

Differentiated Properties of Identified Serotonin Neurons in Dissociated Cultures of Embryonic Rat Brain Stem

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ABSTRACT Serotonin neurons in 14-d embryonic rat brain stem were identified by peroxidase-antiperoxidase immunocytochemistry with an affinity-purified antiserotonin antibody. Brain-stem tissue was dissected from 14- or 15-d embryonic rats, dissociated and grown in cell culture for up to 5 wk, and serotonin neurons were identified by immunocytochemistry. Within 24 h of plating, serotonin immunoreactivity was present in 3.3% of neurons. Immunoreactivity in neuronal cell bodies decreased with time, whereas staining of processes increased. The number of serotonin-immunoreactive neurons remained constant at 3–5% over the first 14 d in culture. From 14 to 28 d, the total number of neurons decreased with little change in the number of serotonin neurons, such that, by day 28 in culture, up to 36% of surviving neurons exhibited serotonin immunoreactivity.

Similar percentages of cultured brain stem neurons accumulating ^3H -serotonin were identified by autoradiography. Uptake was abolished by the serotonin-uptake inhibitor, clomipramine, but was unaffected by excess norepinephrine, or by the norepinephrine-uptake inhibitor, maprotiline. Synthesis of ^3H -serotonin was detected after incubation of cultures with ^3H -tryptophan, and newly synthesized serotonin was released by potassium depolarization in a calcium-dependent manner. More than 95% of serotonin neurons were destroyed after incubation of cultures with 5,6-dihydroxytryptamine.

Brain-stem cultures contained virtually no neurons with the ability to accumulate ^3H -norepinephrine or ^3H -dopamine. Approximately 40% of brain-stem neurons were labeled with gamma-aminobutyric acid (^3H -GABA). However, there was almost no overlap in the surface area of neurons accumulating ^3H -serotonin or ^3H -GABA.

In many instances, the simplified neuronal circuitry and increased accessibility of central neurons maintained in dissociated cell culture have enabled the physiological features of individual neurons to be examined under carefully controlled conditions that are unattainable in vivo. In most regions of the central nervous system (CNS), however, the difficulty involved in identifying and purifying discrete populations of neurons has meant that physiological studies in culture have had to be performed on unidentified neuronal subtypes. With the eventual aim of examining the characteristics of serotonin transmission under defined conditions, we have therefore attempted to maintain and identify central serotonin neurons in dissociated cell culture.

The cell bodies of serotonin-containing neurons in the mam-

malian brain are confined to the raphe and other midline mesencephalic and medullary nuclei (14), whereas the terminal axonal fields of these neurons are distributed throughout the CNS (19, 29). Ionophoretic application of serotonin has been shown to alter neuronal activity in every region of the CNS in which it has been tested, with both excitatory and depressant actions (9). In addition, a number of recent observations suggest that monoamines can influence the development of other central neurons (26, 27, 35). Serotonin neurons themselves appear comparatively early in embryogenesis and express transmitter specific properties on or around the time that they leave the cell cycle (26, 28, 31, 35). Indeed, serotonin histofluorescence in the rat can be detected in cells lying along the midline of the medulla as early as day 12 or 13 of gestation (31). The early

expression of transmitter-specific properties by serotonin neurons seems to provide a functionally relevant set of criteria for identifying the same neurons in culture.

This paper describes the conditions required for the long-term survival of serotonin neurons isolated from embryonic rat brain stem in dissociated cell culture and examines the development, morphological features, and biochemical properties of these neurons.

MATERIALS AND METHODS

Tissue Culture

BRAIN-STEM NEURONS: 13- to 15-d embryos were obtained from timed-pregnant rats (Charles River Breeding Laboratories, Wilmington, Mass.; CD strain) and the exact age of each litter was assessed, using criteria outlined by Shlumpf et al. (35). The brains were removed and placed in calcium-free Eagle's minimum essential medium (MEM, Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) and a 2-mm paramedian sagittal strip of tissue was removed from the level of the tectum to the obex under stereomicroscopic observation (Fig. 1a). The midline brain-stem tissue was then cut into 200- μ m sections in two directions, using a Mellwain tissue chopper (Mickle Laboratory Engineering Co., Ltd., Gomshall, Surrey, England), and incubated in 2 ml of calcium-free MEM containing 0.025% trypsin (Sigma Chemical Co., St. Louis, Mo.; type III) for 10 min at 37°C in an atmosphere of 95% air/5% CO₂. After 10 min, DNase (Sigma Chemical Co.) 80 μ g/ml was added, and incubation was continued for a further 10 min in the presence of trypsin. Tissue was separated from the incubation medium by centrifugation at 180 g for 5 min. Brain-stem tissue was dissociated by repeated trituration, using a fire-polished Pasteur pipette in 3 ml of complete medium (Ham's F12 supplemented with 7.5% heat-inactivated rat serum; 4% 17-d embryonic rat extract; 44 mM glucose, 2 mM glutamine, 1% MEM 100 \times vitamins [Gibco Laboratories], and penicillin/streptomycin 50 IU/ml), until no tissue fragments were visible (usually 20–30 times). The resulting cell suspension was filtered through three layers of sterilized lens paper to remove cellular aggregates (1), and the filtrate was dispensed in 35 mm tissue culture dishes, on one of three different substrates: rat-tail collagen (11) treated with 25 μ g/ml purified human fibronectin (17), collagen gel (4), or on tissue culture

dishes treated overnight with a solution of 40,000 M, poly-L-lysine (100 μ g/ml; Sigma Chemical Co.). This procedure has been shown previously to support the long-term survival of rat cerebellar neurons (13). Cells were plated at a density of 5×10^5 – 1×10^6 cells per 35-mm plate, with a total yield of $\sim 10^6$ cells per embryo. Cultures were treated with cytosine arabinoside (10^{-5} M), for 48 h, 4–5 d after plating, and then fed every 3–5 d.

OTHER NEURONS: Spinal cords were removed from 15-d rat embryos, freed of surrounding pia and dura, then dissociated and plated as described above, at a density of 10^6 cells per 35 mm dish. Dorsal root ganglia were removed from 1- to 3-d postnatal rats and dissociated in calcium-free MEM containing 0.01% collagenase and 0.1% trypsin for 30 min. Cells were plated at a density of 4×10^4 neurons per 35-mm dish in complete medium containing 1 μ g/ml purified nerve growth factor prepared by the method of Varon et al. (40), and treated after 1 d with cytosine arabinoside (10^{-5} M) for 48 h. Neurons from postnatal rat cerebellar cortex were prepared and grown as described previously (13).

NONNEURONAL CELLS: In the absence of treatment with cytosine arabinoside, nonneuronal cells from brain stem, spinal cord, and dorsal root ganglion cultures continued to divide, forming a confluent cell layer. Neurons were removed from these cultures by repeated mechanical agitation, whereas nonneuronal cells remained firmly attached to the substrate. Neuronal outgrowth occurred within 30 min after plating of brain-stem neurons onto a confluent layer of brain-stem nonneuronal cells, with little or no neuronal aggregation. Nonneuronal cells obtained from spinal cord or dorsal root ganglia promoted the attachment of brain-stem neurons, but neuronal aggregation occurred and process outgrowth was retarded. Although, in the virtual absence of nonneuronal cells caused by early treatment with cytosine arabinoside, neurons initially extended processes, their survival rarely exceeded 7–14 d. Experiments to examine the early differentiation of serotonin neurons in culture were therefore performed on neurons maintained on nonneuronal cells obtained from the brain-stem region.

