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Explant cultures of catecholamine-containing neurons from rat brain: Biochemical, histofluorescence, and electron microscopic studies

(locus ceruleus/substantia nigra/tissue culture/norepinephrine/dopamine)

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ABSTRACT **Norepinephrine (NE)-producing cells of the nucleus locus ceruleus and dopamine (DA)-producing cells of the substantia nigra were dissected microscopically from embryonic rat brain, explanted, and maintained in culture for up to 5 weeks. The cultured neurons of both brain regions showed normal maturation of axons and dendrites and formed ultrastructurally defined synaptic contacts. Fluorescence microscopy of cultured neurons from both brain regions showed typical *in situ* cytological features: long axonal processes with multiple varicosities for locus ceruleus cultures, and smooth, wispy nonvaricose processes in the substantia nigra cultures. All cultures processed for fluorescence microscopy contained specific catecholamine-fluorescent cells. By radioenzyme assay for catecholamines, more than half of the locus ceruleus cultures contained measurable (>10 pg) quantities of NE and DA, but, unlike results on intact brains, DA content exceeded NE content. Cultures of substantia nigra neurons retained no NE and very little DA. Media from substantia nigra and locus ceruleus cultures contained substantial quantities of DA. Addition of reserpine (10 μM) to the medium depleted locus ceruleus neurons of both amines.**

The long survival time in culture of locus ceruleus cells, the normal appearance of fluorescent cell bodies and processes, the apparent development of morphologically specialized interneuronal connections, and the ability to synthesize and store NE make these cultures ideally suited for neurophysiological recording as well as morphological, biochemical, and pharmacological experiments.

Many advantages would accrue from the ability to culture specific neuronal nuclei from mammalian brain. The controlled conditions of the culture situation could yield data on the metabolism, pharmacological responsiveness, receptor specificity, and developmental characteristics of defined, homogeneous populations of neurons. To date, neuronal culture systems of mammalian brain have utilized entire brain (1, 2), individual cortical (3–6) or lower mesencephalic (7–11) regions containing heterogeneous cell types, or chemically induced brain tumors (12). This paper describes a modified version of the explant culture technique applied to two regions of rat brain stem that contain neuronal populations utilizing catecholamines as their neurotransmitter. With these modifications, the norepinephrine (NE)-containing neurons of the nucleus locus ceruleus and the dopamine (DA)-containing neurons of the substantia nigra have been successfully cultured for up to 5 weeks. Furthermore, through the use of very sensitive radioenzyme assays for NE and DA, the catecholamine levels of the cultured cells can be measured in the cells and media during *in vitro* maturation. The assay results combined with fluorescence microscopy of the cultures indicate that all explants contained catecholamine-producing cells having characteristics of *in situ* neurons.

MATERIALS AND METHODS

Embryonic Rat Dissection. Embryos were removed after cervical dislocation at 20-days' gestation from albino rats (Zivic–Miller) and kept on ice. Individual embryonic brains placed on sterile gelatin blocks were stabilized with Methocel-enriched medium. Under microscopic control, we dissected the brains, at different anteroposterior levels, into standard slices to which catecholamine-containing cell groups had been localized by fluorescence histochemistry and Nissl stain (cresyl violet) (unpublished data). For the locus ceruleus cells, cuts were made caudal and frontal to the embryonic cerebellum; for substantia nigra, cuts were made at the level of the midbrain flexure and 1–1.5 mm rostral to it. What is referred to here as "substantia nigra" includes, at this stage of development, all of the as yet unseparated mesencephalic DA neuronal groups. The fragments of interest (approximately 3–4 mm^2) were excised from the appropriate slice and placed in 35-mm Falcon dishes freshly coated with collagen (13) and containing approximately 0.5 ml of Methocel medium. The tissue fragment was then teased into 20–30 smaller pieces whose final positions in the dish were stabilized by a minimal amount of Methocel medium. The cultures were incubated at 35° in a 95% air/5% CO_2 atmosphere.

