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Serotonin initiates and autoamplifies its own synthesis during mouse central nervous system development

(hypothalamus/cultured cells/aromatic-L-amino acid decarboxylase/commitment/learning)

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ABSTRACT **Some cells from cultured embryonic mouse hypothalamus were found to express aromatic-L-amino acid decarboxylase (EC 4.1.1.28) activity and serotonin uptake and storage. These neuron-like cells differed from serotoninergic neurons in cultured embryonic mouse brain stem since they did not contain tryptophan hydroxylase. We studied the effect of the serotonin agonist 8-hydroxy-2-[di-(*n*-propyl)amino]tetralin on neuronal differentiation of hypothalamic cells from 12- to 15-day embryos. Repeated treatment of cultures with the serotonin agonist for 10 days resulted in an increased number of serotonin cells containing high levels of decarboxylase activity. Both the increase in cell numbers and the elevated decarboxylase activity could be suppressed by the addition of the serotonin antagonist metergoline to the culture medium. These data show that serotonin (or an agonist), acting on specific receptors, can initiate and amplify its own synthesis in embryonic hypothalamic neurons, as observed in the primitive hypothalamic nerve cell line F7 [De Vitry, F., Catelon, J., Dubois, M., Thibault, J., Barritault, D., Courty, J., Bourgoin, S. & Hamon, M. (1986) *Neurochem. Int.* 9, 43–53]. Such an autocrine-like mechanism may be active during nervous system development and may represent an example of learning at the cellular level.**

During embryogenesis, an ordered sequence of genetically programmed cellular commitments progressively orchestrates the development of a multipotent egg into a complex organism. The expression of the genetic program, however, relies on the availability, in time and space, of adequate environmental epigenetic factors. Their combined action ensures initiation, amplification, and stabilization of each differentiated state. Thus one may predict a cascade of differentiation episodes, each depending upon repetitive external stimuli and driving developmental progression of the embryo to the adult. The purpose of our investigations was to explore whether such differentiation processes could also be achieved in cell culture.

In the central nervous system, neuronal and glial lineages originate from a common precursor, whose time of appearance is still debated (1, 2). If repetitive external stimuli are necessary for the differentiation of neuronal or glial precursor cells into specific types of neurons or glial cells, it should be possible to mimic this mechanism *in vitro*. Initially we used the pluripotent embryonic hypothalamic nerve cell line F7 to initiate distinct neuronal or glial functions by establishing appropriate external signals in the culture environment (3, 4). Thus, it was possible to induce serotonin (5-hydroxytryptamine or 5-HT) synthesis from 5-hydroxytryptophan, L-form, (5-HTP) by repetitive treatment of the F7 cell line with a 5-HT agonist and eye-derived growth factor in appropriate culture conditions (4). In this particular case, an autocrine-like mechanism seemed to be involved in nerve cell differentiation.

In brain, serotoninergic neurons are characterized by their ability to convert tryptophan into 5-HT via two successive enzymatic steps catalyzed by tryptophan hydroxylase and aromatic-L-amino acid decarboxylase (AA decarboxylase; EC 4.1.1.28) (5). These neurons are mainly located within the raphe nuclei at the brain stem level (6), but 5-HT-containing cell bodies can be visualized also in the hypothalamus after an intraventricular perfusion of 5-HT (7, 8) or of 5-HTP (9). This led us to select these two regions from fetal mouse brain as sources of neurons and examine the possible effect of the potent 5-HT agonist 8-hydroxy-2-[di(*n*-propyl)amino]tetralin (8-OH-DPAT) (10) added to their culture medium upon the nerve cell capacity to synthesize and take up 5-HT. The present data provide experimental support for a stimulatory effect of 5-HT, via autocrine or paracrine-like mechanisms, on the expression of specific biochemical markers in serotoninergic neurons.

MATERIALS AND METHODS

Cell Cultures. The method described (11) was used, except that hypothalamic and brain stem cells were cultured on dishes coated with protamine (10 μg/ml of Ham's F12 medium for 1–2 hr at 37°C). The 5-HT agonist 8-OH-DPAT (10 nM) was added to the cultures on days 4, 7, and 9. Where indicated, 1 μM metergoline was added 30 min before each 8-OH-DPAT treatment.

