

Cultures of Striatal Cholinergic Neurons: Phenotypic Changes in the Presence of Muscle Fibers

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Abstract

Striatal cholinergic neurons were purified from 1 to 3 day old rats, using a Nycodenz gradient. The degree of purification was assessed by means of immunohistochemistry and measurement of acetylcholine by chemiluminescent method. Cholinergic striatal cells were cultured in defined medium in the presence, or absence, of fetal tongue myoblasts. In the absence of muscle fibers, cholinergic cells never survived longer than 4 days. Presence of tongue myoblasts enhanced neuronal survival. Contacts between neurons and muscle fibers were observed, with in some cases, muscular contractions. Morphometric parameters of growing cholinergic striatal neurons were affected when cultured with tongue muscle fibers: the number of primary neurites, the number of bifurcations, the total length of neurites as well as the neuronal area were significantly reduced. The spreading index was increased, indicating the growth of one longer neurite under these conditions. These results demonstrate that cholinergic striatal neuronal phenotype can be influenced by epigenetic factors secreted by muscle fibers, giving a neuronal shape similar to that observed for motoneurons. This phenotype transformation is discussed in relation to the trophic influence of muscle cells.

Key words: rat striatal cholinergic neurons, muscle fibers, coculture, morphometric analysis, cholinergic phenotype.

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Cholinergic neurons are present in both the peripheral and central nervous system. Spinal and brainstem motoneurons with long axons, innervate muscle targets, whereas cholinergic striatal interneurons are strictly located in the striatum, making synapses with other interneurons or striato-nigral cells. Activity of cholinergic striatal neurons is controlled by nigro-striatal dopaminergic neurons [4, 37], cortical and thalamic neurons [2, 5].

During ontogenesis, survival, maturation and differentiation of motoneurons are closely related to the expression of various epigenetic factors which modulate the development of their adult shape. Trophic factors, particularly those belonging to the neurotrophin family, are involved in such mechanisms and play an important role in cholinergic motoneuronal phenotype expression [24, 25, 40]. Brain Derived Neurotrophic Factor (BDNF), neurotrophin-3 (NT 3) and neurotrophin-5 (NT 5) have been shown to prevent the death of embryonic rat spinal motoneurons and to act on their development [13]. Furthermore, the messenger RNA coding for these neurotrophins is present in the spinal cord and limb buds and the mRNA for their receptors found in motoneurons [13]. Consequently, growth of

motoneurons is in part related to their interaction with muscle targets.

Effects of neurotrophins were also demonstrated on striatal neurons. Nerve Growth Factor (NGF) was shown to be a neurotrophic factor for striatal cholinergic interneurons based on *in vivo* [31] and *in vitro* [27] studies. BDNF, NT 3 and NT 4-5 were shown to promote survival and biochemical differentiation of striatal neurons *in vitro* [43]. The receptors for tyrosine kinase (TrK B and TrK C), which bind BDNF and NT 3 respectively, were detected in embryonic [7] as well as adult [30] rat striatum. and BDNF, NT 3 and NT 4-5 were shown. Epidermal Growth Factor (EGF) and basic Fibroblastic Growth Factor (bFGF) exert a trophic action on fetal as well as postnatal striatal cells [29, 32, 44]. Finally, Iacovitti [14] has shown that a muscle-derived differentiation factor induces the expression of a cathécholaminergic phenotype in cultured embryonic striatal neurons.

In the present study, our goal was to investigate *in vitro* the effects of the presence of muscle fibers on the survival and maturation of neonatal striatal cholinergic phenotype. At this stage of development, cholinergic striatal in-

terneurons are still expressing embryonic properties and we were interested in the effects of embryonic muscle cells on the morphological characteristics of these neurons. Are they able to express a motoneuronal phenotype under these particular culture conditions? This approach was favoured by the fact that cholinergic striatal neurons, which are the largest cells of the striatum, can be purified as spinal and brainstem motoneurons, using a Nycodenz gradient.

