in vitro*.

## MATERIALS AND METHODS

**Neuronal Cultures in Serum-Free Medium.** Neuronal cultures referred to as "noncoated" were prepared as originally described by Di Porzio *et al.* (12) and modified by ourselves for studies of cAMP formation in striatal neurons (14). Neuronal cultures referred to as "serum-coated" were prepared as follows: striata were removed from 14- to 15-day-old Swiss albino mouse embryos (Iffa Credo, Lyon, France) and mechanically dissociated with a fire-narrowed Pasteur pipette in serum-free medium. Cells were plated ($0.4–0.6 \times 10^6$ cells per ml for morphological studies; $0.8–1.0 \times 10^6$ cells per ml for biochemical studies) in 6-well (2 ml per well) or 12-well (1 ml per well) Costar (Cambridge, MA) culture dishes previously coated successively with poly(L-ornithine) (1.5 μg/ml, $M_r$ 40,000; Sigma) and culture medium containing 10% fetal calf serum. After withdrawing the last coating solution, cells were seeded in serum-free medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 nutrient (GIBCO Europe, Paris) that included glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), and Hepes buffer (5 mM) (all from Sigma). In the place of serum, a defined hormone and salt mixture that included insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and selenium salt (30 nM) (all from Sigma) was added. Using this technique, these cultures were immunocytochemically (with antibodies to neurofilament and glial fibrillary acidic proteins) and morphologically [with transmission electron microscopy (TEM)] defined as purified (>95%) neurons in nature and were examined up to 21 days *in vitro* (DIV).

**Immunofluorescent Detection of Synapsin I.** After 3–13 DIV, neuronal cultures generated on Thermanox glass coverslips (Miles, Naperville, IL) were rinsed in phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$ and 1 mM

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Abbreviations: CNS, central nervous system; DIV, days *in vitro*; GABA, γ-aminobutyric acid; SEM, scanning electron microscopy; TEM, transmission electron microscopy; VIP, vasoactive intestinal polypeptide.

‡To whom reprint requests should be addressed at: Neuroscience Research Unit, Department of Psychiatry, University of Vermont College of Medicine, Burlington, VT 05405.

$MgCl_2$ at 37°C and were immediately fixed with 4% paraformaldehyde in PBS for 30 min at ambient temperature (AT). Coverslips were then rinsed in 0.1 M glycine (pH 7.4) for 30 min at AT and exposed to antisera to synapsin I, 1:100 in Triton buffer (0.3% Triton X-100/0.45 M NaCl/16% goat serum/20 mM sodium phosphate buffer, pH 7.4), for 3 hr at 37°C. Coverslips were washed three times, for 30 min, in Triton buffer and exposed to goat anti-rabbit second antibody conjugated to fluorescein or rhodamine, 1:50 in PBS, for 45 min at AT. After this period coverslips were washed for 30 min in Triton buffer, rinsed with 5 mM phosphate buffer (pH 7.4), mounted on glass slides, and visualized with a Leitz Dialux 20.

**Electron Microscopy.** Cultured striatal neurons, generated on Thermanox *plastic* coverslips, were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.5/120 mM glucose, postfixed in 1% osmium tetroxide in 5 mM veronal buffer, and dehydrated in an ethanol series. Cells were embedded in Epon, cut, and contrasted with uranyl acetate and lead citrate. TEM observations were performed with a Jeol 200 CX electron microscope. Some cultures were treated for scanning electron microscopy (SEM). After glutaraldehyde and osmium tetroxide fixations, coverslips with attached cells were critical point-dried with $CO_2$, mounted on stubs, coated with gold/palladium (400 Å thick), and observed with a Jeol JSM 35 at 20 kV.

**cAMP Determinations.** Intracellular cAMP levels in striatal neurons were determined as described (14).

**Inositol Phosphate Formation.** Inositol phosphate (inositol monophosphate, inositol bisphosphate, and inositol trisphosphate) formation (15) in striatal neurons was determined as described (16).

**Examination of Endogenous γ-Aminobutyric Acid (GABA) Release.** The liberation of endogenous amino acids was carried out according to the method of Gallo *et al.* (17). Amino acid levels in removed media were determined by HPLC with a modification of the method of Hogan *et al.* (18).