Antisera. Antibodies to formaldehyde-linked 5-HT-bovine serum albumin conjugates were raised in rabbits and purified as described (12). The 5-HT antiserum presently used exhibited no crossreactivity with 5-HTP, 8-OH-DPAT, dopamine, norepinephrine, or numerous other related molecules (4, 12). Before use, the 5-HT antiserum was preadsorbed for 1 day with 0.2% bovine serum albumin. The characteristics of the antiserum obtained in rabbits immunized with tyrosine hydroxylase purified from rat pheochromocytoma have been described in detail elsewhere (13). Antiserum to glial fibrillary acidic protein was from DAKO (Santa Barbara, CA).

Antibodies to γ- enolase (EC 4.2.1.11) were a generous gift from L. Legault (Collège de France, Paris).

Immunocytochemical Techniques. *Identification of serotoninergic neuron-like cells.* For brain stem cultures, 5-HT immunoreactivity was detected using the immunoperoxidase technique as described (4). In the case of hypothalamic cultures, cells were first treated with 1 μM 5-HTP for 2 hr at 37°C, then fixed and assayed for 5-HT immunoreactivity.

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Abbreviations: 5-HT, serotonin; 5-HTP, 5-hydroxytryptophan, L-form; AA decarboxylase, aromatic-L-amino acid decarboxylase; 8-OH-DPAT, 8-hydroxy-2-[di(*n*-propyl)amino]tetralin; ED, embryonic day.

Identification of other cell types present in cultures. The presence of astrocytes was assessed in cultures fixed with ethanol/10% (vol/vol) formaldehyde (9:1, vol/vol) at 4°C for 10 min, by indirect immunohistochemistry using a glial fibrillary acidic protein antiserum. Cells with neuronal morphology were identified as neuronal cells using an antiserum against γ-enolase, at a final dilution of 1:1000.

Detection of tryptophan hydroxylase activity. Cells were cultured in the presence or absence of *p*-chlorophenylalanine (1 mM, added on days 4, 7, and 9), a specific inhibitor of tryptophan hydroxylase (5), and then exposed to the monoamine oxidase inhibitor pargyline (10 μM, ref. 5) on day 10, 2 hr before fixation. 5-HT immunoreactivity was assessed as above.

Detection of AA decarboxylase activity. Cells were cultured in the presence or absence of carbidopa (20 μM, added on days 4, 7, and 9), a specific inhibitor of AA decarboxylase (5), and incubated on day 10 with 1 μM 5-HTP and 10 μM pargyline for 2 hr before fixation. Cells were then processed for 5-HT immunoreactivity.

Detection of 5-HT storage. Cells were treated with 1 μM 5-HTP on days 4 and 7 and processed for 5-HT immunoreactivity on day 10.

Uptake of ^{3}H-Labeled 5-HT. Cultures were treated for 30 min with 10 μM pargyline, and then exposed to ^{3}H-labeled 5-HT for a further 30 min (0.1 μM ^{3}H-5-HT for brain stem cultures; 1 μM ^{3}H-labeled 5-HT for hypothalamic cells). Where mentioned, 1 μM citalopram, a specific blocker of 5-HT uptake (5), was added 30 min before ^{3}H-labeled 5-HT. After incubation, cells were rapidly rinsed twice with 2 ml of Ham's F12 and fixed for 1 hr at ambient temperature in 1.5% (vol/vol) glutaraldehyde. Cultures were then washed twice with phosphate buffered saline (PBS; 0.1 M phosphate, pH 7.4) coated with Ilford NTB2 emulsion diluted 1:1 (vol/vol) with distilled water, and exposed for 8 days (brain stem cells) or 15 days (hypothalamic cells) before development with Dektol (Kodak).

Combined Detection of 5-HT Immunoreactivity and ^{3}H-Labeled 5-HT Uptake. Cells were incubated with 1 μM ^{3}H-labeled 5-HT for 30 min, then rinsed with F12 medium and treated for 5-HT immunoreactivity as described above. After a further wash in PBS, the cells were processed for autoradiography. Where used, 1 μM nomifensine, a specific inhibitor of monoamine uptake in catecholaminergic neurons (5), was added 30 min before ^{3}H-labeled 5-HT.