Methods

1) Striatal cholinergic cells in culture

Pregnant rats were purchased from Ifa Credo (L'Arbresle, France) and 1 to 3 day old rats were sacrificed by cervical dislocation. The following dissection steps were carried out in a sterile horizontal laminar flow hood. At all steps siliconized Pasteur pipettes were used. Centrifugations were carried out at room temperature, unless stated otherwise. Brains were rapidly extracted and transferred by spoon to a Petri dish containing calcium- and magnesium-free phosphate buffer saline solution (PBS-). Following separation of the two hemispheres the striata were dissected under a stereomicroscope (12 to 16 striata) and placed in a Petri dish with fresh PBS-. The striata were cut in small pieces (1 mm³) and these fragments were incubated in 0.025% trypsin (Sigma, 110 U/mg) and 0.005% DNase I (Boehringer, 3,000 U/mg) in PBS-, for 20 min at room temperature (25°C). The following steps were performed in a vertical sterile laminar flow hood. The supernatant was carefully removed and replaced by 3 ml of calcium- and magnesium-free Hanks balanced salt solution (HBSS, Gibco Brl.) containing 25 µg/ml gentamycine (Sigma). The tissue was triturated 15-20 times, firstly with a siliconized Pasteur pipette and then with a Pasteur pipette with a smaller tip opening. The volume was filled up to 10 ml of HBSS and cells were triturated again shortly. The cells were then centrifuged for 10 min at 700 g and the pellet was resuspended in 10 ml HBSS containing 0.001% DNase I and incubated for 10 min at 37°C. The cells were again centrifuged for 10 min at 700 g, resuspended in 3 ml of HBSS and layered on the top of 3.5% bovine serum albumin (BSA, Boehringer) in 5 ml HBSS. Cells were centrifuged for 10 min at 200 g to remove debris. The cell pellet was resuspended in 2 ml HBSS and layered on top of a discontinuous Nycodenz gradient (Nyegaard, Norway) in HBSS, composed of two 3 ml layers of densities 1.065 and 1.047 respectively [28]. The cells were then centrifuged in a Sigma K3 centrifuge for 20 min at 700 g, at 4°C, with a programmed soft start and stop. The top 3 ml of the gradient, containing the largest cells, were collected. The cells were diluted into 10 ml HBSS and centrifuged for 10 min at 1,000 g. Cells were resuspended in 500 µl of culture medium and cellular density was determined using a Malassez chamber. 5,000 cells in 100 µl were seeded on 12 x 24 mm glass coverslips coated first with poly-L-Lysine (24 hours, Sigma, 0.005% in H₂O) and with laminin for 24 hours (Sigma, 0.001% in sodium phosphate buffer). The coverslips were placed in 35-mm Petri dishes

(Falcon). Following adhesion (15 to 20 min), 2 ml of culture medium was added. Cultures were incubated in a humid incubator at 37°C with 5% CO₂.

2) Culture medium

The culture medium was a defined medium: DMEM/F12 (Gibco, 1/1: V/V, pH 7.4, osmolarity: 320 mOsm) supplemented with human transferrin (100 µg/ml), insulin (5 µg/ml), putrescine (0.1 mM), sodium selenite (30 nM), progesterone (20 nM), oestradiol (1 pM), D-glucose (33 mM), L-glutamate (2 mM) and gentamycine (100 µg/ml). All additives were purchased from Sigma. Culture medium was changed after 1 day in culture and then every fifth day.

3) Cocultures of purified cholinergic striatal cells and muscles

Purified cholinergic striatal cells were cultured in defined medium in the presence of muscle cells. Myoblasts were obtained from the tongue of E15-E16 rat embryos. Briefly, tongues were cut in small pieces and the tissue was incubated for 30 min, at 37°C, in 10 ml of calcium- and magnesium-free HBSS containing 0.15% of type A collagenase (Sigma). The tissue was triturated with a siliconized Pasteur pipette (15 to 20 times) and the suspension was centrifuged for 10 min at 700 g. The pellet was resuspended in 10 ml of L15 medium (Gibco) and filtered on a 10 µm nylon mesh. Cells were centrifuged (700 g, 10 min) and the pellet (formed off 10 sampled tongues) was resuspended in 2 ml of DMEM/F12 medium supplemented with additives. 200 µl of muscle cells suspension were seeded on coated glass coverslips. The purified cholinergic striatal cells were then plated following proliferation and differentiation of muscular cells (12 to 24 hours, 5,000 to 7,000 purified striatal cells).

4) Characterization of the striatal cholinergic phenotype

The efficiency of the Nycodenz gradient purification was tested by various techniques. Quantitative distribution of the cells and measurement of their diameters were done following ml by ml sampling of the Nycodenz gradient. At the same time, each fraction was prepared for chemiluminescent detection of intracellular acetylcholine (ACh). Five ml of HBSS was added to each fraction and the cells were again centrifuged at 800 g for 10 min. The pellets were placed in 100 µl of 5% trichloroacetic acid and centrifuged at 3,000 g during 5 min. The supernatant was washed four times with 500 µl of ether saturated with water. The last trace of ether was evaporated and the pH of the extract was approximately 4. The chemiluminescent determination of ACh, described in details by Israel and Lesbats [15] and slightly modified by Ternaux and Chamoin [38], is based on the following principle: the enzyme choline oxidase from *Arthobacter globiformis*, catalyses the oxidation of choline, generating betaine and H₂O₂ with consumption of oxygen. The hydrogen peroxide formed is continuously monitored by measuring the light produced by the oxida-

tion of luminol in the presence of peroxidase. The peaks of light produced were proportional to the content of ACh.