Brain Media. The optimal medium as judged by morphologic and biochemical criteria was Dulbecco's modified Eagle's medium with *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid buffer [Grand Island Biological Co. (Gibco), Grand Island, NY] enriched with serum. Ten percent fetal calf serum (Gibco or Reheis Chemical Co., Chicago, IL), 10% heat-inactivated horse serum (Gibco), and 0.4% Methocel (Methocel 60HG, Dow Chemical, Midland, MI) were added for the first 5 days of culture (311 mOsm/kg). After 5 days, the cultures were continued in Dulbecco's modified Eagle's medium supplemented with 10% horse serum (298 mOsm/kg). Experimental variations from this basal medium included addition of nerve growth factor (0.2 μg/ml of medium), ascorbic acid (25 μg/ml final concentration; added daily and 2 hr before harvesting cells), 1 μM 6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride (Calbiochem, San Diego, CA) or 1 μM crude biopterine (Sigma Chemical, St. Louis, MO), 0.1mM or 10 nM $CuSO_4$, and reserpine [dissolved in ascorbic acid solution (250 ng/10 ml) and added to a final concentration of 10 μM].

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Abbreviations: NE, norepinephrine; DA, dopamine.

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Mitosis Inhibitors. We tested 5-fluoro-2'-deoxyuridine (20 μg/ml and 40 μg/ml) at different addition schedules to inhibit thymidylate synthetase (14, 15); we also tested D-arabinofuranosylcytosine (cytosine arabinoside) (16), another DNA synthesis inhibitor (5 μmol or 1.4 μg/ml of medium).

Norepinephrine/Dopamine Determinations. NE and DA levels were measured by a modified radioenzymatic method (17, 18) based on reaction of catecholamines with partially purified catechol-*O*-methyltransferase (*S*-adenosyl-L-methionine:catechol *O*-methyltransferase, EC 2.1.1.6), using *S*-adenosyl[^{3}H]methionine as the methyl donor (19). The sensitivity of the assay was 10 pg for NE and 20 pg for DA. Epinephrine was not separated from NE and appeared in the NE fraction of the assay.

At different times after explantation, cells and media were assayed separately. Replicate brain regions were dissected identically and assayed to determine starting levels of catecholamines *in vivo*.

For each medium assay, 10 μl of concentrated perchloric acid was added to 1 ml of the medium taken from the incubated cultures. Fifty microliters of the acidified sample was taken for the assay. The results were expressed in ng/ml of medium.

For assay of cells, the flattened explants were scraped off the culture dish with a glass pestle into 150 μl of 0.1 M perchloric acid. After centrifugal separation of insoluble material, 50 μl of the supernatant was assayed, and the results were expressed as ng per culture. [The large amount of collagen present precluded using protein levels as a normalizing factor; similarly, DNA measurements for the number of cells may not be accurate (20)].

Freshly dissected tissues, homogenized in 150 μl of 0.1 M perchloric acid, were processed in the same way as cultured cells.

Microscopic Methods. The living cultures were monitored routinely on an inverted phase contrast microscope. In addition, some cultures were processed for histochemical fluorescence or electron microscopy.

For fluorescence studies, cultures (1–4 weeks *in vitro*) were exposed to the monoamine oxidase inhibitor pargyline (0.5 mM, 2 hr) and two doses of ascorbic acid (500 μg/ml of medium; 60 and 120 min) and were then superfused with ice-cold hypertonic formaldehyde solution (21) for 5–15 sec; after drying in an air stream and exposure to formaldehyde vapors (50% humidity, 80°, 60 min), the cultures were viewed without cover slips under epi-illumination (Zeiss Universal, 100 W Hg lamp).

For electron microscopy, the cultures were fixed in cold $KMnO_4$ (4%, 5 min), stained with uranyl acetate (1%), dehydrated, and embedded in Epon. Silver sections were mounted on grids and viewed without further staining (Philips EM 200).

RESULTS

Locus ceruleus and substantia nigra explants survived for several weeks in culture. During the first 5 days *in vitro*, 80–90% of the tissue pieces attached and grew out. Within 6 days, the outgrowth zones of the individual explants became confluent. Addition of 5-fluoro-2'-deoxyuridine prevented overgrowth of the non-neuronal background cells (22) but did not affect the catecholamine levels in these cultures (unpublished data).