## RESULTS

**Morphological Differentiation of Striatal Neurons in Primary Culture.** Cultures of striatal neurons performed on noncoated or serum-coated substrates were compared by SEM and TEM. Striatal neurons on noncoated substrates were poorly developed and generally survived for 6–7 DIV. However, neurons cultured on serum-coated substrates exhibited greatly enhanced cell survival and neurite extension (Fig. 1). Dissociated striatal neurons expressed in culture typical capacities for cell recognition and cell association. Therefore, interneuronal contacts in varied configurations were distinguished (Fig. 2). Whereas axodendritic contacts were frequently observed, the presence of axosomatic, dendrodendritic, and axoaxonic contacts was also noted. On noncoated substrates, these contacts were always characterized by the absence of synaptic vesicles, whereas dense material was sometimes observed consisting of a thickening of one or both sides of the synaptic contact (Fig. 2*a*). However, for the most part, these interneuronal contacts were characterized by a regular intercellular space, without membrane thickening or synaptic vesicles. In contrast, the seeding of dissociated striatal nerve cells on serum-coated culture dishes permitted the development of numerous fully differentiated synapses, revealed by TEM after 9 DIV (Fig. 2 *b–d*). Enlarged axon terminals contained numerous small clear vesicles (30–50 nm) and an irregular smooth reticulum. Symmetric or asymmetric thickenings were often associated with the presence of synaptic vesicles. In addition, along the axons, synaptic vesicles were aggregated in small varicosities (Fig. 2 *e* and *f*).

**Immunolocalization of Synapsin I.** After 3 DIV, synapsin I was found principally in perikarya, with little apparent staining in neurites (Fig. 3*a*). After 6 DIV, less fluorescence was detected in perikarya (although still clearly visible), yet immunostaining was apparent in varicosities in extended neurites (Fig. 3*b*). After 12 DIV, neuronal cell bodies contained no fluorescence, whereas synapsin I was principally found in varicosities and expressed most distinctly in synaptic boutons contacting perikarya (Fig. 3*c*).

**Development of Neurotransmitter-Stimulated cAMP and Inositol Phosphate Formation.** The net increase in cAMP production due to the presence of 0.1 μM vasoactive intestinal polypeptide (VIP) remained constant between 3 and 6 DIV yet increased by 50% after 10–13 DIV (Fig. 4*a*). However, basal levels of cAMP formation and those due to the presence of 0.1 μM forskolin, an activator of the catalytic unit of adenylate cyclase, increased 4-fold in an almost linear fashion between 3 and 13 DIV. As a result, VIP-stimulated cAMP formation, 25- to 30-fold over basal at 3 DIV, decreased sharply during neuronal differentiation to 9- to 10-fold after 10–13 DIV (Fig. 4*b*).

The net increase in inositol phosphate formation due to either 100 μM carbachol or 100 μM noradrenaline rose sharply between 3 and 7 DIV and slightly less thereafter (Fig.

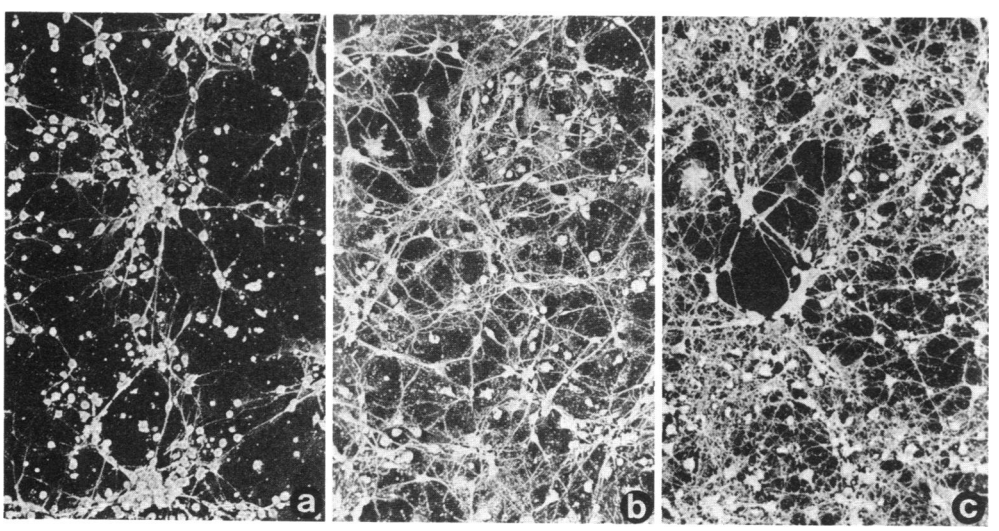


FIG. 1. SEM observations of striatal neurons cultured on noncoated (*a*; ×190) or serum-coated (*b* and *c*; ×190) substrates.