The striatal cholinergic cells were also identified in culture by immunohistochemistry. Following stabilisation of the coverslips at room temperature (25 min), cells from different periods in culture were fixed with 3% paraformaldehyde in 0.1 M sodium phosphate buffer for 30 min and rinsed 3 times with PBS-. For ACh detection, striatal cells were fixed with sodium cacodylate buffer (0.1 M) containing $\text{Na}_2\text{S}_2\text{O}_5$ (10 mg/ml) and glutaraldehyde (3%). Cultures were then incubated, for 1 h at room temperature, in a PBS diluent solution containing: 0.1% Triton X-100, 3% normal serum of the same species as the secondary antibody and 5% BSA. Following 3 washes of 5 min with PBS buffer, the cells were either incubated with a neuron specific enolase antibody (anti-NSE, ICN, 1: 200), a choline acetyl transferase antibody (anti-ChAT, Chemicon, 1: 500), an ACh antibody (anti-ACh, Biosoft, 1: 500), anti-AChE (generous gift from Dr. J. Massoulie, ENS Paris, 1:400) or anti-Islet I (generous gift of Dr. C. Henderson, Marseille, 1: 400). This incubation was performed for 24 h at 4°C. The cells were then rinsed 3 times (5 min each) in PBS buffer and incubated again for 90 min with an anti-rabbit-goat IgG diluted to 1: 100 in the diluent solution. After rinsing (3 times, 5 min each), the cultures were incubated with a rabbit PAP diluted to 1: 400 in the diluent. The cells were again rinsed in PBS (3 times) and once in 0.1 M Tris-buffer (5 min). Immunoreactivity was revealed

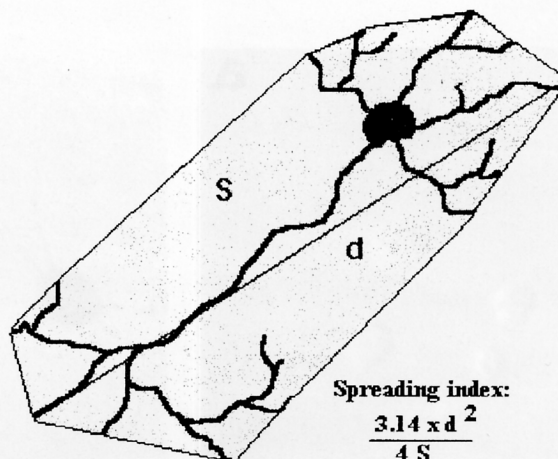


Figure 1. Morphometric analysis of neurons growing "in vitro". Isolated growing cells were photographed and the number of primary neurites emerging from the soma were counted as well as the total number of bifurcations. The total lengths of the neurites was determined using a graphic tablet coupled to a micro-computer. The surface area of the neuron (S) was evaluated by measurement of the surface of a convex polygon drawn at the tip of each neurite ending. The spreading index, which gives an idea of the neuronal shape, was calculated using the formula: $\pi \times d^2 / 4S$, where d is the longest diagonal of the polygon.