The catecholamine levels of the cultures were compared to those obtained from fresh embryonic tissue samples of the two catecholamine-containing brain nuclei (Fig. 1). Whereas 50–60% of the locus ceruleus cultures showed measurable amounts of catecholamines, the percentage of substantia nigra

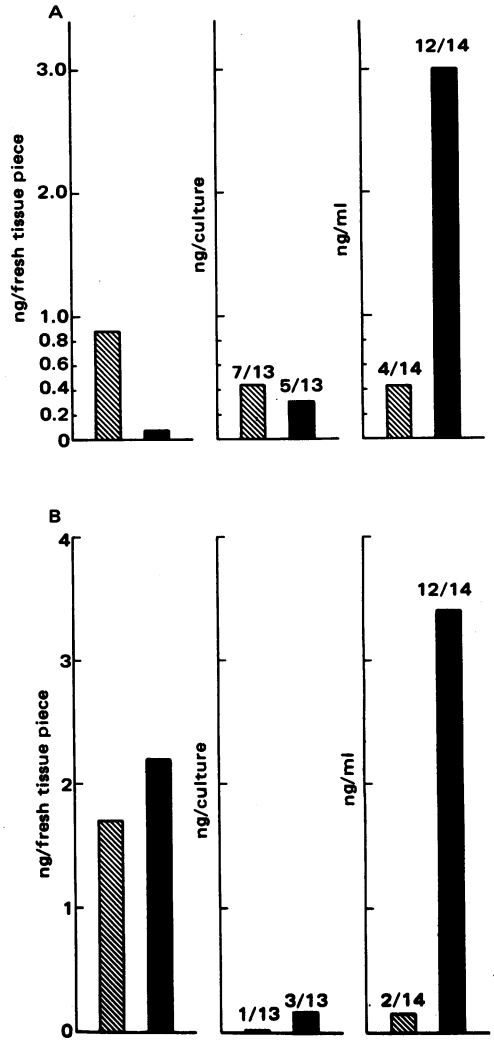

FIG. 1. (*A*) NE and DA levels from 20-day-gestation rat embryonic locus ceruleus (including part of the cerebellum; total weight approximately 2–3 mg) (*Left*) and from the cells (*Middle*) and media (*Right*) after 15 days in culture. (*B*) Catecholamine levels from 20-day gestation embryonic substantia nigra midbrain (approximately 3 mg) (*Left*) and from the cells (*Middle*) and media (*Right*) after 15 days in culture. Concentration in medium (ng/ml) was measured 24 hr after the last medium change. Total medium volume was 1 ml. The fractions above the bars represent the ratio of cultures or media showing measurable amounts of the amine to the total number of cultures assayed—e.g., 4/14 means only 4 of the 14 cultures had a detectable level of that amine. The bars represent the average amine level of all cultures or media including those samples registering zero amount. ▨, NE; ■, DA.

cultures containing catecholamines was lower (<25%). The values represent the average amount of catecholamines obtained from all cultures, including the nonproductive ones. Nevertheless, the average cellular catecholamine content after 2 weeks in culture was about 50% of the level in the initial dissected tissue. No statistical tests were done because of the large amount of variability of the catecholamine levels from culture to culture, with many of the cultures having undetectable levels.

The DA/NE ratio was much higher in the cultured tissue than in the embryonic brain. The culture media also contained considerable amounts of catecholamines. The DA/NE ratio in media was even higher than in cells. Reserpine (10 μM, 3 hr) consistently depleted more than 97% of both NE and DA from the cells of the cultures (2 weeks in culture) but did not alter

Table 1. Influence of ascorbic acid* on cellular content and medium concentration of catecholamines in locus ceruleus and substantia nigra explant cultures after 12–16 days *in vitro*