Immunocytochemistry

IDENTIFICATION OF CELL TYPES PRESENT IN CULTURE: The presence of astrocytes in brain-stem cultures was determined in cultures fixed with 5% acetic acid and 95% ethanol at -20°C for 5 min by indirect immunofluorescence using an antibody directed against glial-fibrillary acidic protein (GFAP) (5) donated by B. Anderton, Department of Immunology, St. George's Hospital Medical School. Cells with typical neuronal morphology were identified as neurons in brain-stem cultures fixed with 4% paraformaldehyde or with 5% acetic

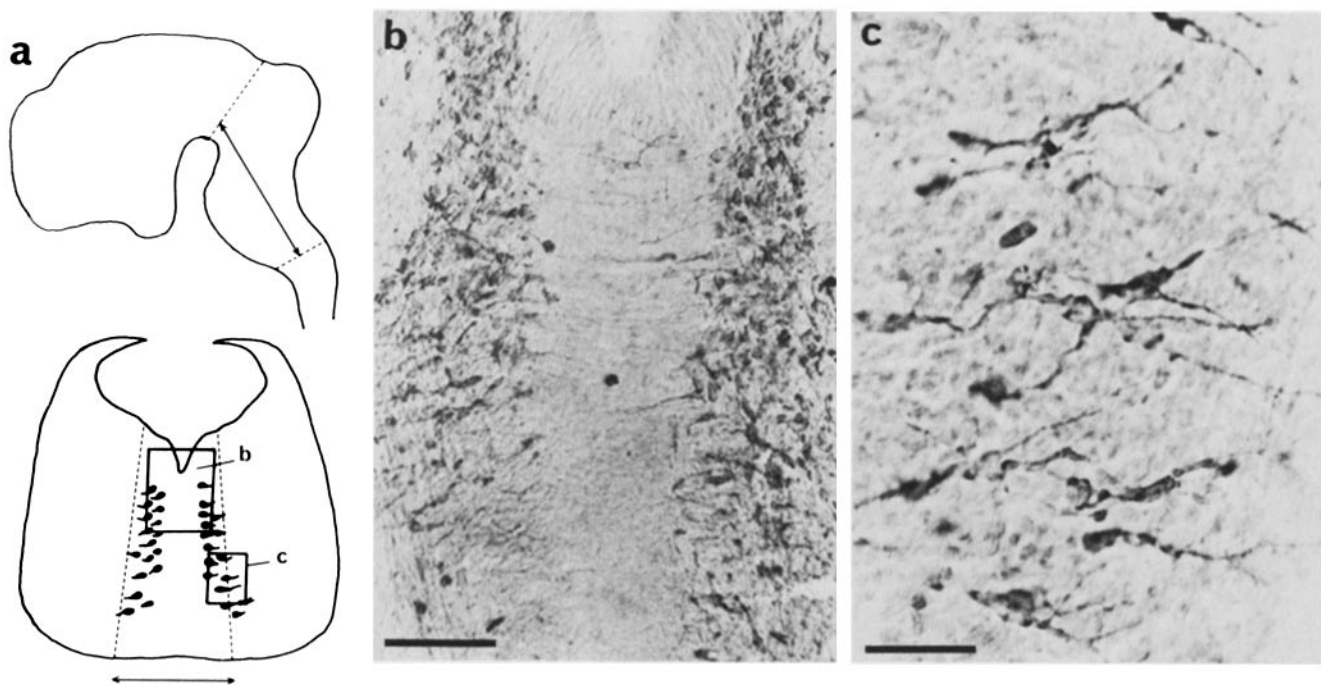


FIGURE 1 Localization of serotonin-immunoreactive neurons in 14-d embryonic rat brain stem. (a) Diagrammatic representation of a side view and transverse section of 14-d embryonic brain stem at the level of the caudal mesencephalon, showing the location of serotonin-immunoreactive neurons and the rostro-caudal and lateral limits of tissue removed for dissociation and plating. (b) Interference contrast micrograph of serotonin-immunoreactive neurons located lateral to the midline of the caudal mesencephalon in 14-d embryonic rat mesencephalon. Bar, 90 μ m. (c) Interference contrast micrograph of serotonin-immunoreactivity within neurons in a 30- μ m fixed section of caudal mesencephalon. The cell bodies of immunoreactive neurons are elongated with tapering processes, bifurcating close to the cell body. Bar, 36 μ m. b, $\times 170$; c, $\times 420$.

acid/95% ethanol by indirect immunofluorescence using a monoclonal antibody (1gG2a subclass) directed against rat brain neurofilaments (41). This antibody exhibits selective staining of central and peripheral neurons in frozen sections and in dissociated cell culture (41).

IDENTIFICATION OF SEROTONIN NEURONS: Antibodies to formaldehyde-linked serotonin/bovine serum albumin conjugates were raised in rabbits and purified as described previously (37). The specificity of this antiserum has been characterized, and the antiserum has been shown to exhibit little or no cross-reactivity, with dopamine or norepinephrine, as assessed by immunofluorescence staining of brain sections (37).

Identification of serotonin neurons in dissociated cell culture was achieved using the unlabeled antibody, peroxidase-antiperoxidase method of Sternberger (38), with all reaction steps carried out at room temperature. Cultures were fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 15–30 min and washed three times with phosphate-buffered saline (PBS) (pH 7.4). Cultures were then incubated with PBS containing 0.2% Triton X-100 for 1–5 min and washed three times with PBS and once with PBS containing 3% normal sheep serum for 15 min to decrease nonspecific binding. Cultures were incubated for 30 min with antiserotonin antiserum diluted 1:1,000 with PBS containing 1% normal sheep serum (PBS-S). Cultures were then washed three times with PBS-S and incubated for 30 min with sheep anti-rabbit IgG (Miles-Yeda, Rehovot, Israel) diluted 1:10 with PBS-S. After washing three times in PBS-S, cultures were incubated for 30 minutes with rabbit peroxidase-antiperoxidase complex (Miles-Yeda) diluted 1:50 with PBS-S. After washing once in PBS-S and three times in 0.05 M Tris-HCl buffer (pH 7.6), cultures were incubated for 20 min in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) activated with 0.002% H₂O₂ in 0.05 M Tris-HCl, rinsed in 0.05 M Tris-HCl buffer and cover-slipped with glycerine/Tris (1:1) and examined.

Increased membrane permeability by treatment with Triton X-100 during immunocytochemical processing often resulted in staining of both the cytoplasm and nucleus of positive cells. Substitution of saponin for Triton X-100 reduced the intensity of staining, although the nuclei of stained neurons remained devoid of reaction product. The same percentage of neurons were stained using Triton X-100 and saponin. To maximize the intensity of reaction product, Triton X-100 was therefore used in all experiments for light microscope immunocytochemistry.

In control experiments, no immunoreactive staining was observed when serotonin antiserum diluted 1:10 with PBS was preabsorbed with 7.5 mM (2.9 mg/ml) serotonin creatinine sulphate for 48 h at 4°C, and used at a final dilution of 1:1,000. Similar or greater concentrations of serotonin have been used to abolish immunoreactive staining in frozen tissue sections (29, 37). Preabsorption of serotonin antiserum with the same concentration of dopamine or norepinephrine did not abolish serotonin immunoreactivity.

Identification of Serotonin Neurons in the Brain of 14-d Embryonic Rats

14-d embryos were placed for 3 h in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and then transferred to 0.1 M phosphate buffer containing 30% sucrose. 30- μ m sections of the brain were cut on a freezing microtome and processed for immunocytochemistry under conditions identical to those employed for tissue culture. After reaction, sections were mounted on glass slides coated with 0.1% gelatin and 0.05% chrome-alum, dehydrated, and mounted in Permount.

Quantitative Estimation of Cell Size and Number

The number of stained and unstained neurons was counted under bright-field or interference-contrast optics and the percentage of immunoreactive neurons calculated from 20 to 25 fields (1.77 mm²) chosen at random. In each culture, the outline of the soma of twelve stained and unstained neurons chosen at random was projected onto drawing paper and its profile traced, cut out, and weighed. From the weight of the paper, the surface area of the profile of each neuronal soma was then calculated.