Drugs. Drugs were obtained from the following sources: pargyline (Abbott); protamine sulfate (Calbiochem); 5-HTP and 5-hydroxytryptamine, creatine sulfate (5-HT) (Sigma); metergoline (Farmitalia); *p*-chlorophenylalanine methylester (Labkemi A B, Västra Frölunda, Sweden); carbidopa (CIBA–Geigy); citalopram (H-Lundbeck & Co., Copenhagen-Valley, Denmark); 8-OH-DPAT (Sandoz Pharmaceutical); nomifensine (Hoechst UK Ltd, Hounslow, Middlesex, UK); ^{3}H-labeled 5-HT (generally labeled, 15.9–26 Ci/mM (1 Ci = 37 GBq; Amersham International or New England Nuclear).

RESULTS

Presence of Immunoreactive 5-HT-Containing Cell Bodies in Cultures from the Embryonic Mouse Hypothalamus. Slight 5-HT immunoreactivity could be observed in the cytoplasm and small processes of some hypothalamic neuron-like cells

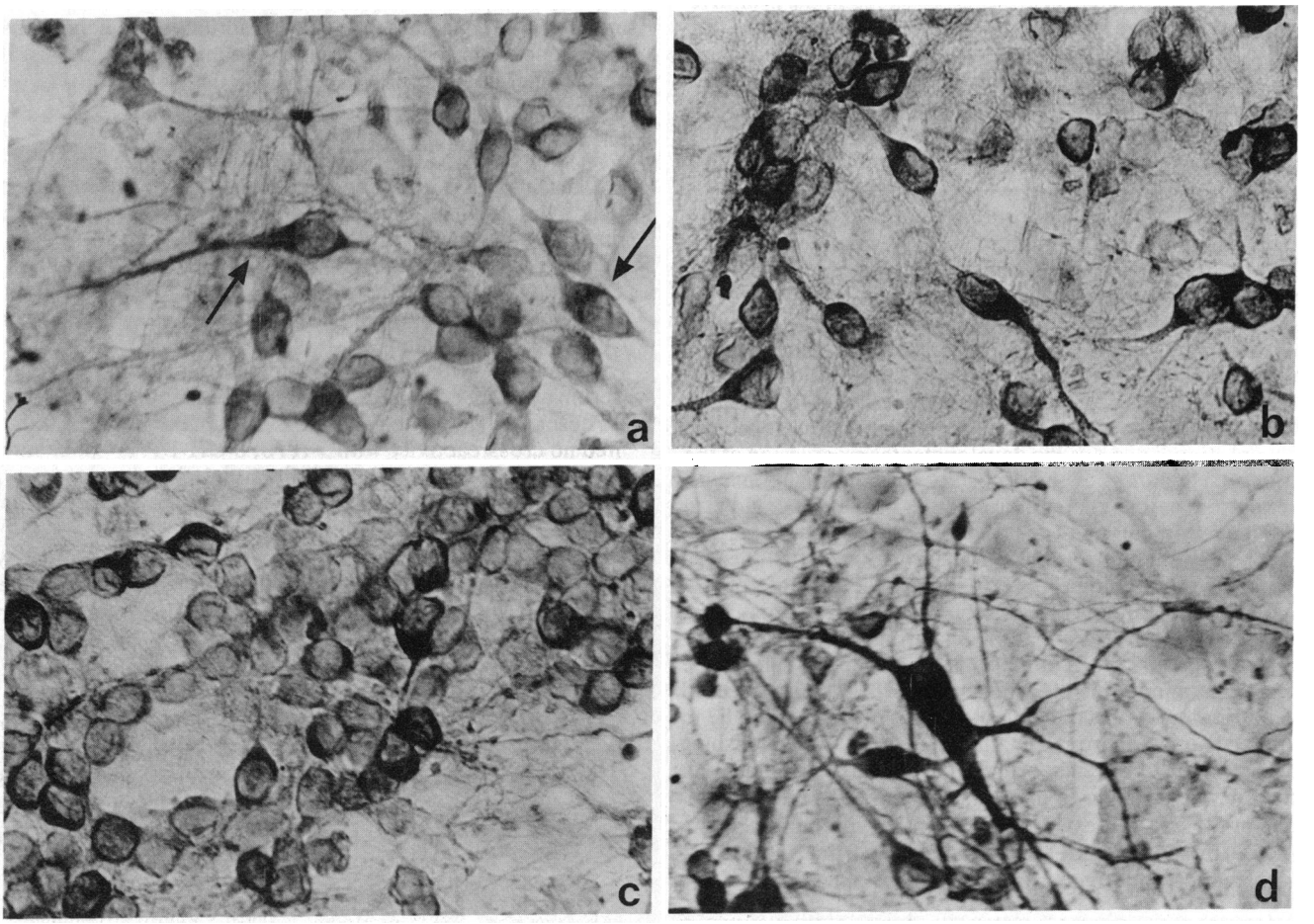

FIG. 1. Effect of 8-OH-DPAT on 5-HT immunoreactivity in hypothalamus cultures from ED 14 embryos. (*a*) Control cells. (Note bipolar neuron-like cells indicated by the arrow.) (*b*) 8-OH-DPAT-treated neuron-like cells. (*c*) Recruited immunoreactive ovoid cells. (*d*) Multipolar brain stem neuron. (×350.)

as early as embryonic day 12 (ED 12) provided cells were incubated with 5-HTP (Fig. 1) but not with tryptophan. The absence of tryptophan hydroxylase but the presence of 5-HTP decarboxylase in these cells was indirectly confirmed using 20 μM carbidopa, a specific AA decarboxylase inhibitor: no immunostaining was detected when the culture medium was supplemented with this drug. In the absence of carbidopa, the number of positive cells increased gradually up to ED 14 and remained stable thereafter (Fig. 2), representing 1.5–2.5% of total neurons present, as assessed by γ-enolase immunoreactivity. 