	Locus ceruleus		Substantia nigra	
	Control	+ ascorbic acid	Control	+ ascorbic acid
Cells:				
NE, ng/culture	0.379 (16)	0.345 (18)	0.003 (16)	0.015 (10)
DA, ng/culture	0.975 (16)	0.299 (18)	0.098 (16)	0.186 (10)
Medium:				
NE, ng/ml	0.143 (17)	0.582 (18)	0.038 (16)	0.197 (10)
DA, ng/ml	3.80 (17)	4.48 (18)	3.80 (16)	2.77 (10)

Number of determinations shown in parentheses.
* Ascorbic acid was added to the cultures at a dose of 500 μg per culture daily; the final addition was 2 hr before harvesting the cells.

levels in the media. Decay curves of catecholamines under our incubation conditions [obtained by adding the amines to medium alone (pH 7.2) and incubating the mixture at 35°] indicated a half-life of less than 1 hr. Thus, the high catecholamine content in the media and the rapid decay of catecholamines at incubator conditions suggest that cultured neurons are capable of vigorous synthetic capacity.

Cofactors (biopterin, 6,7-dimethyl-5,6,7,8-tetrahydropterine). To evaluate the adequacy of media and supplement for synthesis, we studied the possible effect of the pteridine cofactors biopterine and its synthetic congener 6,7-dimethyl-5,6,7,8-tetrahydropterine. Neither compound (1 μM) enhanced the catecholamine levels over control cultures.

Ascorbic Acid. Dopamine β-hydroxylase [dopamine β-monooxygenase, 3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1], the enzyme that converts DA into NE in noradrenergic neurons, requires a reducing cosubstrate, such as ascorbic acid (26). The Dulbecco's modified Eagle's medium used to grow central nervous system neurons did not contain ascorbic acid. However, intracellular NE levels in locus ceruleus cultures did not rise after ascorbic acid supplementation (25 ng/ml daily and 1–2 hr before harvesting) whereas intracellular DA levels in both central cultures clearly increased (Table 1). In the media, ascorbic acid supplementation consistently increased NE concentrations by 4- to 5-fold for both locus ceruleus and substantia nigra cultures, whereas DA levels were unchanged. The higher NE levels in the media together with the higher DA levels in the cells suggest that the major effect of ascorbate may be its antioxidant action rather than its enhancement of β-hydroxylation.

Copper. We investigated the influence of Cu^{2+}, a known prosthetic group for dopamine β-hydroxylase (23), on catecholamine production by cultured brain neurons (Fig. 2). No effects of Cu^{2+} (10 nM) were seen on NE levels in 9-day cultures; although DA levels were somewhat higher, they were within the usual range of variability. Higher Cu^{2+} concentration (0.1 mM, 19-day cultures) resulted in 90% decrease of both amine levels. This result could be due to high doses of Cu^{2+} inhibiting dopamine β-hydroxylase activity (24) or other catecholamine synthetic enzymes, to direct cellular toxicity, or to more rapid chemical oxidation to catecholamines.

Morphology. *Phase contrast microscopy.* For the first 5 days *in vitro*, the cultures were disturbed minimally in order not to interfere with the attachment procedure. After day 6, the cultures were examined routinely under a phase contrast microscope. Growth and differentiation of neuron-like cells could be followed daily. With time, there was an increase in cell diameter, in the development of complex processes, and in length of fibers (Fig. 3).