Synthesis of ³H-Serotonin

Cultures were incubated in Ham's F12 medium containing 44 mM glucose and 0.1% bovine serum albumin (F12/GA) and 10⁻⁴ M pargyline hydrochloride for 15 min at 37°C. After rinsing, cultures were incubated with F12/GA containing 10⁻⁶ M ³H-tryptophan (Amersham Corp., Arlington Heights, Ill.; 111 GBq/mmol) for 15, 30, and 60 min at 37°C. Cultures were washed five times with Earle's balanced salt solution (BSS) (Gibco Laboratories) at 4°C. 1 ml of ice-cold distilled water was added and cells were disrupted with a Teflon scraper and centrifuged at 12,000 g for 1 min. The supernate was passed through a 3 cm

× 1 cm Sephadex G-10 column and eluted with 6 ml of H₂O followed by 3 × 1 ml of 0.5 M formic acid (10). The radioactivity in each fraction was estimated by liquid scintillation spectrometry with a counting efficiency of 36%. ³H-Tryptophan and ³H-5-hydroxytryptamine creatinine sulphate (Amersham, TRK 223; 550 GBq/mmol) were used as standards. Previous studies have shown that the formic acid fraction contains ³H-serotonin but not serotonin or tryptophan metabolites (10). In one experiment, eluted samples were lyophilized and reconstituted with 100 μ l of pyridine acetate buffer (pH 1.9) for separation of radioactivity by high-voltage paper electrophoresis (23) on Whatman No. 1 chromatography paper. Unlabeled indoleamine standards, ³H-tryptophan, and ³H-serotonin were also chromatographed. Samples were run at 3–6 kV for 90 min and dried, and reference compounds were visualized after spraying with ninhydrin or by observation under UV light. Chromatograms containing experimental samples were cut into 1 cm strips and eluted overnight in 2 ml of ethoxyethanol and counted. The position of radioactive peaks after electrophoresis of water and formic acid fractions was compared with indoleamine standards and with ³H-tryptophan and ³H-serotonin.

Release of Newly Synthesized ³H-Serotonin

Cultures maintained in vitro for 14 d were incubated with F12/GA containing 10⁻⁴ M pargyline for 15 min at 37°C. After rinsing, cultures were incubated with 10⁻⁶ M ³H-tryptophan for 60 min. Cultures were then washed four times with F12/GA and incubated for successive 5-min periods in medium containing (in mM): Na⁺, 130; K⁺, 4; Ca⁺⁺, 1.8; Mg⁺⁺, 0.8; Cl⁻, 123; SO₄²⁻, 0.8; HEPES, 25; glucose, 44; BSA, 0.1 mg/ml; pH 7.4. High-K⁺ solutions were prepared by substituting KCl (to 40 mM) for NaCl isosmotically. Cobaltous chloride (2 mM) was substituted for calcium chloride in some incubations. At the end of each 5-min incubation period, the bathing solution was removed and a fresh solution added to the cultures. At the end of the experiment, cells were extracted with 1 ml of water at 4°C and the cellular and released ³H-serotonin were measured as described above. Release of ³H-serotonin was calculated as the percentage of total cellular ³H-serotonin at each incubation time.

Autoradiography

Cultures were incubated with BSS containing 44 mM glucose and 0.1% BSA and 10⁻⁴ M pargyline for 15 min at 37°C, followed by incubation with ³H-serotonin (10⁻⁷ M) for 30 min. After incubation, cultures were rinsed rapidly with 3 × 2 ml of BSS at 4°C and fixed in 0.12 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde at 4°C, with gradual warming to room temperature over 15 min. Cultures were washed with 2 × 2 ml of BSS and 2 × 2 ml of water, dried, and coated with Ilford L4 emulsion diluted 1:1 with water, gelled on ice and stored at 4°C in a light-tight box in the presence of silica gel. After exposure for 8 or 11 d, the autoradiographs were developed with Kodak D-19 developer at 22°C for 2.5 min, rinsed, and fixed in Ilford Hypam fixative for 5 min. After washing, cultures were cover-slipped with glycerine/water (1:1) and viewed under bright-field phase-contrast, and interference-contrast optics. In some experiments, cultures were incubated, as described above, for 30 min with 5 × 10⁻⁷ M ³H-dopamine (Amersham, TRK 312; 222 GBq/mmol) with 10⁻⁷ M ³H-noradrenaline (Amersham, TRK-584; 740 GBq/mmol) or 5 × 10⁻⁸ M (³H-GABA; Amersham, TRK-527; 2.6 GBq/mmol). Experiments using ³H-GABA were performed after a 15-min preincubation with, and in the presence of, 10⁻³ M β -alanine to inhibit GABA uptake into nonneuronal cells (36).

RESULTS

Distribution of Serotonin Neurons in 14-d Embryonic Rat Brain

In 14-d embryonic rat brain, serotonin-immunoreactive neurons were found bilaterally from 100 to 300 μ m lateral to the midline and extending from the floor of the fourth ventricle to the ventral surface of the brain stem (Fig. 1a and b). Stained neurons were observed most rostrally at the level of the caudal mesencephalon, and caudally at all levels of the medulla to the obex. The cell bodies of the majority of stained neurons were fusiform and measured from 5 to 10 μ m in diameter (Fig. 1c). Neurons with bifurcating processes extended over considerable distances throughout the field (Fig. 1c). Primary migration of serotonin neurons from the ventricular zone had already occurred at this stage, and occasional stained neurons were observed close to the midline (Fig. 1b). For tissue culture, the

dissection of fresh tissue included all regions that were observed in fixed sections to contain serotonin-immunoreactive neurons (Fig. 1a).

Culture Conditions

In dissociated cell culture, neuronal survival was most consistent from 14-d embryos (crown-rump length: 11–12 mm; total embryonic weight: 110–130 mg), although tissue from 15-d embryos also produced reasonable cultures. Neuronal survival from 12- and 13-d embryos was poor. Optimal cultures were obtained using 35-mm dishes coated with rat-tail collagen and treated with 25 $\mu\text{g}/\text{ml}$ purified human fibronectin for 30–60 min immediately before plating. Less consistent growth was obtained on a substrate of collagen alone or poly-L-lysine. Although collagen gel supported neuronal growth, it was incompatible with the present immunocytochemical techniques, because primary and secondary antibodies used in the peroxidase-antiperoxidase procedure were absorbed by the gel. When plated on a combined substrate of collagen and fibronectin, the freshly dissociated cells soon aggregated into small clumps. After 2–3 d in culture, the gradual outgrowth of nonneuronal cells caused the neuronal aggregates to disperse and the morphology of individual neurons could then be determined (Fig. 2a). Not all of the morphologically identifiable classes of nonneuronal cells supported neuronal survival. Astrocytes, determined by GFAP immunoreactivity (5), appeared to provide the optimal substrate for neuronal survival, whereas the proliferation of more rapidly dividing nonneuronal cells led to the displacement of neuronal processes and cell death. Treatment of the cultures at day 4–5 with 10^{-5} M cytosine arabinoside produced a reasonably selective cytotoxicity of rapidly dividing cells and did not appear to affect the survival of astrocytes.

Almost all nonneuronal cells present in brain-stem cultures exhibited GFAP immunoreactivity. The cell body and processes of cells with typical neuronal morphology were almost invariably stained by a monoclonal antineurofilament antibody (41). It is unlikely, therefore, that a significant proportion of cells with neuronal appearance are, in fact, oligodendrocytes.