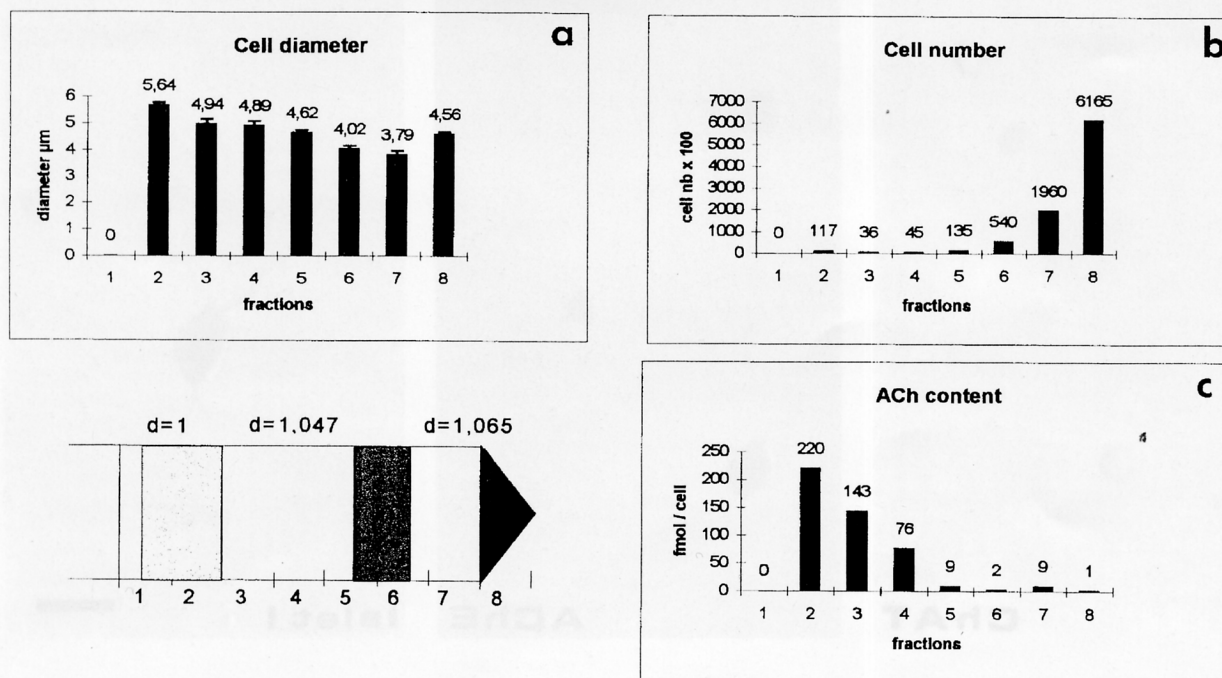


Figure 2. Purification of striatal cholinergic cells using a Nycodenz gradient. An example of cell analysis performed following centrifugation on a Nycodenz gradient. Fractions of 1 ml were sampled from top to bottom of the gradient and aliquotes were used to count the cells using a Malassez chamber. Diameters were measured from photographs (inverted microscope equipped with Hofmann contrast, magnification $\times 600$, $n = 80$ cells for each fraction). ACh content was determined using a chemiluminescent assay and results were expressed in fmol / cell.

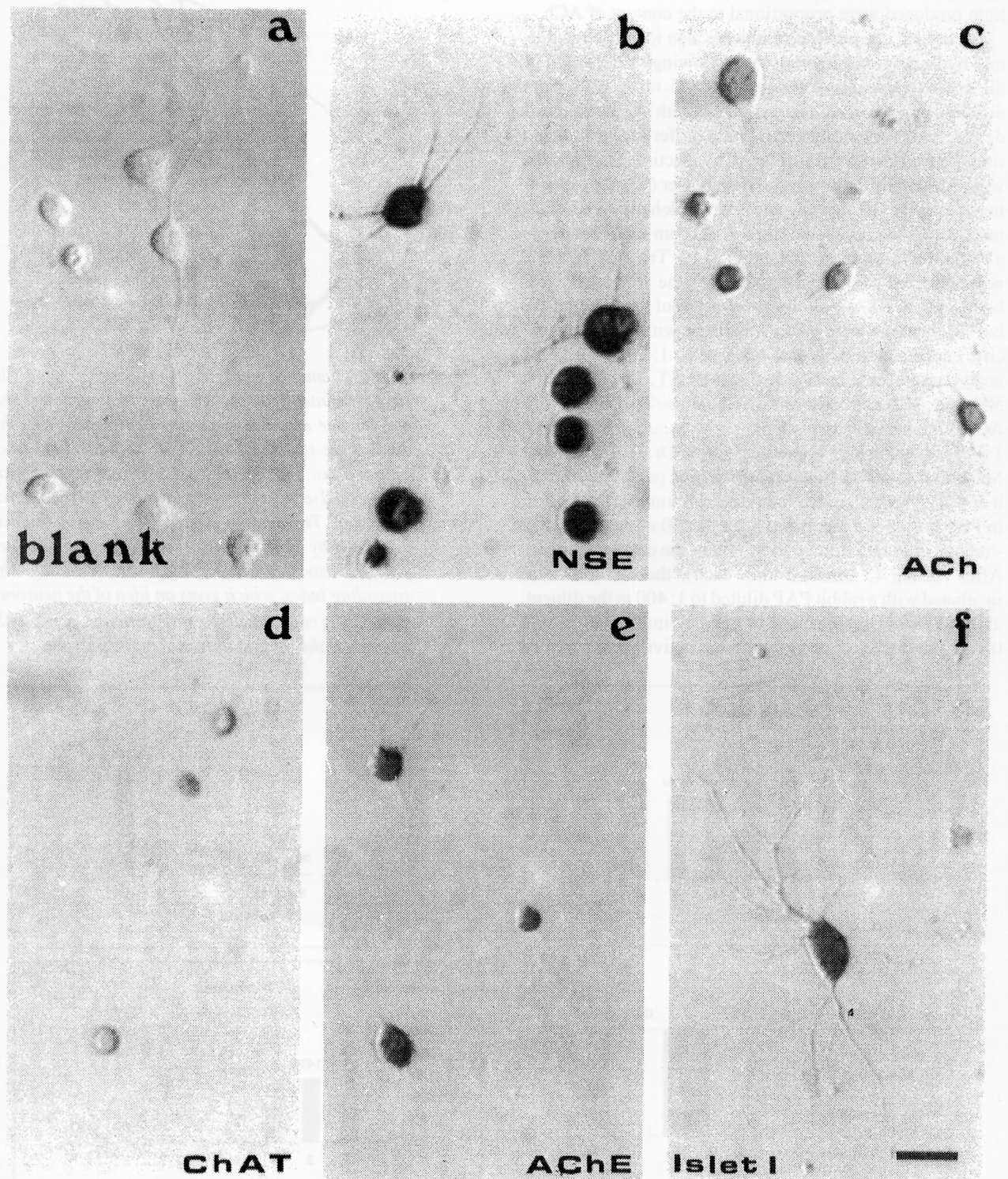


Figure 3. Immunohistochemical identification of purified striatal cells. Cultures of purified striatal cells were fixed at various times after plating and grown in defined medium. b) Immunoperoxidase staining with an antibody raised against NSE (3 days in culture). c) Staining with anti- ACh (1 day in culture). d) Revelation of ChAT in striatal cells (1 hour after plating). e) AChE labelling (1 day in culture). f) Staining obtained with an antibody raised against Islet I (4 days in culture). a) Blank was obtained by omission of the primary antibody (3 days in culture). Scale bar: 10 μ m.