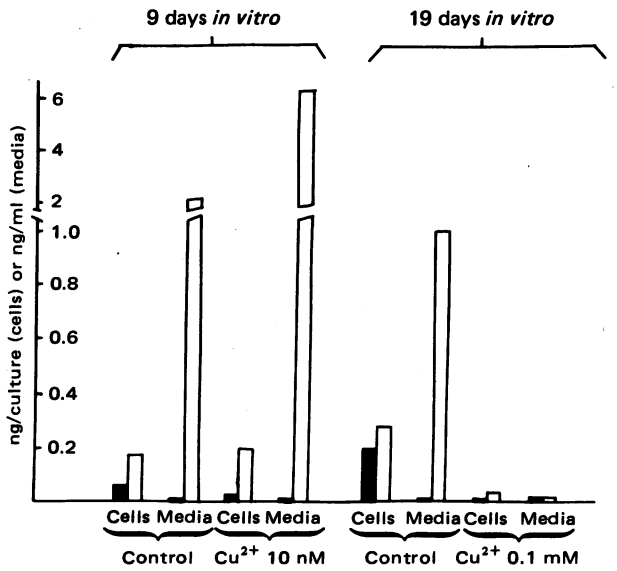

FIG. 2. Effects of Cu^{2+} (10 nM and 0.1 mM) on NE and DA levels in cultures of catecholamine-containing nuclei (A1–A6) of the lower brain stem. The bars represent means of at least five cell levels (ng per culture) and media concentrations (ng/ml). ■, NE; □ DA.

Fluorescence microscopy. All locus ceruleus and substantia nigra cultures processed for fluorescence microscopy contained one or two large groups of brightly fluorescent perikarya as well as two or three scattered smaller groups containing several cells each.

In locus ceruleus cultures, the fluorescent processes were radially oriented around the large group of tightly packed cell bodies. An abundance of fine fluorescent fibers appeared to emanate from the larger cell groups (Fig. 4A). Occasionally,

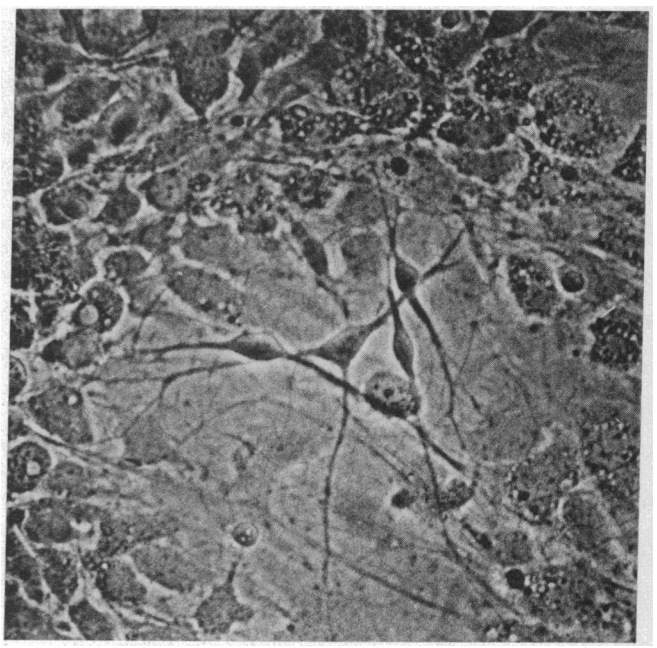

FIG. 3. Phase contrast microscopy of living cells of the substantia nigra at 8 days in culture. The outline of the somata and the pattern of the processes are suggestive of neurons. These elongated cells (12 × 22 μm), frequently with two or three processes, are commonly observed at the border of an explant outgrowth zone.