Identification of Serotonin Neurons in Culture by Immunocytochemistry

Serotonin immunoreactivity could be observed in the cell body and processes of brain-stem neurons 24 h after plating onto a confluent layer of brain-stem nonneuronal cells (Fig. 2b). At this time, some immunoreactive processes were observed more than 1 mm from the cell body. 1 d after plating, the number of neurons present in cultures ranged between 15,000 and 20,000/ cm^2 and 3–4% of these exhibited serotonin immunoreactivity (Fig. 3c).

Over the 1st mo in culture, the total number of serotonin-immunoreactive neurons gradually declined, with about half of the number originally present surviving at day 28 (Fig. 3b). The total number of neurons declined much more dramatically, and by day 28 only 7% of the original neuronal population remained (Fig. 3a). The percentage of serotonin neurons in culture therefore increased markedly after 14 d, and by day 28 up to 36% of surviving neurons contained serotonin (Fig. 3c).

Although serotonin-immunoreactive neurons exhibited a wide variety of profiles, certain morphological features predominated. Frequently, stained somata appeared to be either pyramidal, with multipolar processes, or fusiform, with bipolar processes (Fig. 2c). Many of the neuronal somata were ex-

tremely elongated, and the transition from soma to process was often poorly defined (Fig. 2d).

At all times after plating, the mean area of the somatic profile of serotonin neurons was significantly larger than that of unstained neurons. This difference in surface area became more marked at longer survival times (Fig. 4).

Although at 10 d in culture, serotonin-immunoreaction product was most dense in the cell body and proximal parts of the processes of neurons (Fig. 2b and c), by the 3rd wk the density of immunoreactivity within the processes had increased dramatically and the use of dark-field optics revealed each of the individual neurons to have extremely elaborate processes (Fig. 5). Staining within the processes was frequently associated with varicosities (Fig. 5) and, in contrast to the lightly stained cell body, intense staining was often observed near the termination of each of the processes (Fig. 2e).

After incubation of 14-d cultures with 5,6-dihydroxytryptamine (5,6-DHT) (3×10^{-5} M) in complete medium containing 10^{-4} M pargyline for 24 h, the serotonin immunoreactivity was confined to <5% of neurons as compared with cultures incubated with pargyline alone. The immunoreactive cells were invariably rounded with short processes (Fig. 2f). A lower concentration of 5,6-DHT ($1-3 \times 10^{-6}$ M) did not decrease the number of stained neurons as compared with controls. Rather, the cell bodies appeared more intensely stained, and the immunoreactive processes from these neurons showed signs of degeneration. However, after treatment with 3×10^{-5} M 5,6-DHT, the total number of neurons was not significantly altered, and the morphology of unstained neurons was not visibly affected (Fig. 2f).

Spinal cord and dorsal root ganglion neurons grown in culture for 3–4 wk exhibited no serotonin-immunoreactive cells.

Identification of Serotonin Neurons in Culture by Autoradiography

After incubation of 1-d-old cultures with ^3H -serotonin (10^{-7} M) for 30 min at 37°C , autoradiographs revealed 2.1% of neuronal somata to be labeled with silver grains (Fig. 6a). Nonneuronal cells were devoid of labeling. Although the density of silver grains over cell bodies varied from cell to cell, labeled neurons were clearly distinguishable from unlabeled cells and, in every example of somatic labeling, silver grains were also visible over the processes and growth cones of the labeled cell. In some instances, however, only processes appeared to be labeled.

By day 14, the number of neurons labeled with ^3H -serotonin had risen to 4.0% (Fig. 8b), while experiments on sister cultures revealed 5.1% of neurons to exhibit serotonin immunoreactivity. In 2-wk-old cultures, the density of neuronal processes labeled with ^3H -serotonin was quite striking (Fig. 6b) and careful tracing of the processes from individual labeled cells showed them to extend for several millimeters. The surface area ($340 \pm 25 \mu\text{m}^2$, mean \pm SEM, $n = 50$ neurons) and morphology of labeled cell bodies (Fig. 6c) were similar to those found by immunocytochemistry.

In 14-d cultures, decreasing the ^3H -serotonin incubation period from 30 to 10 min caused a marked decrease in the number of labeled cell bodies (to <0.2% of the total neuronal population), although labeling of processes was clearly evident (Fig. 6d).

Incubation of 14-d cultures with 10^{-7} M ^3H -serotonin in the presence of the selective serotonin-uptake inhibitor, clomipra-