after 10 min by 3-3'-diaminobenzidine tetrahydrochloride (0.05% in the presence of 0.01% H_2O_2 in 0.1 M Tris-buffer). The reaction was stopped by rinsing with 0.1 M sodium phosphate buffer. The coverslips were then dehydrated in increasing alcohol concentrations and mounted in Entellan (Merck). Controls were made by omission of the primary antibody.

5) Quantitative morphometric analysis of growing striatal cholinergic cells

Cultures were viewed with an inverted microscope (IMT2, Olympus) equipped with phase- and Hofmann contrasts. Isolated, purified striatal cholinergic neurons were photographed with an OM 4 Olympus camera or with a video CCD camera (Sony) coupled to a video graphic printer (Sony UP 850). Quantitative morphometric analysis was performed on video images using a graphics tablet (Summasketch III, Summagraphics) coupled to a PC computer. Morphometric software (designed at University College, London) allows the direct measurement of lengths (μm) and surface area (μm^2). Morphometric analysis of striatal cholinergic cells during "in vitro" development was performed daily following plating. In each experiment 30 cells were randomly selected and the following parameters were measured: the number of primary neurites emerging from the soma, the total number of bifurcations, the total length of the neurites and the surface area of the neuron and spreading index (Fig. 1). For each parameter studied, results were expressed as the mean $\pm$ SEM. Statistical analysis of the results was performed using the Student *t* test.

Results

1) Identification of purified striatal cholinergic cells

As shown in Fig. 2a, cells which were found in the two first ml at the top of the gradient (fraction 2) have the largest diameters ($5.64 \mu m$). Cell diameters decreased from the top to the bottom of the gradient, except for the last fraction where cells with a diameter of $4.56 \mu m$ were detected. The largest cells which were detected at the interface between HBSS and Nycodenz solution (at a density of 1.047) represent 1.7 to 2.3% of the cell population layered on the gradient (Fig. 2b). Measurement of ACh in each fraction clearly showed that the highest concentrations of ACh were detected in the three first fractions of the gradient, attesting that these fractions contain large diameter cholinergic neurons (Fig. 2c).

Immunohistochemistry of purified neurons from the top of the gradient (fractions 2 and 3) revealed that more than 95% of these neurons are immunoreactive to antibodies raised against NSE, ChAT, ACh, AChE and Islet I (Fig. 3) verifying that these cells are cholinergic neurons expressing embryonic markers such as Islet I.

2) Growth and survival of purified striatal cholinergic cells

When purified striatal cholinergic cells were cultured in

defined medium, growth of neurites was observed within the first hours following plating. Nevertheless, more than 50% of the cells died within 4 days. At 5 days after plating, living cells were about 10% compared to starting density.

Coculturing with tongue myoblasts clearly promoted the survival of purified striatal cholinergic neurons. More than 60% of the cell were present after 8 days in culture and some cells survived for more than 2 weeks. As shown in Fig. 4, cells grown either in the presence of defined medium or in the presence of tongue myoblasts had different shapes: cholinergic striatal cells cocultured with muscle fibers had fewer neurites, one of which was longer than the others. Contacts between differentiated muscle fibers and cholinergic neurons were observed. Poly- as well as mono-"innervations" were detected (Fig. 5a, 5d). In some cases, pictures of reinnervation were accompanied with regular muscular contractions (Fig. 5e, 5f).

3) Morphometric analysis of cholinergic striatal neurons grown in the absence or presence of muscle fibers

As described in Fig. 6 and 7, the morphometric parameters of striatal cholinergic cells were significantly affected by the presence of tongue muscle fibers. The number of primary neurites springing from the cell soma was reduced (Fig. 6a) and the total number of bifurcations was decreased by about 50% (Fig. 6b, 6c). The total length of