5-HT-immunoreactive cells had big soma as compared to other hypothalamic neurons and were often fusiform with bipolar processes or pyramidal with tripolar processes (Fig. 1). The accumulation of 5-HT newly synthesized from 5-HTP was observed only when its catabolism was inhibited by pargyline. In contrast brain stem embryonic serotoninergic neurons exhibited 5-HT immunoreactivity, even in the absence of 5-HTP and pargyline (Fig. 1), as do serotoninergic neurons containing active tryptophan hydroxylase in the raphe nucleus of adult mouse. As expected, brain stem cultures treated with *p*-chlorophenylalanine contained only a limited number of neurons with positive but low 5-HT immunoreactivity (not shown).

The 8-OH-DPAT-Induced Increase in the Number of 5-HT-Immunoreactive Cultured Embryonic-Mouse Hypothalamus Cells. A significant increase of 5-HT immunoreactivity within described hypothalamic neuron-like cells was found after repetitive treatment with 8-OH-DPAT followed by a 2-hr incubation with 5-HTP (Fig. 1). As illustrated in Fig. 2, this treatment also increased the number of 5-HT-containing cells, particularly in cultures from ED 14 hypothalamus. Under this condition, up to 5–6% of hypothalamic neuronal cells exhibited 5-HT immunoreactivity. Ovoid neuroblast-like cells, often grouped in the same culture area and occasionally with short processes, were among these recruited immunoreactive cells (Fig. 1 *b* and *c*). As already mentioned for hypothalamic neuronal cells cultivated in the absence of 8-OH-DPAT (Fig. 1*a*), the 5-HT immunoreactivity directly resulted from the activity of AA decarboxylase within cells, since no immunostaining was found when the medium containing 8-OH-DPAT was supplemented with 20 μM carbidopa. Direct biochemical measurement of 5-HTP decarboxylation in cultures exposed to 8-OH-DPAT (as that performed with the primitive nerve cell line F7, ref. 4) was not possible as the substrate ^{3}H-labeled 5-HTP is no longer available from any commercial source. Nevertheless, immunocytochemical data were highly reproducible using cultures from ED 14 embryos, and the same developmental stage was chosen for further characterization of the possible effects of 8-OH-DPAT upon 5-HT uptake and accumulation in hypothalamic cells.