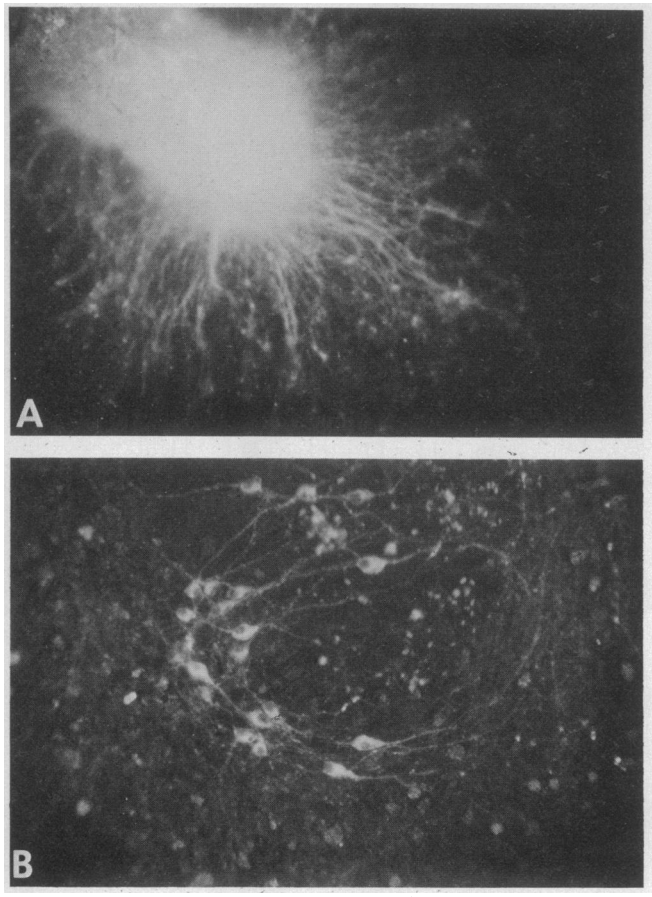

FIG. 4. Fluorescence microscopy. (*A*) Whole-culture mounts from locus ceruleus cultures grown for 1 week *in vitro*, showing a thick cluster of fluorescent cells apparently forming several long processes. (*B*) A green fluorescent cell group from a locus ceruleus culture grown for 8 days *in vitro*, showing large ovoid cells with clearly visible nuclei forming very long thin and varicose processes.

fluorescent varicosities could be seen even at considerable distances from the nucleus of cell bodies.

When separated from the large nucleus of fluorescent cells, individual neurons could be identified (Fig. 4*B*). In size (30 × 22 μm) and cell body and process morphology, these fluorescent neurons bore a strong resemblance to the cells of the nucleus locus ceruleus *in situ*. Substantia nigra cultures revealed cells less densely packed and having thick nonvaricose processes proximal to the perikaryon, similar to the *in situ* appearance of this nucleus.

Six of six cultures washed with saline before exposure to formaldehyde solution were devoid of catecholamine-specific fluorescent cells and fibers, although these saline-washed cultures still contained the same number of yellow autofluorescent cells.

Electron microscopy. Electron microscopy of the cultures revealed a cellular morphology similar to that of brain *in situ* (Fig. 5). Neuronal cell bodies, axons, dendrites, and synapses could easily be identified in the brain stem cultures. We found several different types of vesicles in the processes and in bouton-like structures; however, using $KMnO_4$ fixation we were unable to identify any small granular vesicles characteristic of NE storage (25, 26) in the small sample we examined.

DISCUSSION

The objective of these experiments was to determine optimal conditions for growth and differentiation *in vitro* of brain

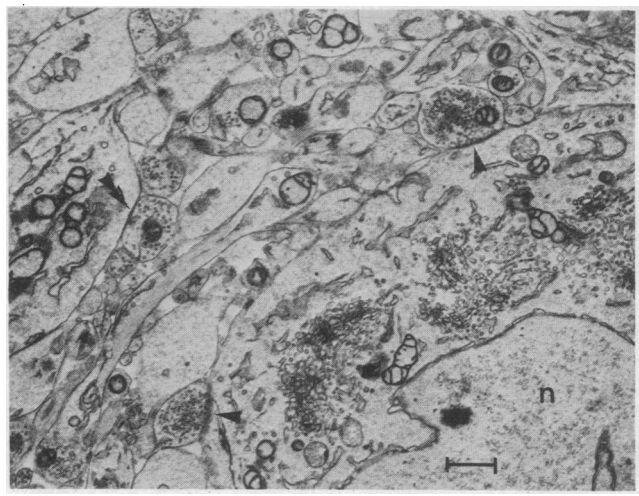

FIG. 5. Electron micrograph (permanganate stain) from locus ceruleus culture grown for 32 days *in vitro*, showing a cell with nucleus (n), endoplasmic reticulum, mitochondria, and several synaptic contacts (single arrow). In addition, synaptic contacts can be seen in the neuropil region (double arrow). (Bar represents 2 μm.)

neurons that utilize catecholamines as neurotransmitters.