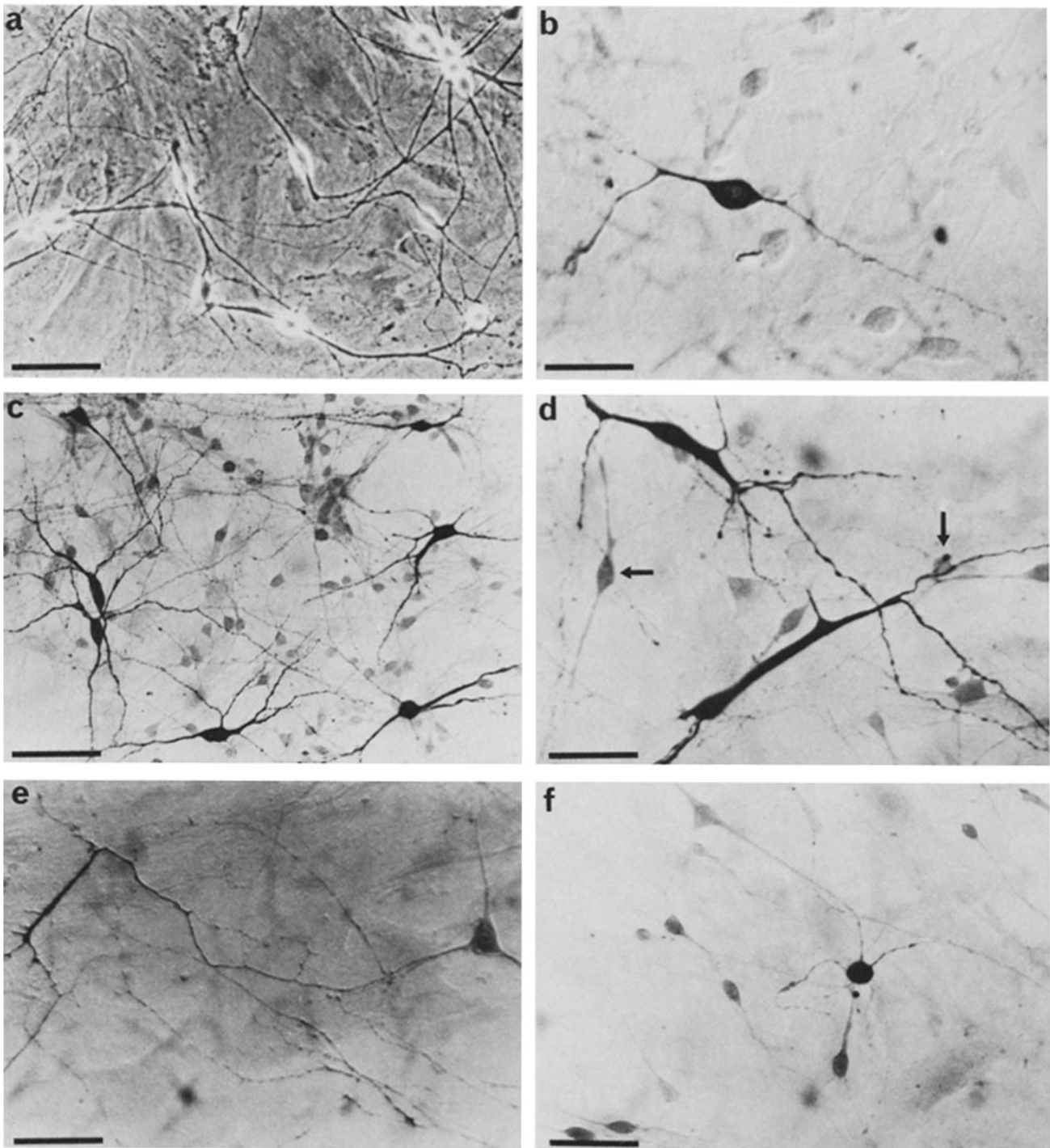


FIGURE 2 Immunocytochemical localization of serotonin-containing neurons in dissociated cultures of rat brain stem. (a) Phase-contrast micrograph of brain stem neurons grown in culture for 28 d: phase-bright cell bodies and neuronal processes can be observed growing on a confluent layer of nonneuronal brain-stem background cells. Bar, 100 μ m. (b) Serotonin-immunoperoxidase staining in the cell body and processes of a neuron removed from a 14-d embryonic rat brain stem and grown in culture for 24 h on a confluent layer of brain-stem nonneuronal cells. The cell body and processes of five other brain-stem neurons are unstained. Bar, 30 μ m. (c) Bright-field micrograph showing serotonin-immunoreactivity in the cell body and processes of seven brain-stem neurons grown in culture for 14 d. Numerous unlabeled neurons and background cells can be observed. Bar, 75 μ m. (d) Serotonin-immunoreactivity in two elongated brain-stem neurons grown in culture for 14 d. Immunoreactive processes frequently contact other, smaller unstained neurons (arrows). Bar, 40 μ m. (e) Interference-contrast micrograph showing dense serotonin immunoreactivity in the process of a single brain-stem neuron grown in culture for 26 d. The cell body and initial processes of the neurons are stained less intensely. Bar, 56 μ m. (f) Bright-field micrograph showing immunoperoxidase staining of a rounded neuron with degenerated processes, 24 h after 5,6-DHT treatment. The processes of the unstained neurons show no apparent signs of degeneration. Bar, 45 μ m. a, \times 150; b, \times 500; c, \times 200; d, \times 375; e, \times 270; f, \times 330.

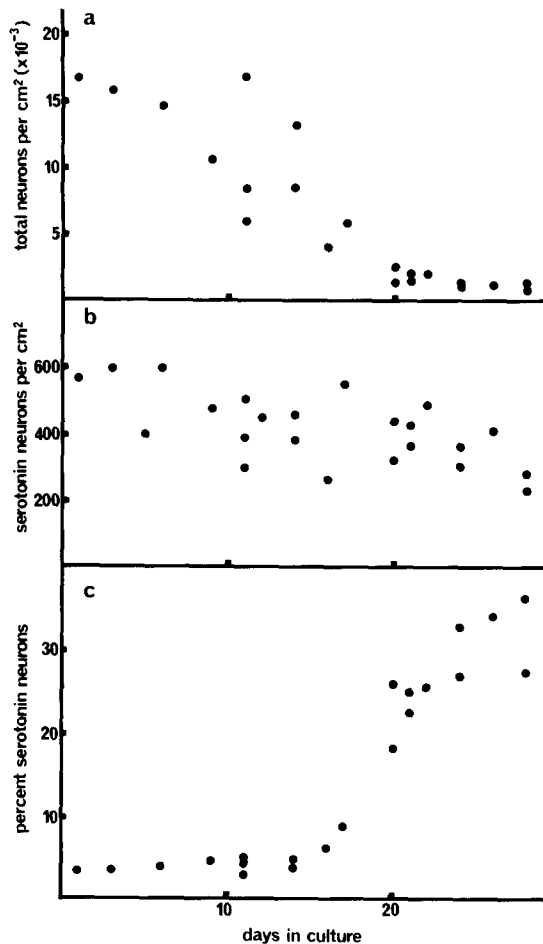


FIGURE 3 Survival of serotonin neurons in dissociated cultures of embryonic rat brain stem: (a) Survival of cells with neuronal morphology over 28 d in culture. (b) Survival of serotonin neurons detected by immunoperoxidase staining of neuronal cell body. In a and b each point represents the total number of neurons and immunoreactive neurons counted in 20–25 fields (1.77 mm^2) chosen at random, in cultures obtained from five separate platings. (c) Immunoreactive neurons as a percentage of the total number of neurons.

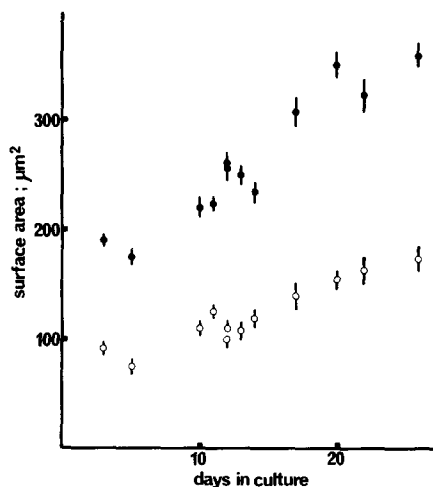


FIGURE 4 Surface area of the profile of brain-stem neuronal cell bodies in culture. Closed circles represent the mean surface area of serotonin-immunoreactive neurons grown for different times in culture; open circles represent the mean surface area of unstained neurons. Each point represents the mean area \pm SEM of 10–20 neurons chosen from four separate platings.

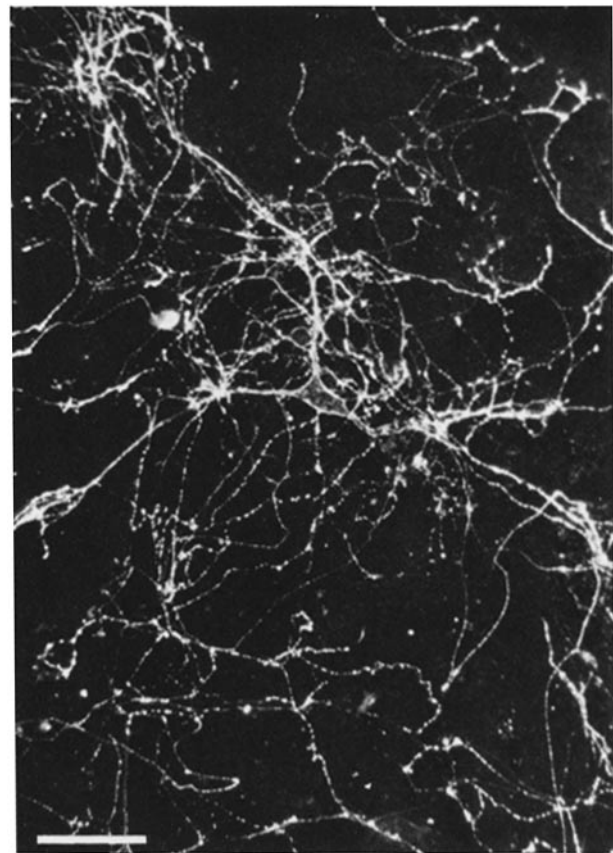


FIGURE 5 Dark-field micrograph showing intense serotonin immunoreactivity in varicosities along the processes of a single brain-stem neuron grown in culture for 28 d. Bar, $68 \mu\text{m}$. \times , 220.

mine, at a concentration of 10^{-6} M almost completely abolished neuronal labeling (Fig. 6e). Parallel cultures harvested after incubation of ^3H -serotonin in the presence and absence of clomipramine and counted by liquid scintillation spectrometry, showed ^3H -serotonin uptake to be inhibited by 87%. Neuronal labeling was also abolished after incubation of cultures with ^3H -serotonin (10^{-7} M) in the presence of 10^{-5} M serotonin. Neuronal labeling by ^3H -serotonin was unaffected by incubation of cultures in the presence of the selective norepinephrine-uptake inhibitor, maprotiline (10^{-6} M) (Fig. 6f), or 10^{-5} M norepinephrine (Fig. 6g).