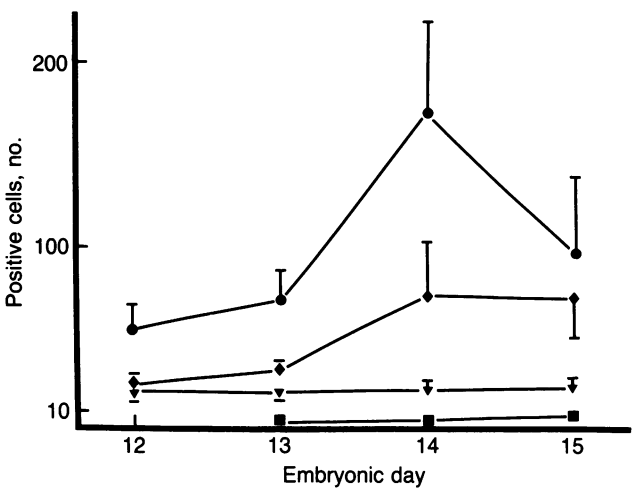

FIG. 2. Effects of 8-OH-DPAT on the number of cells synthesizing 5-HT from 5-HTP, accumulating ^{3}H-labeled 5-HT, or containing tyrosine hydroxylase in primary cultures from embryonic hypothalami. Cultures were made from hypothalami of mouse embryos at ED 12–15 as indicated on the abscissa. 8-OH-DPAT (10 nM) was added to the culture medium on days 4, 7, and 9; and each marker [5-HT-immunoreactivity, ^{3}H-5-HT, tyrosine hydroxylase (TH)-immunoreactivity] was examined on the 10th day of culture. ●, 5-HT-immunoreactive cells in cultures with 5-HTP/8-OH-DPAT. ◆, 5-HT-immunoreactive cells in cultures with 5-HTP alone. ▼, ^{3}H-labeled 5-HT-labeled cells in cultures with 5-HTP. The addition of 8-OH-DPAT exerted no effect on the number of ^{3}H-labeled 5-HT-labeled cells at all embryonic ages tested. ■, TH-immunoreactive cells in cultures with 5-HTP. 8-OH-DPAT in the culture medium did not affect the number of TH-containing cells at any age considered. Each point is the mean ± SEM of the number of positive cells per dish corresponding to four hypothalamus in six different experiments for ED 12, 13, and 15, and in 10 experiments for ED 14.

In these serum-containing cultures, some nonneuronal cells exhibited glial fibrillary acid protein immunoreactivity. The bodies and processes of cells with typical neuronal morphology were almost invariably stained using a γ-enolase antiserum. Most flat cells underlying neurons exhibited neither γ-enolase nor glial fibrillary acid protein immunoreactivity.

Evidence for 5-HT Receptor-Mediated 8-OH-DPAT-Induced Increase in the Number of Cells Containing 5-HT in Cultured Embryonic Mouse Hypothalamus. As shown in Fig. 3, the 8-OH-DPAT-induced increase in the intensity of 5-HT immunostaining and in the number of 5-HT-immunoreactive neuronal-like cells was abolished in the presence of 1 μM metergoline, a specific 5-HT receptor antagonist (5). Control experiments indicated that metergoline alone had no effect on hypothalamic cell viability.

8-OH-DPAT Treatment Had No Effect on 5-HT Uptake and Storage by Hypothalamic Neuron-Like Cells. When exogenous ^{3}H-labeled 5-HT was supplied to ED 14 hypothalamic cells, autoradiography revealed that neuronal-like soma labeled with silver grains were less numerous than cells synthesizing 5-HT from 5-HTP, suggesting that not all neuronal-like cells containing AA decarboxylase were able to take up ^{3}H-labeled 5-HT. Silver grains were generally located on the soma and the processes of labeled cells (Fig. 4 *a* and *c*), but in some instances only long processes and occasionally growth cones were labeled. These few labeled neuronal-like cells were found as early as ED 12, indicating that ^{3}H-labeled 5-HT uptake and AA decarboxylase appeared