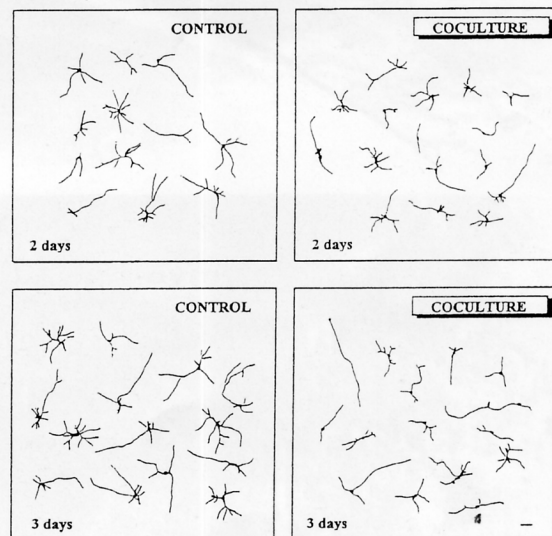


Figure 4. Maturation of purified cholinergic striatal neurons in defined medium and in coculture with tongue myoblasts. Drawings of isolated purified cholinergic striatal neurons sampled 2 and 3 days after plating. Cells were cultured in defined medium in the presence (COCULT) or absence (CONT) of tongue myoblasts. Cholinergic striatal neurons grown in the presence of tongue myoblasts exhibit fewer neuritic bifurcations than the controls. In addition, the presence of myoblasts induces the growth of one longer neurite which gives the striatal neurons a shape similar to that of motoneurons. Scale bar: $30 \mu m$.

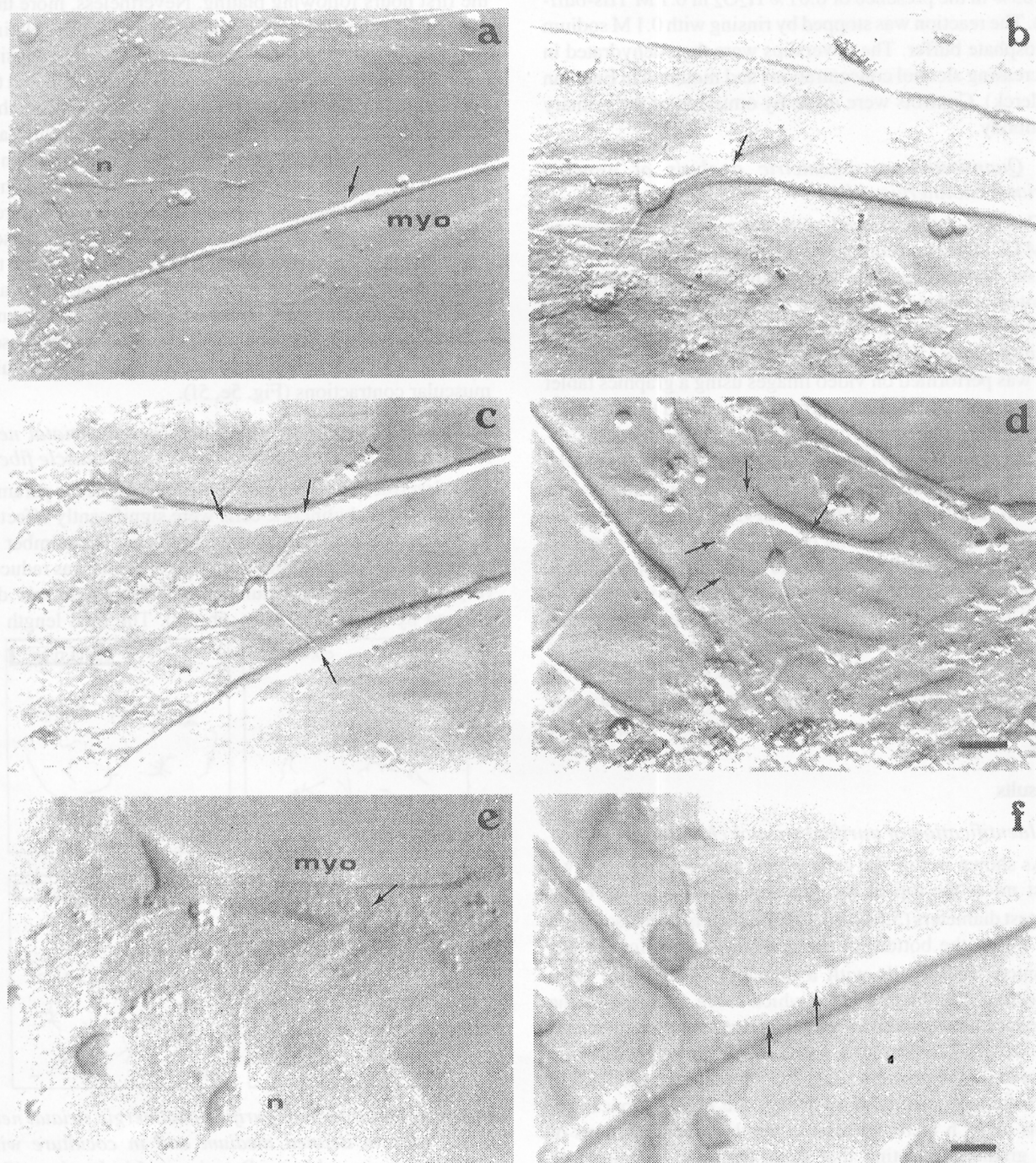


Figure 5. Striatal cholinergic neurons in coculture with tongue myoblasts. Examples of neuromuscular contacts between cholinergic striatal neurons and tongue muscle cells grown in defined medium. *a* and *b*: 2 days in culture. *c* and *d*: 4 days in culture, neurites from a given neuron are seen to contact one or two muscle fibers. *e*: 1 day in culture. *f*: 8 days in culture, in this case muscular fiber exhibited regular contractions, suggesting a functional innervation. Arrows indicate contacts between neurons and muscle fibers. Scale bar, for *a*, *b*, *c*, *d*: 20 μ m; for *e* and *f*: 10 μ m.

