

Neuroblast Mitosis in Dissociated Culture: Regulation and Relationship to Differentiation

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Abstract. Although neuron generation is precisely regulated during ontogeny, little is known about underlying mechanisms. In addition, relationships between precursor proliferation and the apparent sequence of developmental processes, including cell migration, neurite elaboration, transmitter expression and synaptogenesis remain unknown. To address these issues, we used a fully defined neuronal cell culture system derived from embryonic rat sympathetic ganglia (DiCicco-Bloom, E., and I. B. Black. 1988. *Proc. Natl. Acad. Sci. USA.* 85:4066–4070) in which precursors enter the mitotic cycle. We now find that, in addition to synthesizing DNA, neuroblasts also underwent division in culture, allowing analysis of developmental relationships and mitotic regulation.

Our observations indicate that mitotic neuroblasts

expressed a wide array of neuron-specific characteristics including extension of neuritic processes with growth cones, elaboration of neurotransmitter enzyme, synthesis and transport of transmitter vesicles and organization of transmitter release sites. These data suggest that neuroblasts in the cell cycle may simultaneously differentiate. Consequently, the apparent sequence of ontogenetic processes is not an immutable, intrinsic neuronal program. How, then, are diverse developmental events coordinated? Our observations indicate that neuroblast mitosis is regulated by a small number of epigenetic factors, including insulin and EGF. Since these signals also influence other processes in developing neurons, epigenetic regulation normally may synchronize diverse ontogenetic events.

DEVELOPMENT of the nervous system proceeds as a complex of well-defined and apparently sequential ontogenetic processes (28, 43). These seemingly discrete processes, including proliferation, cell migration, differentiation, axonal elongation, and synaptogenesis have each been characterized extensively (3, 10, 15, 21, 28, 40–42, 52). Relationships among them, however, are largely undefined due to lack of a suitable experimental model (5, 46, 47, 66). In typical nonneuronal systems, such as developing myoblasts in vitro, relationships have been studied and suggest that differentiation proceeds only after withdrawal of cells from the mitotic cycle (32). In addition, epigenetic signals play a critical role in regulating cell participation in these developmental events (9, 36, 39). However, for neuronal precursors, ontogenetic relationships and regulation are virtually unknown (17, 20, 45, 65, 67).

In vivo, previous extensive study has documented the chronology and geometry of neuroblast generation in many areas of the nervous system (7, 13, 19, 23, 24, 28, 31, 41). Although neuroblast production occurs in apparent excess, precursor mitosis is highly reproducible quantitatively, suggesting that the process is rather tightly constrained (7, 13, 19, 23, 24, 31). Nevertheless, regulatory mechanisms and ontogenetic relationships have been difficult to define in the complex in vivo situation.

To examine relationships in neuronal ontogeny, we have developed a fully defined, dissociated sympathetic neuronal culture system in which precursors or neuroblasts¹ enter the mitotic cycle (14). The embryonic population was identified by visualizing two distinct phenotypic markers immunocytochemically, the catecholamine transmitter enzyme, tyrosine hydroxylase (TH),² and a neuron-specific intermediate filament. Use of the simplified culture system has indicated that neuroblast thymidine incorporation occurs in vitro, and has helped identify specific regulatory factors (14). With further characterization, we have now documented ongoing neuroblast cell division. In turn, we now describe the relationship of DNA synthesis to characteristic mitotic processes, such as karyokinesis and cytokinesis, and define mitotic regulation.

In addition, we have exploited the system to examine the

1. To avoid ambiguity, we define sympathetic precursors as ganglion cells that will give rise to mature neurons. For those precursors still capable of undergoing mitosis, we use the term neuroblast to connote this blastic potential. This definition does not conform to that of Ramón y Cajal, for whom neuroblasts were immature, postmitotic neurons (43).

2. *Abbreviations used in this paper:* CMFSalG, Ca⁺⁺/Mg⁺⁺-free Puck's saline G; DCV, dense-core vesicle; LI, labeling index; NGF, nerve growth factor; TH, tyrosine hydroxylase.

relationship of mitosis to other developmental processes. Our goal is to determine whether aspects of neuronal differentiation constrain, or even prevent mitosis, analogous to observations in developing myoblasts (9, 32, 36, 39). Different neuronal cellular events may influence mitosis in different manners. For example, must neuroblast mitosis cease before axon elongation and synaptogenesis can commence? Does the apparently strict sequence of developmental processes *in vivo* reflect an immutable, intrinsic ontogenetic program? Alternatively, these different ontogenetic activities may be independent, allowing different sequences under different circumstances; the timing and sequence of ontogenetic processes in neuronal development may ultimately depend upon epigenetic influences.