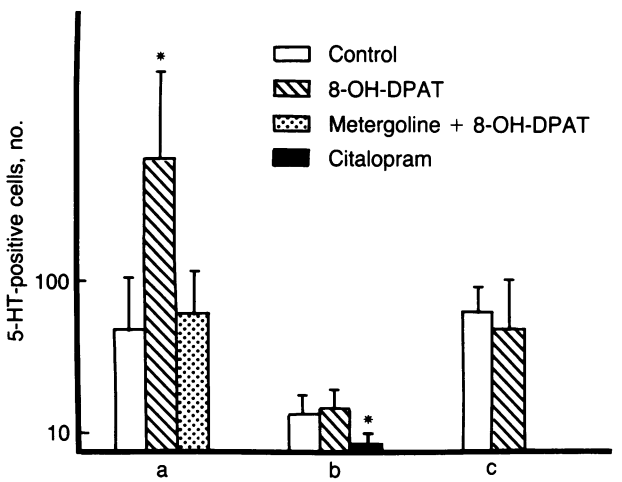

FIG. 3. The effect of 8-OH-DPAT on the number of neurons in hypothalamus cultures. 5-HT-immunoreactive neurons (*a*); neurons showing specific ^{3}H-labeled 5-HT uptake (*b*); neuron-like cells with 5-HT accumulation in cultures from ED 14 embryos (*c*). Each bar is the mean ± SEM of nine different experiments.