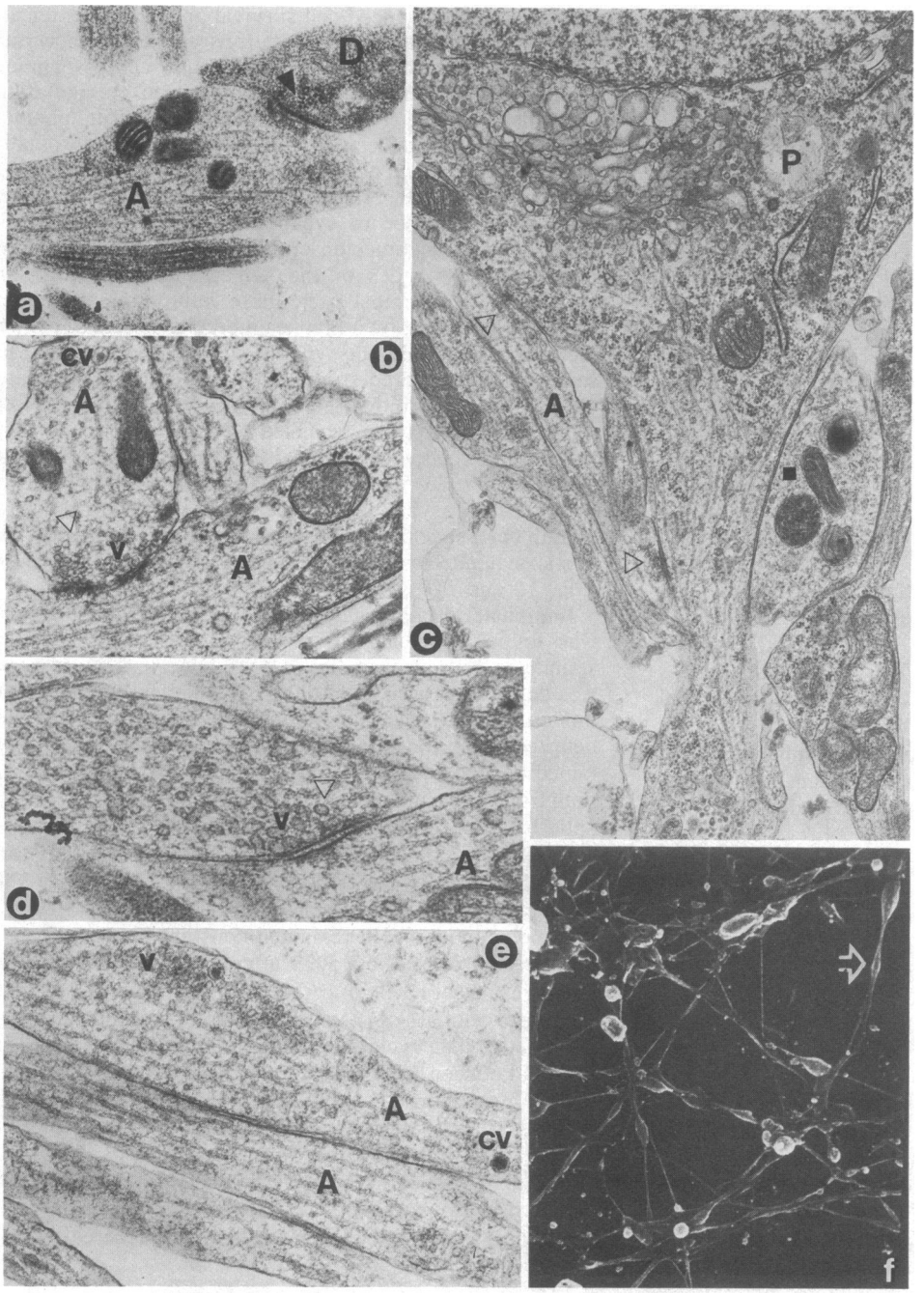


FIG. 2. TEM observations of striatal neurons. (*a*) When plated in poly(L-ornithine)-coated culture dishes, neurons exhibit immature synapses with membrane thickening, yet lacking synaptic vesicles. In this axodendritic contact (▲) dense material is more or less symmetrically distributed along the membrane. (*b–d*) When plated in culture dishes coated successively with poly(L-ornithine) and serum (subsequently withdrawn), striatal neurons differentiate and form mature synapses (△). Small round or elliptical electron-luscent vesicles (v) are observed in presynaptic nerve terminals as well as thickening of pre- and postsynaptic membranes. Neuronal contacts characterized by regular intercellular spaces (■) were observed in noncoated (not shown) and serum-coated (*c*) cultures. (*e*) Numerous small electron-luscent vesicles are also present in nonsynaptic areas along axons. Large cored vesicles (cv) are less frequent but appear along the axon (*e*) as well as in nerve terminals (*b*). (*f*) Varicose swellings (open arrow) along neurites without synaptic contacts (observed with SEM). P, perikaryon; A, axon; D, dendrite. (*a*, ×19,000; *b*, ×30,000; *c*, ×15,000; *d*, ×41,000; *e*, ×30,000; *f*, ×1,500.)

4*c*); basal levels rose up to 7 DIV and afterward they remained constant. However, in contrast to VIP-stimulated cAMP formation, neurotransmitter-stimulated inositol phosphate formation (fold stimulation over basal) increased during neuronal differentiation (Fig. 4*d*). Carbachol stimulation, 3-fold at 3 DIV, increased and saturated at 5-fold between 10 and 14 DIV. The response to noradrenaline, not apparent at 3 DIV, increased in an almost linear fashion to 3-fold at 14 DIV.