Synthesis and Release of Serotonin

Brain-stem neurons grown in culture for 14 d were shown to synthesize ^3H -serotonin by incubating them with 10^{-6} M ^3H -tryptophan and separating the radioactivity by Sephadex G-10 chromatography (Fig. 7a). Incubation of neurons with ^3H -tryptophan in the presence of *p*-chlorophenylalanine (10^{-4} M) inhibited the synthesis of ^3H -serotonin by 31–47% (Fig. 7a). Neuronal cultures prepared from the dorsal root ganglia or cerebellum of 2- to 4-d postnatal rats grown at the same cell density for 14 d did not synthesize measurable amounts of ^3H -serotonin after incubation for 60 min with ^3H -tryptophan (Fig. 7a).

In one experiment, cells were incubated in 10^{-6} M ^3H -tryptophan for 60 min and harvested, and the radioactivity, eluted first by water and then by formic acid was subjected to high-voltage paper electrophoresis. More than 90% of radioactivity eluted with water comigrated with ^3H -tryptophan

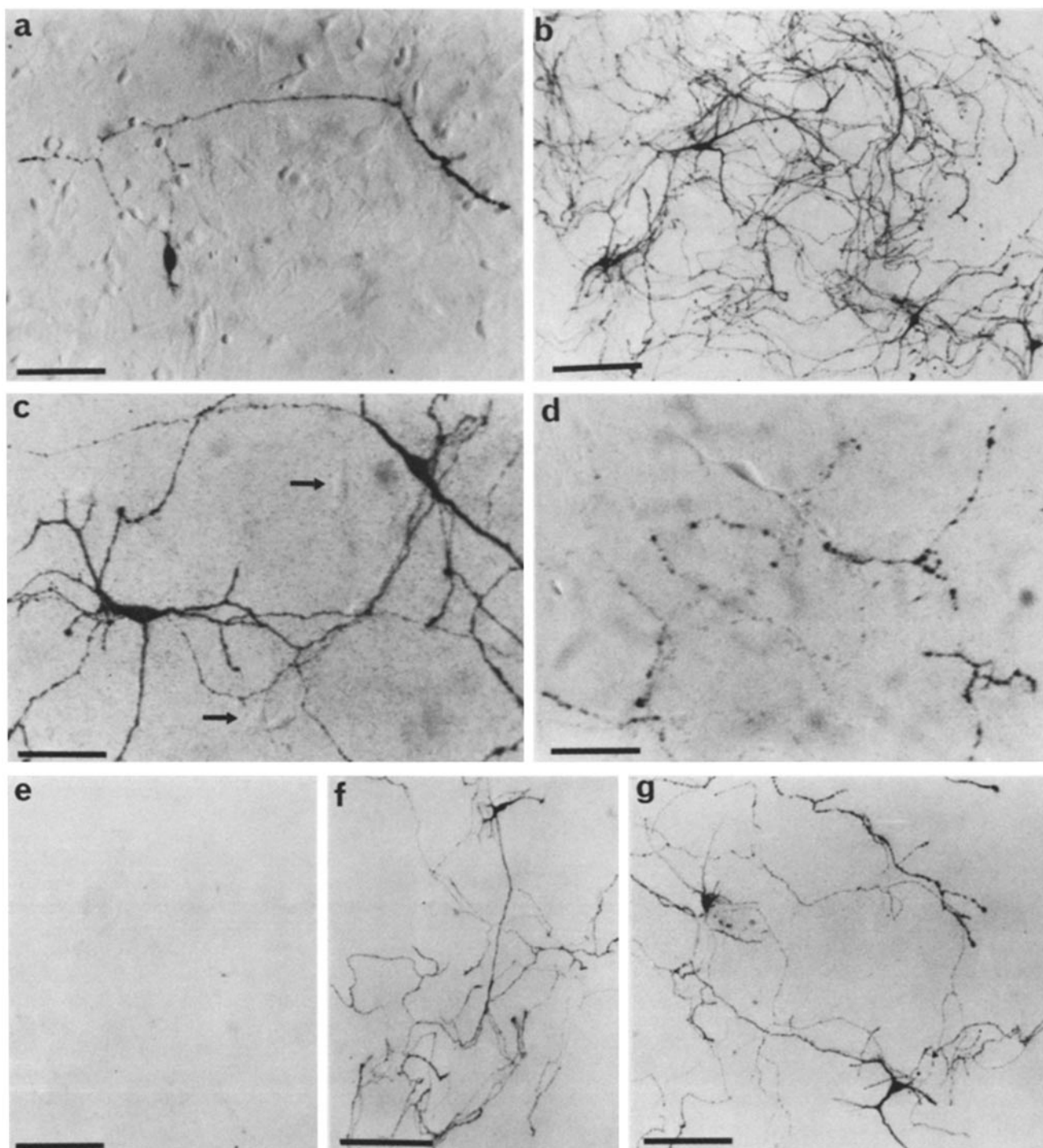


FIGURE 6 Autoradiographic localization of neurons in brain-stem cultures exhibiting high-affinity serotonin uptake. (a) Interference-contrast micrograph showing accumulation of silver grains over the cell body and processes of a single brain-stem neuron grown in culture for 24 h on a confluent layer of nonneuronal cells. Other cell bodies are devoid of silver grains. Exposure time, 8 d. Bar, 65 μm . (b) Bright-field micrograph showing silver grains overlying the cell body and processes of brain-stem neurons grown in culture for 14 d. Exposure time, 11 d. Bar, 150 μm . (c) Interference-contrast micrograph showing the accumulation of silver grains over the cell body and processes of two brain-stem neurons. Arrows indicate unlabeled neurons. Exposure time, 11 d. Bar, 50 μm . (d) Accumulation of silver grains over the processes of brain-stem neurons grown in culture for 14 d after incubation with 10^{-7} M ^3H -serotonin for 10 min. Labeling is confined to the processes of neurons, while neuronal cell bodies are devoid of silver grains. Exposure time, 8 d. Bar, 50 μm . (e) Sparse labeling of 14-d brain-stem neurons after incubation with 10^{-7} M ^3H -serotonin in the presence of 10^{-6} M clomipramine. Exposure time 8 d. Bar, 120 μm . (f) Labeling of the cell body and processes of 14-d brain-stem neurons after incubation with 10^{-7} M ^3H -serotonin in the presence of 10^{-6} M maprotiline. Exposure time, 8 d. Bar, 120 μm . (g) Labeling of the cell body and processes of a single 14-d brain-stem neuron after incubation with 10^{-7} M ^3H -serotonin in the presence of 10^{-6} M unlabeled norepinephrine. Exposure time, 8 d. Bar, 100 μm . a, $\times 230$; b, $\times 100$; c and d, $\times 300$; e and f, $\times 125$; g, $\times 150$.

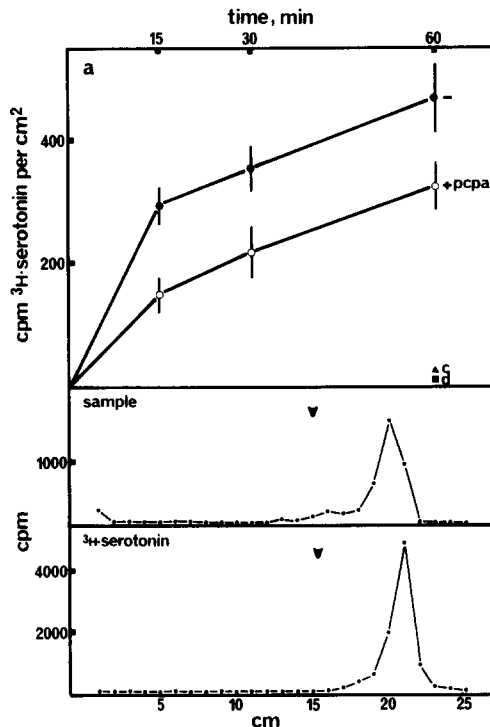


FIGURE 7 Synthesis of ^3H -serotonin from ^3H -tryptophan in brain-stem neuronal cultures. (a) Accumulation of ^3H -serotonin in the absence (closed circles) and presence (open circles) of 10^{-4} M *p*-chlorophenylalanine (*pcpa*) after incubation with 10^{-6} M ^3H -tryptophan. Accumulation of ^3H -serotonin was negligible in cerebellar (triangle, c) or dorsal root ganglion cultures (square, d). Each point represents the mean \pm SEM of four cultures. Lower two panels show high-voltage paper electrophoretic separation of radioactivity present in cultures after incubation with 10^{-6} M ^3H -tryptophan for 60 min, in comparison with authentic ^3H -serotonin. Arrowheads on lower panels indicate migration of ^3H -tryptophan.

standards, while more than 95% of radioactivity eluted by 0.5 M formic acid comigrated with ^3H -serotonin (Fig. 7).