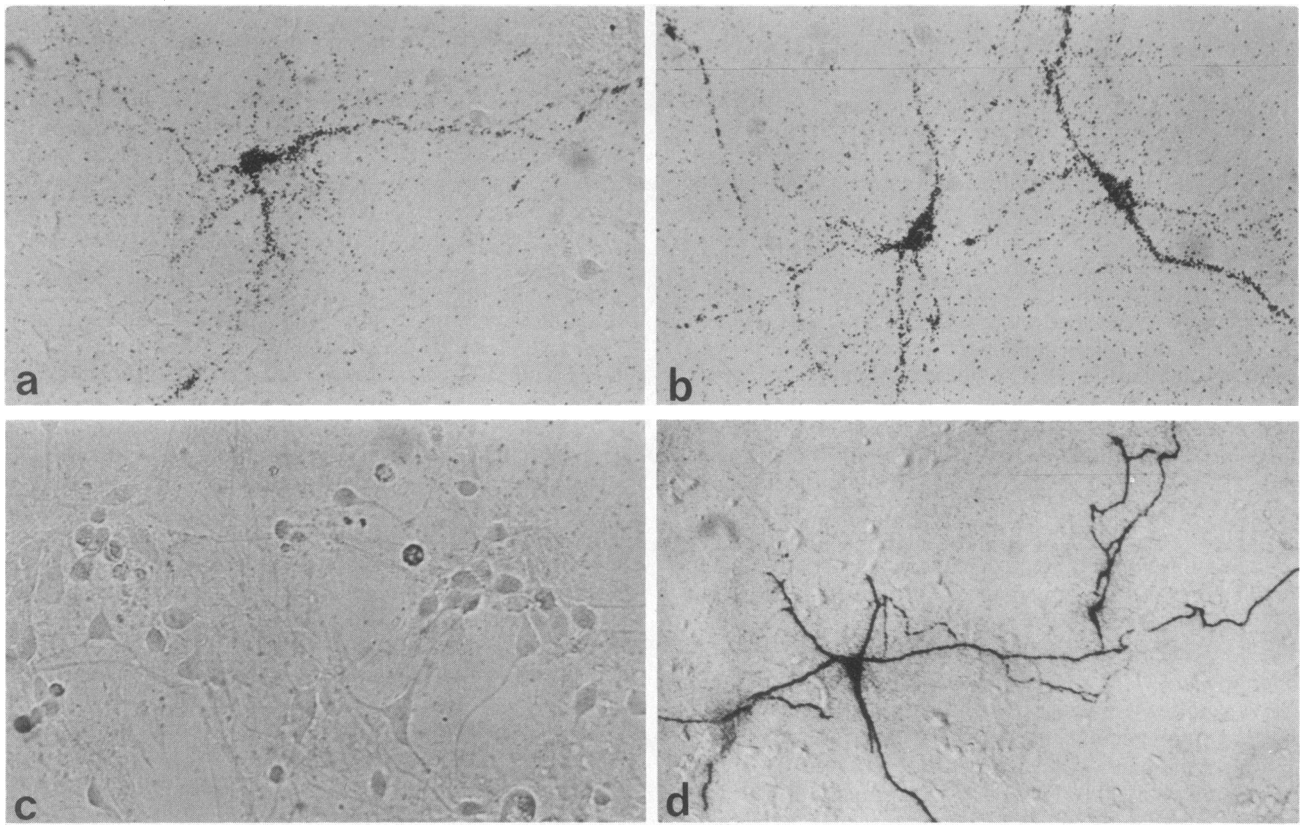

FIG. 4. Autoradiographic localization of ^{3}H-labeled 5-HT uptake into cultures from ED 14 embryos. Hypothalamic neuron-like cell (×220) (*a*); control in the presence of 10 μM citalopram (*c*), or nomifensine (*b*) (×220); brain stem neurons (×90) (*d*) were labeled after incubation with 1 μM (hypothalamus), or 0.1 μM ^{3}H-labeled 5-HT (brain stem).

simultaneously (Fig. 2). In cultured cells from embryonic hypothalamus or brain stem, ^{3}H-labeled 5-HT uptake was abolished in the presence of citalopram, a specific blocker of 5-HT uptake, but was unaffected by nomifensine, a specific blocker of dopamine and norepinephrine uptake. These results showed that uptake capacity of hypothalamic 5-HT neuronal-like cells was pharmacologically identical with that of typical serotoninergic neurons and distinct from that of catecholaminergic cells.

As shown in Fig. 3, intracellular storage of 5-HT was found in hypothalamic cultures grown for 7 days in the presence of 1 μM 5-HTP and assayed 3 days later for 5-HT immunoreactivity. Repeated exposure to 8-OH-DPAT modified neither ^{3}H-labeled 5-HT uptake nor 5-HT storage in cultures of ED 14 hypothalamic cells.

Coexistence of Endogenous 5-HT Storage and ^{3}H-Labeled 5-HT Uptake in Hypothalamic Neuron-Like Cells. By combining radioautography, to reveal neurons capable of ^{3}H-labeled 5-HT uptake, and immunohistochemistry, to detect endogenous 5-HT, we found that the same neuron-like cells exhibited both properties. As shown in Fig. 5*a*, silver grains accumulated over the cell body and processes of a few neuron-like cells that were immunoreactive for 5-HT. However, numerous other 5-HT immunoreactive somas were devoid of silver grains.

Evidence That Hypothalamic Neuron-Like Cells Containing Tyrosine Hydroxylase Were Distinct From ^{3}H-Labeled 5-HT Accumulating Cells. Tyrosine hydroxylase immunoreactivity could not be detected in hypothalamic cell cultures initiated from ED 12. At ED 13–15, only a few neurons were positively stained (Fig. 2). To examine whether neurons containing tyrosine hydroxylase were distinct from ^{3}H-labeled 5-HT-accumulating cells or not, double labeling experiments were performed at ED 13 and 15. As shown in Fig. 5*b*, evidence was obtained that dopaminergic neurons containing tyrosine hydroxylase were distinct from cells labeled with ^{3}H-labeled 5-HT. As expected from these observations, ^{3}H-labeled 5-HT