**Endogenous GABA Release from Striatal Neurons.** Amino acids, released into the extracellular medium subsequent to a 3-min $K^+$-mediated depolarization of striatal neurons after 7–18 DIV, were derivatized with *o*-phthalaldehyde and subjected to HPLC analysis (Fig. 5). $K^+$ (56 mM) depolarization resulted in a 2-fold increase in GABA release, 50% of which was $Ca^{2+}$-dependent, after 7–11 DIV. This effect was readily reversible within minutes of return to the resting state. Between 11 and 14 DIV, release evoked by 56 mM $K^+$ increased up to 5-fold, 75% of which was $Ca^{2+}$-dependent.

## DISCUSSION

In preliminary experiments, cultures were generated on surfaces that had been precoated with a polycation [poly(L-ornithine)] prior to seeding mechanically dispersed cells in a serum-free medium, originally described by Bottenstein and Sato (5). Although pure neuronal cultures deprived of non-neuronal cells were obtained, striatal neurons rarely survived for >7 days and were not capable of undergoing synaptogenesis. A simple modification (7), consisting of precoating the culture dishes with serum prior to the seeding of the cells, permitted striatal neuronal differentiation. Neurons underwent rapid neurite proliferation and a highly dense network was obtained after 10–14 DIV. Light and electron microscope observations as well as biochemical analysis reveal that these

