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 mammalian 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). Iontophoretic 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-mated 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. 1 a). The midline brain-stem tissue was then cut into 200-μm sections in two directions, using a McIlwain 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 μg/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% 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. 1 a). The midline brain-stem tissue was then cut into 200-μm sections in two directions, using a McIlwain 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 μg/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 x 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 tissue fragments, and the filtrate was dispensed in 35 mm tissue culture dishes, on one of three different substrates: rat-tail collagen (11) treated with 25 μg/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 μg/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 x 10⁵-1 x 10⁶ cells per 35-mm plate, with a total yield of ~10⁶ cells per embryo. Cultures were treated with cytosine arabinoside (10⁻⁵ M), for 48 h, 1-5 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 10⁶ 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 x 10⁵ neurons per 35-mm dish in complete medium containing 1 μg/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 acid.

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 μm. (c) Interference contrast micrograph of serotonin-immunoreactivity within neurons in a 30-μm fixed section of caudal mesencephalon. The cell bodies of immunoreactive neurons are elongated with tapering processes, bifurcating close to the cell body. Bar, 36 μm. b, x 170; c, x 420.
acid/95% ethanol by indirect immunofluorescence using a monoclonal antibody (IgG2a 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).

Release of Newly Synthesized \(^{3}H\)-Serotonin

Cultures maintained in vitro for 14 d were incubated with F12/ GA containing \(^{10}\) M pargyline for 15 min at 37°C. After rinsing, cultures were incubated with \(^{10}\) M \(^{3}H\)-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\(^{2+}\), 1.8; Mg\(^{2+}\), 0.8; Cl\(^{-}\), 123; SO\(_4\)\(^{2-}\), 0.8; HEPES, 25; glucose, 4.4; BSA, 0.1 mg/ml; pH 7.4. High-K solutions were prepared by substituting KCl for NaCl isosmotically. Colchicine 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 \(^{3}H\)-serotonin were measured as described above. Release of \(^{3}H\)-serotonin was calculated as the percentage of total cellular \(^{3}H\)-serotonin at each incubation time.

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-μm 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 10 random sampled fields (1.77 mm\(^2\)) 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 \(^{3}H\)-Serotonin

Cultures were incubated in Ham's F12 medium containing 44 mM glucose and 0.1% bovine serum albumin (F12/GA) and \(^{10}\) M pargyline hydrochloride for 15 min at 37°C. After rinsing, cultures were incubated with F12/GA containing \(^{10}\) M \(^{3}H\)-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. A 1-ml aliquot of biopsy diluent was added and cells were disrupted with a Teflon scraper and centrifuged at 12,000 rpm for 1 min. The supernate was passed through a 3 cm x 1 cm Sephadex G-10 column and eluted with 6 ml of H\(_2\)O followed by 3 x 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%. \(^{3}H\)-Tryptophan and \(^{3}H\)-5-hydroxytryptamine creatinine sulphate (Amersham, TRK-527; 2.6 GBq/mmol) were used as standards. Previous studies have shown that the formic acid fraction contains \(^{3}H\)-serotonin but not serotonin or tryptophan metabolites (10).

In one experiment, eluted samples were lyophilized and reconstituted with 100 μl of pyridine acetate buffer (pH 1.9) for separation of radioactivity by high-voltage paper electrophoresis (23) on Whatman No. 1 chromatography paper. Unlabeled indoleamines standards, \(^{3}H\)-tryptophan, and \(^{3}H\)-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 \(^{3}H\)-tryptophan and \(^{3}H\)-serotonin.

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 μm 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 close to the midline (Fig. 1b). For tissue culture, the somata was then calculated.

\(^{3}H\)-tryptophan and \(^{3}H\)-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 \(^{3}H\)-tryptophan and \(^{3}H\)-serotonin.
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 μg/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 antineurolamin 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 extremely 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 x 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 x 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 x 10^{-7} 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 ± 25 μm^2, mean ± 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 μm. (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 μm. (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 μm. (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 μm. (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 μm. (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 μm. a, X 150; b, X 500; c, X 200; d, X 375; e, X 270; f, X 330.
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 mm²) chosen at random, in cultures obtained from five separate platings. (c) Immunoreactive neurons as a percentage of the total number of neurons.

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 ± SEM of 10-20 neurons chosen from four separate platings.

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 μm. x, 220.

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^{-5} M unlabeled norepinephrine. Exposure time, 8 d. Bar, 100 μm. a, × 230; b, × 100; c and d, × 300; e and f, × 125; g, × 150.
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 $5 \times 10^{-8}$ M $^3$H-GABA in the presence of $10^{-3}$ M $\beta$-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 $^3$H-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 $^3$H-GABA remained constant at 40% of the total neuronal population.

In contrast to the long, unbranched projections of neurons typically labeled with $^3$H-serotonin, the processes of most neurons labeled with $^3$H-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 ± 5 $\mu$m$^2$) was 2.7-fold smaller than the mean area of neuronal cell bodies labeled with $^3$H-GABA. Moreover, in 14-d-old cultures, there was almost no overlap in the surface area of the somatic profile of neurons labeled with $^3$H-GABA or $^3$H-serotonin (Fig. 10).

Incubation of 14-d cultures with $10^{-7}$ M $^3$H-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 µm. (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 µm. a, x 150; b, x 440.

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 x 10^6 cells per 35-mm dish, ~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 vitro 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 ^{3}H-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 ^{3}H-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 ^{3}H-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 ^{3}H-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, thyrotropin-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 monooamine and peptide transmitter candidates within the same neurons.

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