Whereas certain regions of the embryonic brain, such as cerebellum and cerebral cortex, grow well as dispersed cells (3, 27, 28), our own experience is that brain stem and midbrain regions do not. This may be due to the susceptibility of these cells to injury by the dissociation procedures (trypsinization, mechanical disruption, etc.).

Brain stem and diencephalic regions of the embryonic brain, however, grow well when explanted intact rather than dissociated (9–11). Neuronal survival is much higher in explant type cultures, perhaps because the neuron–neuron and neuron–supporting cell interactions remain relatively intact (29).

Our modified explant technique for brain stem regions produced certain advantages: long-term survival of brain stem cells in culture (at least 5 weeks); improved microscopic observation, both in the living state (phase contrast) and fixed (fluorescence histochemistry); and greater amounts of tissue per culture, desirable for quantitative biochemical determinations. The key to our procedure is, we believe, the handling of the embryonic tissue. The region to be explanted is gently disrupted (by teasing apart, not by cutting) into very small pieces (less than 0.5 mm^3). This procedure is carried out in the culture dish, so that no further transfers or media exchanges are necessary for several days. In the dish, the small tissue pieces tend to flatten into layers two to three cells deep, resulting in much improved visualization.

Although we have characterized and optimized the catecholamine-synthesizing ability of the neurons in these culture preparations, currently only 5–20% of the entire culture can be identified as catecholamine-containing cells.

There are several indications of a more labile state for intracellular catecholamines in cultures of brain neurons compared to peripheral sympathetic neurons. The first piece of evidence is that washing brain cultures (after pretreatment with ascorbic acid and pargyline) with physiological solutions results in the total loss of catecholamine fluorescence. This situation does not occur when using peripheral noradrenergic neurons (30). The lability of catecholamine storage in cultured brain may be due to disabled active reuptake with a failure to reaccumulate released amine. Additional evidence for the limited storage capacity of immature brain noradrenergic neurons is supplied by the work of Coyle and Axelrod (31, 32). Using ho-

mogenates of rat brain from 18 days' gestation to adult, they observed a minimal amount of uptake ability in the immature brain which increased 300-fold by adulthood. The underdeveloped reuptake of catecholamines from the medium could account for both the low intraneuronal storage capacity and the excessive amounts of neurotransmitters we found in the media.

The situation of the substantia nigra cells in culture is more difficult to evaluate. These cells presumably are DA synthesizing; the NE in the initial explanted piece (Fig. 1*B*, *left*) was most likely contained in fibers passing through and not to intrinsic noradrenergic cell bodies. Although the cellular amine content of most substantia nigra cultures fell below the level of sensitivity of the chemical assay (Fig. 1*B*, *middle*), the fluorescence histochemistry indicated that catecholamines were present in neurons of all cultures observed. Further, the detection of large amounts of DA in the media of most substantia nigra cultures (Fig. 1*B*, *right*) would suggest that these cells produce large amounts of DA and that the intracellular fluorescing compound is probably DA.

Using reaggregation cultures of 18-day embryonic mouse midbrain, Levitt *et al.* (33) also found that DA is the predominant catecholamine synthesized after 3 days in culture. Their 3-day culture results are comparable in age and DA content to our starting material, but these authors did not report DA levels or fluorescence microscopy beyond 3 days of culturing, so that further comparison is difficult. Because fluorescence histochemistry reveals more normal DA content in cultured nigral cells than can be detected by radioenzyme assay, fluorescence microscopy may be the optimal tool at present for assessing the synthetic capacity of these cells.

Locus ceruleus explant cultures, on the other hand, offer at present a suitable preparation for *in vitro* studies of the development and characterization of NE-mediated synaptic events in the central nervous system.