Four additional cultures were incubated for 60 min in 10^{-6} M ^3H -tryptophan, washed extensively with BSS, and placed in a water bath at 37°C , and the release of newly synthesized ^3H -serotonin in successive aliquots was measured by column chromatography, as described above. The spontaneous efflux of newly synthesized ^3H -serotonin during the first two collection periods declined from 2.4 to 1.3% of the total tissue content (Fig. 8). Increasing the potassium concentration in the incubation medium to 44 mM resulted in a 2.5-fold increase in release of ^3H -serotonin, which returned to prestimulus levels in the next collection period. Repeating the potassium pulse in the presence of 2 mM Co^{++} and absence of calcium completely abolished the potassium-evoked release of serotonin (Fig. 8). Restoration of the calcium concentration to 2 mM resulted in a 4.3-fold increase in ^3H -serotonin release in response to 44 mM potassium. Over 50 min of incubation, $45 \pm 2\%$ (mean \pm SEM; $n = 4$ cultures) of newly synthesized ^3H -serotonin was released from brain-stem neurons. One culture exposed to this release protocol was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer and processed for immunocytochemistry to determine the total number of serotonin neurons present. Serotonin immunoreactivity was clearly detectable within neurons after the release experiment and a similar percentage of immunoreactive neurons was found when compared with parallel cultures processed solely for immunocytochemistry. Approxi-



FIGURE 8 Potassium-evoked release of newly synthesized ^3H -serotonin from cultured brain-stem neurons. Cultures were incubated for two 5-min periods in low potassium solution (4 mM K^+) followed by exposure for 5 min to incubation solution containing 44 mM K^+ . Cultures were then incubated in solution containing 2 mM cobalt, and the potassium depolarization was repeated. A third 44 mM K^+ incubation was performed after removal of cobalt and restoration of the calcium concentration to 2 mM. At the end of the experiment, cells were harvested and the newly synthesized ^3H -serotonin released and that remaining in the cells were determined (8). Histogram is expressed as the percentage of total cellular stores released per minute. Each column represents the mean \pm SEM from four cultures.

mately 100 cpm of ^3H -serotonin were synthesized per serotonin-immunoreactive neuron in 60 min under these conditions.

Identification of Other Neuronal Populations in Brain-stem Cultures

Brain-stem neurons were grown for 14 d in culture and then incubated for 30 min with 5×10^{-8} M ^3H -GABA in the presence of 10^{-3} M β -alanine and processed for light-microscope autoradiography. Many neurons were heavily labeled, whereas nonneuronal cells were devoid of silver grains (Fig. 9a and b). At this age, 40% of neurons accumulated ^3H -GABA.

The density of somatic labeling varied from neuron to neuron, but labeled and unlabeled neurons could clearly be distinguished (Fig. 9b). In 25-d-old cultures, the number of neurons labeled with ^3H -GABA remained constant at 40% of the total neuronal population.

In contrast to the long, unbranched projections of neurons typically labeled with ^3H -serotonin, the processes of most neurons labeled with ^3H -GABA were short and extensively branched and usually confined to the immediate vicinity of the labeled cell body. (Fig. 9a and b). The mean surface area ($127 \pm 5 \mu\text{m}^2$ (mean \pm SEM); $n = 50$ neurons) of the profile of neuronal cell bodies in 14-d-old brain-stem cultures labeled with ^3H -GABA, was 2.7-fold smaller than the mean area of neuronal cell bodies labeled with ^3H -serotonin. Moreover, in 14-d-old cultures, there was almost no overlap in the surface area of the somatic profile of neurons labeled with ^3H -GABA or ^3H -serotonin (Fig. 10).

Incubation of 14-d cultures with 10^{-7} M ^3H -norepinephrine

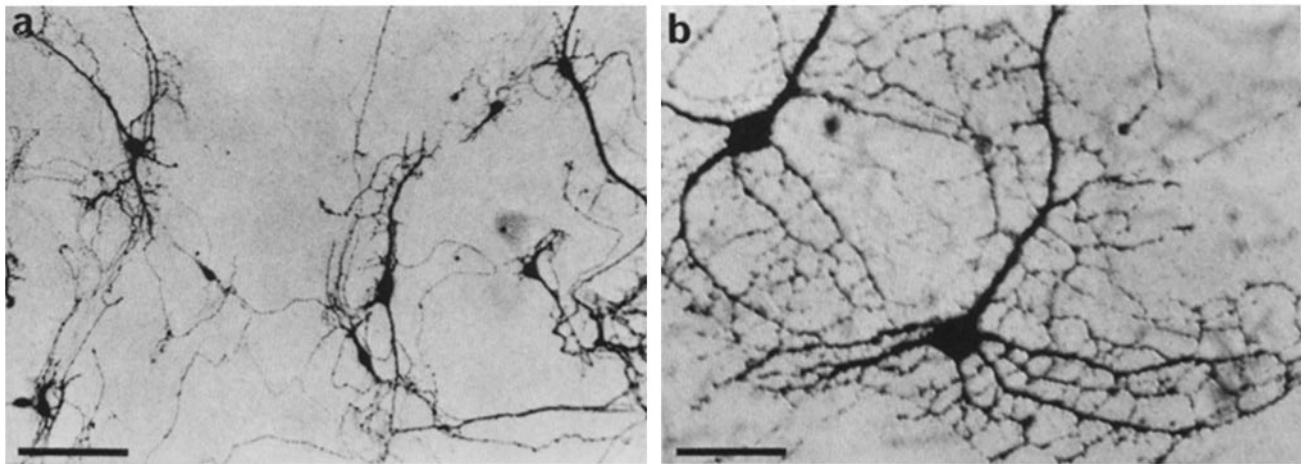


FIGURE 9 Autoradiographic localization of ^3H -GABA uptake into brain-stem neurons in culture. (a) Bright-field photomicrograph shows silver grains that overlie the cell body and processes of neurons. Exposure time, 8 d. Bar, $100\ \mu\text{m}$. (b) Interference-contrast micrograph showing silver grains over short branched processes in the immediate vicinity of labeled cell bodies. Exposure time, 8 d. Bar, $34\ \mu\text{m}$. a, $\times 150$; b, $\times 440$.