Our observations indicate that sympathetic neuroblasts not only incorporated thymidine, but also underwent cytokinesis, suggesting that cell division itself transpires in culture. Second, neuroblasts in the mitotic population expressed multiple differentiated neuronal traits, including varicose neurites, neurotransmitter vesicles, and even synapses. Consequently, neuroblast mitosis and neuronal differentiation may occur virtually simultaneously. Finally, multiple epigenetic factors, including insulin, EGF, and basic FGF, stimulated DNA synthesis. These studies allow formulation of tentative schemata describing the relationship of mitosis to other developmental events in the nervous system.

Materials and Methods

Preparation of Cultures

Serum-free cultures of dissociated sympathetic precursors or neuroblasts were obtained from superior cervical ganglia of 15.5-d embryonic rats as previously described (14). Briefly, after preincubation in $\text{Ca}^{++}/\text{Mg}^{++}$ -free Puck's saline G (CMFSAIG), ganglia were exposed to trypsin (0.1 mg/ml) for 20 min at 37°C and trypsin inhibitor (0.2 mg/ml) for 5 min at 21°C before gentle trituration in defined medium. Aliquots of dissociated cells were added to poly(D-lysine)-coated, 35-mm culture dishes containing defined medium and fibronectin (5 $\mu\text{g}/\text{ml}$). Defined medium consisted of a 1:1 (vol/vol) mixture of Ham's F12 and DME (Gibco Laboratories, Grand Island, NY) containing penicillin (50 U/ml) and streptomycin (50 $\mu\text{g}/\text{ml}$) and supplemented with transferrin (100 $\mu\text{g}/\text{ml}$) (Calbiochem-Behring Corp., San Diego, CA), putrescine (100 μM), progesterone (20 nM), selenium (30 nM), glutamine (2 mM), glucose (6 mg/ml), guanosine (200 μM), and BSA (10 mg/ml). Unless otherwise stated, insulin was present at 10 $\mu\text{g}/\text{ml}$. EGF was from Collaborative Research (Lexington, MA) and basic FGF was a gift from D. Gospodarowicz (University of California, San Francisco, CA). The following investigators generously supplied other reagents: Drs. C. Russo and M. Weckler, IL-1 and IL-2, and Drs. T. McCaffrey and B. Weckler, PDGF and ECGF (Cornell Medical College, NY); Dr. H. Moses, TGF- β (Vanderbilt University, Nashville, TN). Other materials were obtained from Sigma Chemical Co. (St. Louis, MO) or commercial suppliers. Cultures received growth factors at time zero and were maintained for up to 48 h in a humidified 5% $\text{CO}_2/95\%$ air incubator at 37°C.

Thymidine Incorporation Assays

DNA synthesis was used as a marker for cells in the mitotic cycle (2, 51). After addition of [^3H]thymidine (1 $\mu\text{Ci}/\text{ml}$) at 24 h of incubation, incorporation into culture acid precipitates on filter paper disks was assayed at 48 h by scintillation spectroscopy as previously described (14). For experimental blanks, cultures were incubated with isotope for 1 min.

Morphologic analysis of [^3H]thymidine incorporation was performed by determining the labeling index (LI). After incubation with [^3H]thymidine as above, cultured cells were processed for combined immunocytochemical visualization of neurotransmitter enzyme, TH, and autoradiography as previously described (14). The LI represents the proportion of the TH-positive population that specifically incorporated thymidine in the nucleus. For experimental groups of three dishes, 100 TH-positive cells in three or four

random, nonoverlapping areas were scored (at 400 \times) for silver grains on each dish, until 1,000 cells were counted. A Leitz Orthoplan microscope equipped with incident illumination for fluorescence and transmitted light for visualization of silver grains was used.

Cell Counting and Time-lapse Photography

Living cultures were examined with a Wild inverted phase microscope at 200 \times . Cells in 3% of the culture dish area were counted as described (14). For each of three time-lapse experiments, three to five randomly selected fields in three dishes were serially photographed at $\sim 4, 8, 16, 24,$ and 42 h. Photographic fields were located by microscope stage micrometer ordinates and scalpel marks incised into culture dish surfaces.