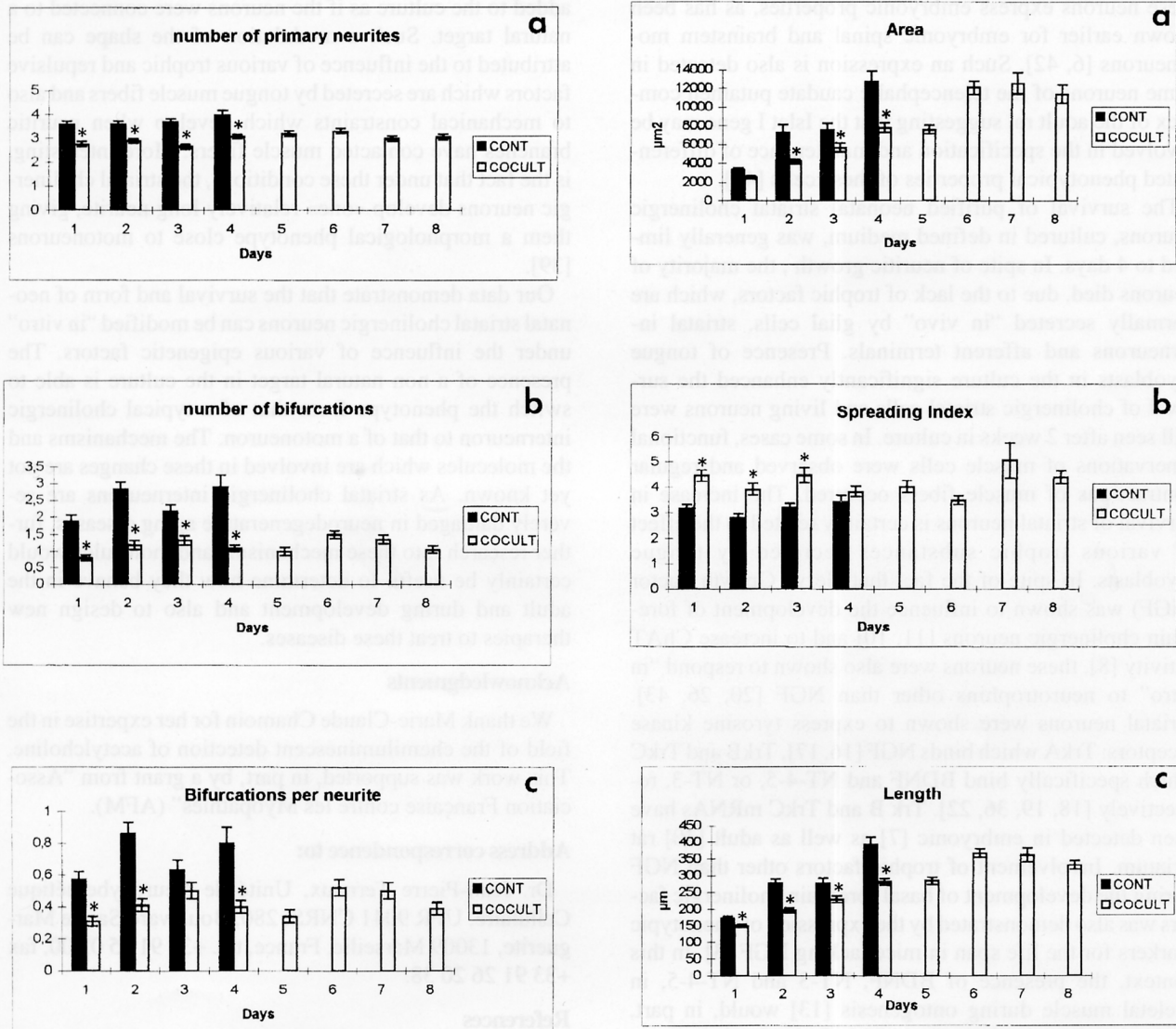


Figure 6 and 7. Morphometric analysis of striatal cholinergic neurons grown in the absence or presence of muscle fibers. Morphometric parameters were determined as described in "Material and methods". Each value is the mean $\pm$ SEM of 90 neurons from 3 cultures. * Statistical difference versus defined medium. * $P < 0.001$. 6: evolution of the number of primary neurites, number of bifurcations and bifurcations per neurite. 7: evolution of neuronal surface area, spreading index and total length of neurites.

neurites was significantly increased in defined medium (Fig. 7c) and the neuronal surface area was reduced in presence of muscle fibers (Fig. 7a). Finally, the spreading index (Fig. 7b) indicates that striatal cholinergic neurons, grown in presence of muscle, possess a neurite of greater length than the others.