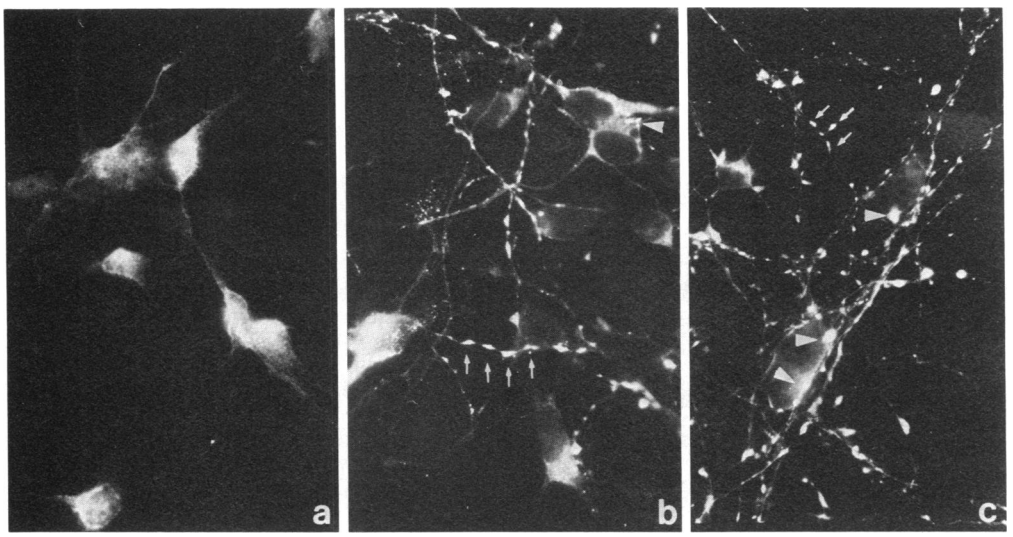


FIG. 3. Indirect immunofluorescent localization of synapsin I after 3 (*a*), 6 (*b*), and 12 (*c*) DIV (×450). Note the decrease and eventual disappearance of immunoreactivity in perikarya and increasing presence of synapsin I in varicosities (arrows) and synaptic boutons (arrowheads).

neurons remain viable for 14–21 DIV. Our findings are somewhat in contrast with those of Tixier-Vidal and colleagues. Whereas immunocytochemical and TEM observations confirmed the absence of nonneuronal cell types (<5%) in our striatal cultures, differentiation of hypothalamic neurons was achieved on a monolayer of glial cells (7). This difference may be due to the fact that hypothalamic structures were dissociated in the presence of serum or more likely may be due to a structure-specific difference in glial proliferation under these experimental conditions. It is clear that components of the extracellular matrix are essential for neuronal maturation (19). In addition, there is some evidence that laminin (20) and fibronectin (21), as coating substrates, promote neurite outgrowth. There is little doubt that certain factors remaining after the serum precoating promoted neuronal survival and differentiation. Their identification may deeply enhance our understanding of neuronal ontogenesis.

Similar to findings described for the neostriatum of developing rabbits (22), synaptogenesis in cultured striatal neurons was derived principally from axodendritic or axoaxonic contacts. Synapses "en passant" were numerous—in particular, at the level of bulbous processes. These synaptic terminals contain small electron-luscent vesicles and exhibit symmetrical or asymmetrical synaptic thickenings. In addition, varicosities—i.e., axonal swellings containing vesicles but not exhibiting junctional complexes—were also frequently observed. These bulbous processes were often not connected to their adjacent axon. Such varicose swellings, containing synaptic vesicles, were identified by TEM (Fig. 2) as well as by the appearance of synapsin I (Fig. 3). These varicosities may be considered as storage sites, periodically distributed along the axon, that allow the gathering of organelles, small and/or dense core vesicles. In addition, they may represent nonsynaptic sites for neurotransmitter release (22).

Synapsin I has been demonstrated to be associated with the cytoplasmic side of small synaptic vesicles found principally in nerve terminals (23), phosphorylated by synaptic activity (24), and expressed during ontogenesis with the start of neuronal synapse formation (25). In cultures of striatal neurons, synapsin I was immunocytochemically localized initially in cell bodies after 3 DIV and eventually in varicosities and nerve endings, where synaptic vesicles were observed by TEM. These findings suggest that the localization