Flow Cytometry

Groups of 50–60 ganglia were treated with trypsin and trypsin inhibitor as above, and triturated in 1.8 ml of CMFSAIG. After large pieces settled, the upper three-quarters of dissociate was transferred and centrifuged at 1,000 rpm for 10 min. The cell pellet was resuspended in 200 μl of CMFSAIG and diluted with an equal volume of CMFSAIG containing 0.015% Triton X-100. 30 s later, 100 U of RNase A (Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN) was added in a 100 μl volume and cells were incubated for 20 min at 37°C. Subsequently, 500 μl of CMFSAIG containing propidium iodide and BSA was added, yielding final concentrations of 5 $\mu\text{g}/\text{ml}$ and 10 mg/ml, respectively. In two separate experiments, measurements were performed on an Epics V cell sorter interfaced to an Easy 88 computer (Coulter Electronics Corp., Hialeah, FL) operated by the Cornell University Medical College Flow Cytometry Core Facility as previously described (18).

Electron Microscopy

Cells maintained in culture for 24 and 48 h were examined with conventional electron microscopy. Cells were plated either into culture dishes as described above or into dishes fitted with a glass coverslip according to the method of Bray (4). Three different fixative procedures were used. (a) For 24-h cultures, cells were fixed in 2% glutaraldehyde and postfixed in 2% osmium tetroxide. (b) For 48-h cultures, cells were fixed in 2.5% glutaraldehyde, 2.5% paraformaldehyde, and 0.05% picric acid (27), and postfixed in a solution of 1% osmium tetroxide and 1.5% potassium ferrocyanide (29), and en bloc stained with uranyl acetate. (c) Alternatively, some cultures were prepared by a modified chromaffin procedure to optimize visualization of biogenic amine-containing vesicles (61): cells were fixed in 1% glutaraldehyde, 0.4% formaldehyde and 0.1 M sodium chromate/potassium dichromate, then stored in 0.2 M sodium chromate/potassium dichromate, and postfixed in a solution of 2% osmium tetroxide and 0.1 M sodium chromate/potassium dichromate. Dehydration and embedding were performed in the plastic dishes by omitting the use of propylene oxide as previously described (35). Thin sections, stained with uranyl acetate and lead citrate, were examined in a JEOL 100CXII electron microscope.

Results

Characterization of Neuroblast Mitosis in Culture

To study neuronal ontogeny, we used a virtually pure neuronal cell culture system in which presumptive precursors enter the mitotic cycle (14). Cultures were derived from dissociated superior cervical sympathetic ganglia of 15.5-d embryonic rats and were grown in a fully defined, serum-free medium (14). Previous study indicated that neuroblasts in this system incorporated [^3H]thymidine, a marker for DNA synthesis (2, 14, 51). Is thymidine incorporation by neuroblasts *in vitro* accompanied by other characteristic mitotic processes such as cytokinesis? Phase microscopic examination of the living cultures revealed numerous phase-bright round cells occurring as singlets and doublets (Fig. 1, *a* and *b*), suggesting that single cells were dividing.

To determine whether cultured neuroblasts were undergoing cytokinesis, we performed time-lapse photography. 23%