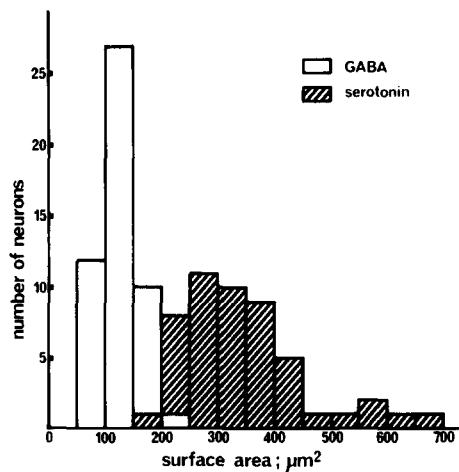


FIGURE 10 Histogram showing the distribution of the surface areas of the profiles of brain-stem neuronal cell bodies grown for 14 d in culture and labeled with ^3H -GABA or with ^3H -serotonin. Autoradiographs were exposed for 11 d, and the density of labeling over cell bodies was similar in cultures labeled with ^3H -GABA (open columns) and ^3H -serotonin (hatched columns). Measurements were performed on neurons chosen at random from cultures obtained from three separate platings.

or $5 \times 10^{-7}\ \text{M}$ ^3H -dopamine for 30 min resulted in extremely few, lightly labeled processes and no labeled cell bodies.

DISCUSSION

Central monoamine neurons are known to express transmitter properties extremely early in embryogenesis, on or around the time of final mitotic division (26, 28, 31, 35), and sensitive immunocytochemical and biochemical markers are now available for the detection of monoamine transmitters and their synthetic enzymes (25, 37). The results of the present studies provide evidence that serotonin neurons removed from the embryonic rat brain stem soon after the onset of differentiation survive in culture and express many of the transmitter-specific features of the same neurons *in vivo*.

Serotonin-containing neurons obtained from embryonic guinea-pig myenteric plexus (15, 16) and from explants of

neonatal rat brain stem (22) have been grown in organotypic cultures. Central dopamine- and norepinephrine-containing neurons have also been localized in explant cultures of substantia nigra and locus coeruleus (34) and, more recently, have been identified in dissociated cultures of embryonic mouse mesencephalon (32, 33).

In our experiments, serotonin-immunoreactive neurons were readily identifiable within the brain stem of 14-d embryonic rats and also in cultures of embryonic brain stem <24 h after plating, without pharmacological treatments to enhance neuronal serotonin content. Endogenous catecholamine fluorescence in embryonic mesencephalic neurons grown in culture, however, cannot be detected by glyoxylic acid fluorescence histochemistry until the 3rd wk in culture (33).

With a yield of 10^6 cells per embryonic rat brain stem and a plating density of 5×10^5 cells per 35-mm dish, $\sim 5,500$ serotonin-immunoreactive neurons could be detected in a single culture, 24 h after plating. From these values it seems likely that the brain stem of a 14-d embryonic rat contains at least 11,000 serotonin neurons. During the 1st wk in culture, serotonin-immunoreactive staining appeared to be most dense in the cell body. With increasing age, the apparent density of immunoreactivity in the cell body diminished, whereas process staining increased. Developing serotonin neurons *in vivo* have also been shown to undergo a gradual decrease in fluorescence intensity within the cell soma around the time that terminal fluorescence can first be detected (28).

The early appearance of serotonin in cultured neurons was accompanied by the presence of a specific high-affinity serotonin-uptake system. After 24 h in culture, immunocytochemistry and ^3H -serotonin uptake identified a similar number of neurons. In both 1- and 14-d cultures incubated with ^3H -serotonin for 30 min, labeled processes could be observed in the absence of somatic labeling, suggesting that uptake sites are more prominent in neuronal processes. In support of this, decreasing the incubation time resulted in almost no cell body labeling, whereas process labeling remained intense. Early developing enteric serotonergic neurons have also been reported to exhibit a selective accumulation of ^3H -serotonin into axonal processes (18, 20). The somatic labeling of serotonin neurons *in vitro* and *in vivo* may therefore partly reflect the

retrograde transport of ^3H -serotonin (21, 39) after uptake into processes.

Although we attempted to confine dissection of the brain stem to regions known to contain serotonin cell groups, the virtual absence of catecholamine labeling seems surprising, because occasional catecholamine neurons have been reported within midline brain-stem nuclei (30, 31). When cultured neurons prepared from embryonic mouse mesencephalon were incubated with ^3H -dopamine under almost identical conditions, many neurons were labeled (32). It is possible, therefore, that central catecholamine neurons dissociated from 14-d embryonic rat brain stem fail to survive our dissociation procedure or culture conditions.

The virtual absence of serotonin-immunoreactive neurons 24 h after treatment with 3×10^{-5} M 5,6-DHT provides further evidence that the biochemical properties of membrane uptake sites for serotonin are retained *in vitro*. Exposure of cultures to lower 5,6-DHT concentrations, or to the same concentration for shorter times, resulted in the retraction of processes and an intensification of somatic staining, suggesting that the absence of serotonin-immunoreactive neurons after 5,6-DHT represents a true neuronal degeneration rather than an interruption of serotonin synthesis. Although the specificity of serotonin neurotoxins *in vivo* is somewhat suspect, 5,6-DHT has been reported to provide a greater discrimination between serotonin and other monoamine neurons (3, 6). Because <5% of the total neuronal population exhibited serotonin immunoreactivity after 14 d in culture, we were not able to detect a significant loss in the total number of neurons at this stage. In 28-d cultures, the same concentration of 5,6-DHT reduced the number of serotonin-immunoreactive neurons by 92% and the total number of neurons by 31%, which is consistent with the greater proportion of serotonin neurons observed in older cultures (Jessell and Yamamoto, unpublished observations).

Brain-stem neurons were able to synthesize serotonin from labeled precursors by day 14 in culture, the earliest time examined. Although almost half the cellular content of the newly synthesized ^3H -serotonin was released over a 50-min incubation period, there was no discernible decrease in the number of immunoreactive neurons or in the intensity of immunoreactivity within individual neurons after release experiments. Newly synthesized ^3H -serotonin may, therefore, be released preferentially from serotonin neurons in culture, whereas much of the endogenous serotonin detected by immunocytochemistry does not appear to be immediately available for release.

Over the 1st mo in culture, the total number of neurons decreased by ~90%, whereas the percentage of serotonin-immunoreactive neurons increased 10-fold, suggesting that under the present culture conditions a preferential survival of serotonin neurons occurs. It seems unlikely that other neurons acquire the ability to synthesize and store serotonin with time in culture. The overall number of serotonin neurons decreased gradually with time, and serotonin immunoreactivity is absent from spinal cord or dorsal root ganglion neurons grown in culture. The proportion of neurons exhibiting a high-affinity GABA-uptake system remained constant from 2 to 4 wk in culture, indicating that the number of GABA neurons decreased in proportion to the decrease in total neuronal number. We have not attempted to examine the factors responsible for the preferential survival of serotonin neurons, although central serotonin neurons are known to retain considerable regenerative properties after transplantation of defined fragments of

embryonic brain-stem tissue into ectopic sites in rat brain (7, 8).

In essence then, we have found that brain-stem cultures grown for 3–4 wk contain a high percentage of serotonin neurons that have a significantly greater area than other neuronal subpopulations and often possess a characteristic somatic morphology. These observations suggest that it may be possible to identify likely serotonin-containing neurons solely on the basis of phase-contrast microscopy of living cultures. Intracellular recording from serotonin neurons would enable the physiological properties and transmitter sensitivity of these neurons to be examined under carefully controlled conditions that are unattainable *in vivo*, followed by the unequivocal identification of the serotonergic nature of the neuron by immunocytochemistry or autoradiography. Enriched cultures of serotonin neurons grown in coculture with identified target neurons from the central or peripheral nervous system would provide a powerful model with which to examine the role of serotonin in neuronal differentiation and the physiology of central serotonergic synaptic transmission.

The cell bodies of some serotonin neurons in the nucleus raphe magnus and nucleus paragigantocellularis of adult rats have been shown to contain the peptides substance P, thyrotrophin-releasing hormone, and enkephalin (2, 12, 24). Central serotonin neurons maintained in culture may, therefore, also provide a convenient preparation with which to examine the development and function of monoamine and peptide transmitter candidates within the same neurons.

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