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1. Godfrey, E. W., Nelson, P. G., Schrier, B. K., Breuer, A. C. & Ranson, B. R. (1975) *Brain Res.* **90**, 1–21.
2. Schrier, B. K. (1973) *J. Neurobiol.* **4**, 117–124.
3. Lasher, R. S. & Zagon, I. S. (1972) *Brain Res.* **41**, 482–488.
4. Wolf, M. K. & Dubois-Dalcq, M. (1970) *J. Comp. Neurol.* **140**, 261–280.
5. Kim, S. U. (1972) *Exp. Neurol.* **35**, 305–321.
6. Seil, F. J., Kelly, J. M. & Leiman, A. L. (1974) *Exp. Neurol.* **45**, 435–450.
7. Hosli, E., Meier-Ruge, W. & Hosli, L. (1971) *Experientia* **27**, 310.
8. Coyle, J. T., Jacobowitz, D., Klein, D. & Axelrod, J. (1973) *J. Neurobiol.* **4**, 461–470.
9. Geller, H. M., Cechner, R. L. & Fleming, D. G. (1972) *J. Neurobiol.* **2**, 154–161.
10. Masurovsky, E. B., Benitez, H. H. & Murray, M. (1971) *J. Comp. Neurol.* **143**, 263–277.
11. Sobkowicz, H. M., Bleier, R. & Monzain, R. (1974) *J. Comp. Neurol.* **155**, 355–376.
12. Schubert, D., Heinemann, S., Carlisle, W., Tarikas, H., Kimes, B., Patrick, J., Steinbach, J. H., Culp, W. & Brandt, B. L. (1974) *Nature* **249**, 224–227.
13. Bornstein, M. (1973) in *Tissue Culture Methods and Application*, eds. Kruse, P. F. & Patternson, M. K. (Academic Press, New York and London), pp. 89–90.
14. Rueckert, R. R. & Mueller, G. C. (1950) *Cancer Res.* **20**, 1584–1591.
15. Pollack, R. E., Green, H. & Todaro, G. J. (1968) *Proc. Natl. Acad. Sci. USA* **60**, 126–133.
16. Fishbach, G. G. (1972) *Dev. Biol.* **28**, 407–429.
17. Coyle, J. T. & Henry, D. (1973) *J. Neurochem.* **21**, 61–67.
18. Palkovits, M., Brownstein, M., Saavedra, J. M. & Axelrod, J. (1974) *Brain Res.* **77**, 137–149.
19. Axelrod, J. (1963) *Science* **140**, 499–500.
20. Shashoua, V. E. & Wolf, M. K. (1971) *J. Neurochem.* **18**, 1149–1152.
21. Azmitia, E. & Henriksen, S. J. (1976) *J. Histochem. Cytochem.* **24**, 1286–1288.
22. Schrier, B. K. & Shapiro, D. L. (1974) *J. Neurobiol.* **5**, 151–159.
23. Kaufman, S. & Friedman, S. (1965) *Pharmacol. Rev.* **17**, 71–100.
24. Molinoff, P. B. & Orcutt, J. C. (1973) in *Frontiers in Catecholamine Research*, eds. Usdin, E. & Snyder, S. (Pergamon Press, Oxford), pp. 195–200.
25. Hokfelt, T. (1967) *Z. Zellforsch. Mikrosk. Anat.* **79**, 110–117.
26. Hokfelt, T. (1968) *Z. Zellforsch. Mikrosk. Anat.* **91**, 1–74.
27. Sensenbrenner, M., Boehrer, J. & Mandel, P. (1971) *Z. Zellforsch. Mikrosk. Anat.* **117**, 559–569.
28. Yavine, E. & Menkes, J. H. (1973) *J. Cell Biol.* **57**, 232–237.
29. Giller, E. L., Breakefield, X. O., Christian, C. N., Neale, E. A. & Nelson, P. G. (1975) in *Golgi Centennial Symposium Proceedings*, ed. Santini, M. (Raven Press, New York), pp. 603–623.
30. Mains, R. E. & Patterson, P. H. (1973) *J. Cell Biol.* **56**, 329–345.
31. Coyle, J. T. & Axelrod, J. (1971) *J. Neurochem.* **18**, 2061–2075.
32. Coyle, J. T. (1974) in *The Neurosciences*, eds. Schmitt, F. O. & Worden, F. G. (MIT Press, Cambridge), pp. 877–884.
33. Levitt, P., Moore, R. Y. & Garber, B. (1976) *Brain Res.* **111**, 311–320.