Discussion

The immunohistochemical staining as well as the determination of ACh content with chemiluminescent methods demonstrate that the upper fraction of the Nycodenz gradient is enriched with cholinergic striatal interneurons. Our data indicate that the purification method, using the Nycodenz gradient, designed by Martinou et al. [28] to purify rat spinal motoneurons and brainstem motoneurons [39], is suitable to harvest isolated striatal cholinergic interneurons (which are the largest cells in the striatum). The percentage of cholinergic striatal cells obtained under these conditions was comparable to that determined in the whole striatum following immunohistological analysis. Stainings obtained with the antibodies raised against intermediates in the ACh metabolic pathway indicate that the cultured cells contain ACh as well as the synthesizing (ChAT) and the catabolic enzyme (AChE). The presence of Islet I antigene in neonatal striatal cholinergic cells reveals that

these neurons express embryonic properties, as has been shown earlier for embryonic spinal and brainstem motoneurons [6, 42]. Such an expression is also detected in some neurons of the telencephalic caudate putamen complex of the adult rat suggesting that the Islet I gene may be involved in the specification and maintenance of differentiated phenotypical properties of these cells [41].

The survival of purified neonatal striatal cholinergic neurons, cultured in defined medium, was generally limited to 4 days. In spite of neuritic growth, the majority of neurons died, due to the lack of trophic factors, which are normally secreted "in vivo" by glial cells, striatal interneurons and afferent terminals. Presence of tongue myoblasts in the culture significantly enhanced the survival of cholinergic striatal cells and living neurons were still seen after 2 weeks in culture. In some cases, functional innervations of muscle cells were observed and regular contractions of muscle fibers occurred. The increase in survival of striatal neurons is certainly related to the effect of various trophic substances secreted by tongue myoblasts. In spite of the fact that Nerve Growth Factor (NGF) was shown to influence the development of forebrain cholinergic neurons [11, 10] and to increase ChAT activity [8], these neurons were also shown to respond "in vitro" to neurotrophins other than NGF [20, 26, 43]. Striatal neurons were shown to express tyrosine kinase receptors: TrkA which binds NGF [16, 17], TrkB and TrkC which specifically bind BDNF and NT-4-5, or NT-3, respectively [18, 19, 36, 22]. Trk B and TrkC mRNAs have been detected in embryonic [7] as well as adult [30] rat striatum. Involvement of trophic factors other than NGF during the development of basal forebrain cholinergic factors was also demonstrated by the expression of phenotypic markers for the life span in mice lacking NGF [3]. In this context, the presence of BDNF, NT-3 and NT-4-5, in skeletal muscle during ontogenesis [13] would, in part, explain the increased survival of purified striatal cholinergic neurons cocultured with muscular fibers. In addition, influence of the Insulin Growth Factors (IGF I and IGF II) which are present at high concentration in the embryonic tongue [1] can also be suspected. Finally, cholinergic trophic influences both from muscles and from the neuronal compartment itself cannot be excluded, since ACh and AChE influence the survival and growth of various neurons [23, 33, 34, 35] and ACh release from cultures of satellite muscle cells [9, 21] has been shown to promote the differentiation of proliferating myoblasts into myotubes.

Not only do muscle cells induce longer survival, but they also modify the form of purified striatal cholinergic neurons. The presence of myocytes induced a significant decrease in the number and branching of primary neurites. It reduced both total length of the neurites and the neuronal surface area. However the spreading index was significantly increased in presence of tongue muscles. These results indicate that the morphological properties of striatal cholinergic neurons are stabilized when muscle cells are

added to the culture as if the neurons were connected to a natural target. Such a modulation of the shape can be attributed to the influence of various trophic and repulsive factors which are secreted by tongue muscle fibers and also to mechanical constraints which develop when neuritic branches have contacted muscle fibers. More interesting, is the fact that under these conditions, the striatal cholinergic neurons develop «one» relatively long neurite, giving them a morphological phenotype close to motoneurons [39].

Our data demonstrate that the survival and form of neonatal striatal cholinergic neurons can be modified "in vitro" under the influence of various epigenetic factors. The presence of a non natural target in the culture is able to switch the phenotype from that of a typical cholinergic interneuron to that of a motoneuron. The mechanisms and the molecules which are involved in these changes are not yet known. As striatal cholinergic interneurons are severely damaged in neurodegenerative aging diseases, further research into these mechanisms and molecules would certainly be useful to determine how they behave in the adult and during development and also to design new therapies to treat these diseases.

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