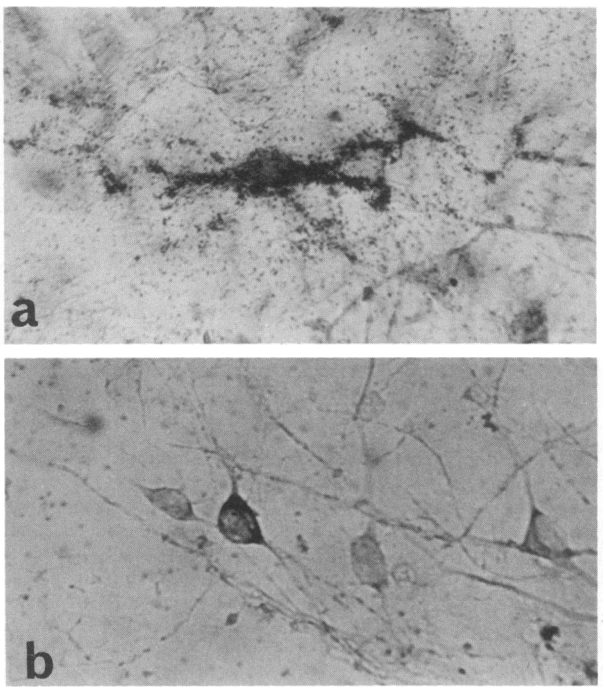

FIG. 5. Combined radioautography of ^{3}H-labeled 5-HT uptake and immunohistochemistry on hypothalamic cells. 5-HT antibody (*a*), note the colocalization of endogenous 5-HT immunoreactivity and silver grains on same ED 13 neuron-like cell; tyrosine hydroxylase antibody (*b*), note that immunoreactive ED 13 neuron is devoid of silver grains. ^{3}H-labeled 5-HT uptake was measured using 1 μM of the labeled substrate. (×350.)

uptake was blocked by citalopram, but not by nomifensine, which inhibits monoamine uptake in dopaminergic neurons.

DISCUSSION

This study revealed that neuron-like cells from embryonic hypothalamus, from ED 12 and on, express some of the characteristics typical of serotoninergic neurons: AA decarboxylase activity and 5-HT uptake and storage are present in the same neuron-like cells, which also express the γ-enolase antigen, a specific marker of neuronal cells in these cultures. However, such neuron-like cells containing 5-HT differ from brain stem embryonic serotoninergic neurons (Fig. 1; see also ref. 14), since they lack the specific enzyme tryptophan hydroxylase. They differ also from catecholaminergic embryonic neurons since they lack tyrosine hydroxylase, as revealed by immunocytochemistry using tyrosine hydroxylase antiserum.

Cells able to accumulate exogenous 5-HTP or ^{3}H-labeled 5-HT have been visualized in the hypothalamus of adult rats (7–9), but the simultaneous presence of AA decarboxylase activity and 5-HT uptake in the same cells is poorly documented. Cells containing immunoreactive AA decarboxylase have already been visualized early in development in the mesencephalon (15). In the present study, the use of an appropriate double labeling technique allows us to conclude that AA decarboxylase catalyzing the conversion of 5-HTP into 5-HT, and ^{3}H-labeled 5-HT uptake are actually present within the same hypothalamic neuron-like cells.

It has been suggested that monoamines can play a neurotrophic role in early ontogenesis (16, 17), probably via the stimulation of specific receptors on target cells (17). In the present study, the involvement of specific 5-HT receptors on neuron-like cells can be proposed on account of the effects of the potent 5-HT agonist 8-OH-DPAT and their blockade by the 5-HT antagonist metergoline (5). The 8-OH-DPAT-induced increase in the number of 5-HT neuron-like cells probably did not result from a better survival of neurons, as assessed by direct counting of cells in control and 8-OH-DPAT-treated cultures. Furthermore, cell attachment was not affected by the addition of 10 nM 8-OH-DPAT to the culture medium 24 hr after cell seeding (unpublished observations). Therefore, these data provide evidence that the 5-HT agonist 8-OH-DPAT could act by stimulating the synthesizing capacity of cells already committed—at least partially—to the serotoninergic phenotype. Such autocrine or paracrine-like mechanisms for self-stimulation may be of critical importance during early development of the nervous system.

These results also support and extend the conclusion of our studies (4) that showed that under appropriate culture conditions, repetitive treatment with a 5-HT agonist and eye-derived growth factor, irreversibly induced AA decarboxylase activity in the primitive hypothalamic cell line F7: such induction, reinforcement, and stabilization of the commitment of one cell have been defined as a "learning" process at the cellular level (4). The capacity of embryonic nerve cells to synthesize and respond to their own neurotransmitter, and the stimulatory effect of a neurotransmitter, such as 5-HT, on the neuron commitment to a given neurochemical specificity, could be an important concept in determining the functional link between neurotransmitters and brain development.

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