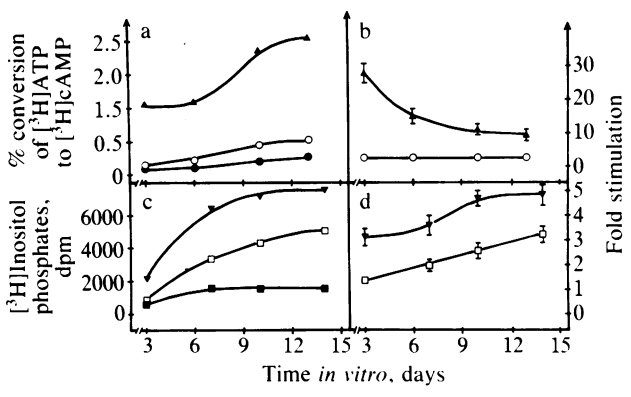


FIG. 4. Development of neurotransmitter-stimulated cAMP (*a* and *b*) and inositol phosphate (*c* and *d*) formation in striatal neurons. Representative experiments are shown. (*a*) % conversion of [$^3$H]ATP to [$^3$H]cAMP examined with no additions (●), in the presence of 0.1 μM forskolin (○), or in the presence of 0.1 μM forskolin and 0.1 μM VIP (▲). (*c*) [$^3$H]Inositol phosphate formed when examined with no additions (■), in the presence of 100 μM noradrenaline and 1 μM alprenolol (□), or in the presence of 100 μM carbachol (▼). The fold stimulation over basal levels of cAMP (*b*) and inositol phosphates (*d*) was determined.

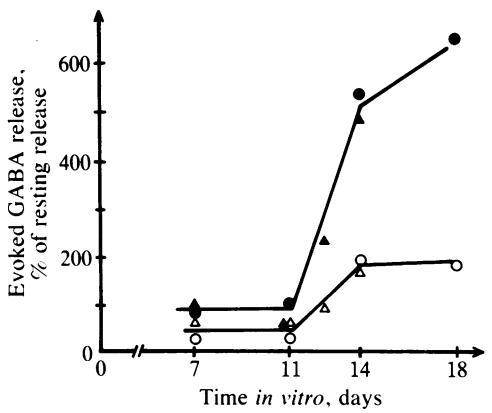


FIG. 5. Liberation of GABA from developing striatal neurons. GABA release evoked by 56 mM $K^+$ was examined in the absence (○, △) and presence (●, ▲) of 2.7 mM $Ca^{2+}$ at 7–18 DIV. Different symbols represent the individual experiments.

of synapsin I in nerve cell bodies may reveal its site of synthesis. Whether or not synapsin I migrates in neurites associated with synaptic vesicles is not known.

Cultures of striatal and cortical neurons, devoid of non-neuronal elements, examined 5–6 DIV after coating with only poly(L-ornithine) (no synapse formation) have been used by us (14, 26) and others (27) to study neurotransmitter receptors coupled to adenylate cyclase. It appears, therefore, that most of the receptors linked to adenylate cyclase are present and functional prior to synapse formation. In fact, in this study we observed that VIP stimulation (fold over basal) was greatest after 3 DIV (Fig. 4*b*) and decreased progressively thereafter, whereas stimulation of the catalytic unit remained constant. Such a pattern of ontogenetic development has been described for the postnatal neurotransmitter-stimulated adenylate cyclase in rat striatum (28). There is a body of evidence suggesting that cAMP may modulate synapse formation (29). Whether our findings reflect a particular role for cAMP during neuronal differentiation remains to be determined.

Interestingly, in direct contrast to that for cAMP formation, neurotransmitter-stimulated inositol phosphate formation (fold over basal) increased significantly during the differentiation of striatal neurons in primary culture. In fact, in the case of noradrenaline, there was no effect on inositol phosphate formation after 3 DIV and before 14 DIV the response had not yet saturated (Fig. 4*d*). The carbachol response, however, was quite evident at 3–6 DIV and saturated at 10–14 DIV. Thus, it would appear that differentiated neurons are necessary for full expression of all receptors that regulate intracellular inositol phosphate formation. In addition, it is possible that these two signal transduction mechanisms (cAMP and inositol phosphate) may have separate functions during neuronal differentiation.

In cultured neurons, release of neurotransmitters appears to be a function that is highly correlated with the final stage of neuronal differentiation (7). In striatal neurons in primary culture, $Ca^{2+}$-dependent GABA release was barely detectable until 11 DIV, increasing very rapidly thereafter (Fig. 5), similar to the development of $Ca^{2+}$-dependent GABA release in cultures of cortical neurons (30). It seems clear that this process appeared 1–2 DIV subsequent to the establishment of vesicle-containing synapses.

In conclusion, the primary culture of striatal neurons in a serum-free, defined medium and in the absence of non-neuronal cell types is able to undergo maturation and differentiation and provides an ideal model for examining the anatomical, biochemical, and physiological processes in nerve cell function.

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