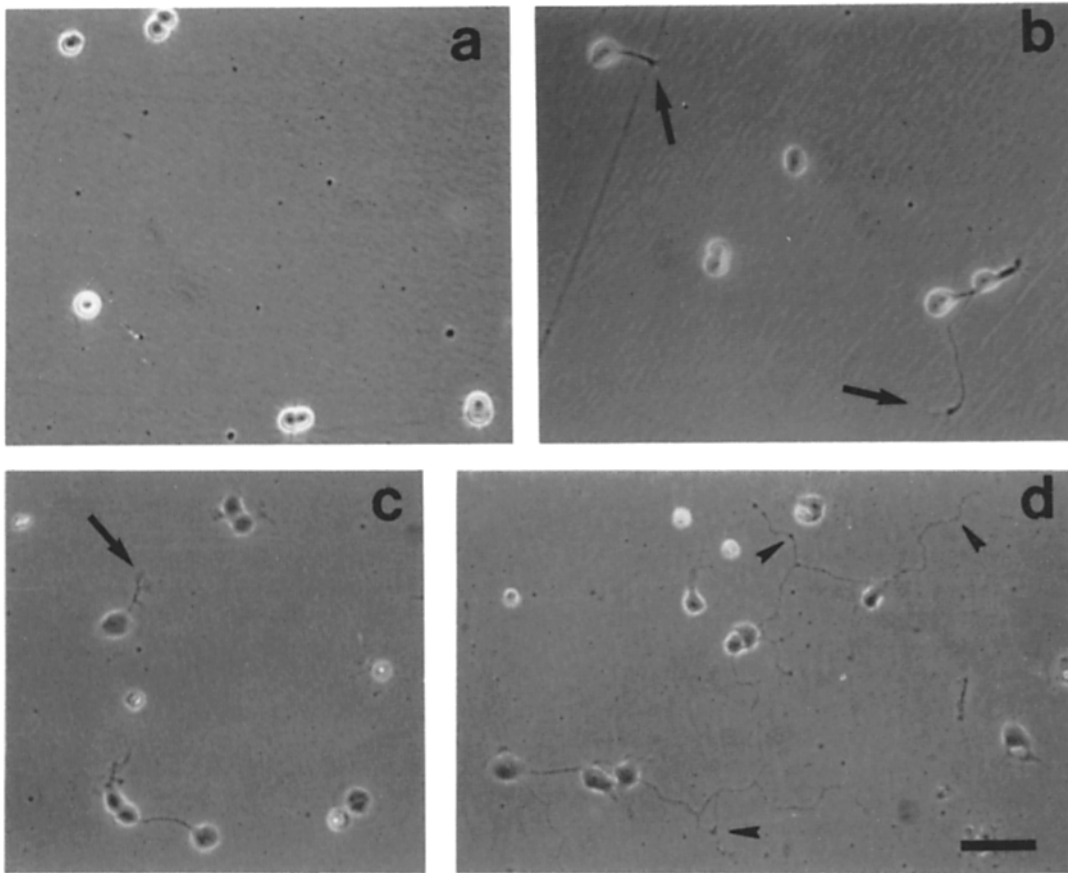


Figure 1. Behavior of sympathetic neuronal precursors in culture. Sympathetic ganglia at embryonic day 15.5 were dissociated and cultured in fully defined, serum-free medium. Living cultures were photographed at 5 (a), 12–20 (b), 24 (c), and 48 h (d). At early times, cells appear as singlets and doublets (a and b). Characteristic neuritic processes terminating in growth cones (arrows) are elaborated during the first day of culture (b and c). Processes progressively elongate and demonstrate typical varicosities (arrowheads) by 48 h (d). Bar, 50 μ m.

of the cells first observed at 4 h had divided by 48 h (Fig. 2), indicating that presumptive neuroblasts underwent cell division *in vitro*. Since previous work indicated that $\sim 30\%$ of cultured neuroblasts incorporated thymidine (reference 14; see also Fig. 11), our observations suggested that precursors participated in all stages of the mitotic cycle *in vitro*. To directly examine this question, pulse-labeling experiments were performed. After a 2-h [3 H]thymidine pulse at 4 h of culture, cells were further incubated for 1 or 2 d and subsequently processed for combined TH-immunocytochemistry and autoradiography. TH, the rate-limiting enzyme in catecholamine biosynthesis, served to identify these cells as neuronal precursors (14). Indeed, cultures exposed to a [3 H]thymidine pulse at 4 h exhibited silver grain-labeled doublets at later times of incubation (Fig. 3). Moreover, addition of colchicine (2 μ g/ml) after the pulse, at a dose that blocked neurite outgrowth but not total [3 H]thymidine incorporation, inhibited doublet formation by $> 75\%$ (data not shown). These observations indicate that a population of neuroblasts in the culture synthesized DNA and subsequently divided.

Relationship of Neuroblast Division *In Vitro* to Development *In Vivo*

Although developmental studies *in vivo* suggest that sympathetic precursors are dividing at this gestational age (26, 48), tissue culture conditions may artificially induce mitosis

in some populations (2). To establish the relationship of neuroblast division *in vitro* to mitosis *in vivo*, freshly dissociated ganglion cells were analyzed by flow cytometric analysis for relative DNA quantity (2, 18). Cells that are synthesizing DNA (S phase) or that have completed chromosome replication (G_2) have a $> 2n$ complement of cellular DNA (2). The results demonstrated that 17–19% of the dissociated cells were in the S phase while $\sim 10\%$ had completed chromosome replication (Fig. 4), indicating ongoing mitosis *in vivo*. The occurrence of mitosis *in vivo* suggested that neuroblast division in culture faithfully reflected developmental events in the animal. Further, since cells were in all stages of the cell cycle at plating, a similar diversity of mitotic stages was likely to be present *in vitro*. Moreover, there was a striking correlation of the percentage of cycling cells *in vivo* with that *in vitro*: the proportion of mitotic cells *in vivo* approximated the percent undergoing division in culture. Finally, a similar percentage of cells entered the mitotic cycle on day 2 of culture, as indicated by [3 H]thymidine labeling (Fig. 11; also see reference 14), suggesting that autoradiography reliably estimates mitosis during neuronal ontogeny.

Relationship of Neuroblast Mitosis to Process Elaboration

The occurrence of mitosis in culture permitted study of the relationship of cell division to other developmental events,

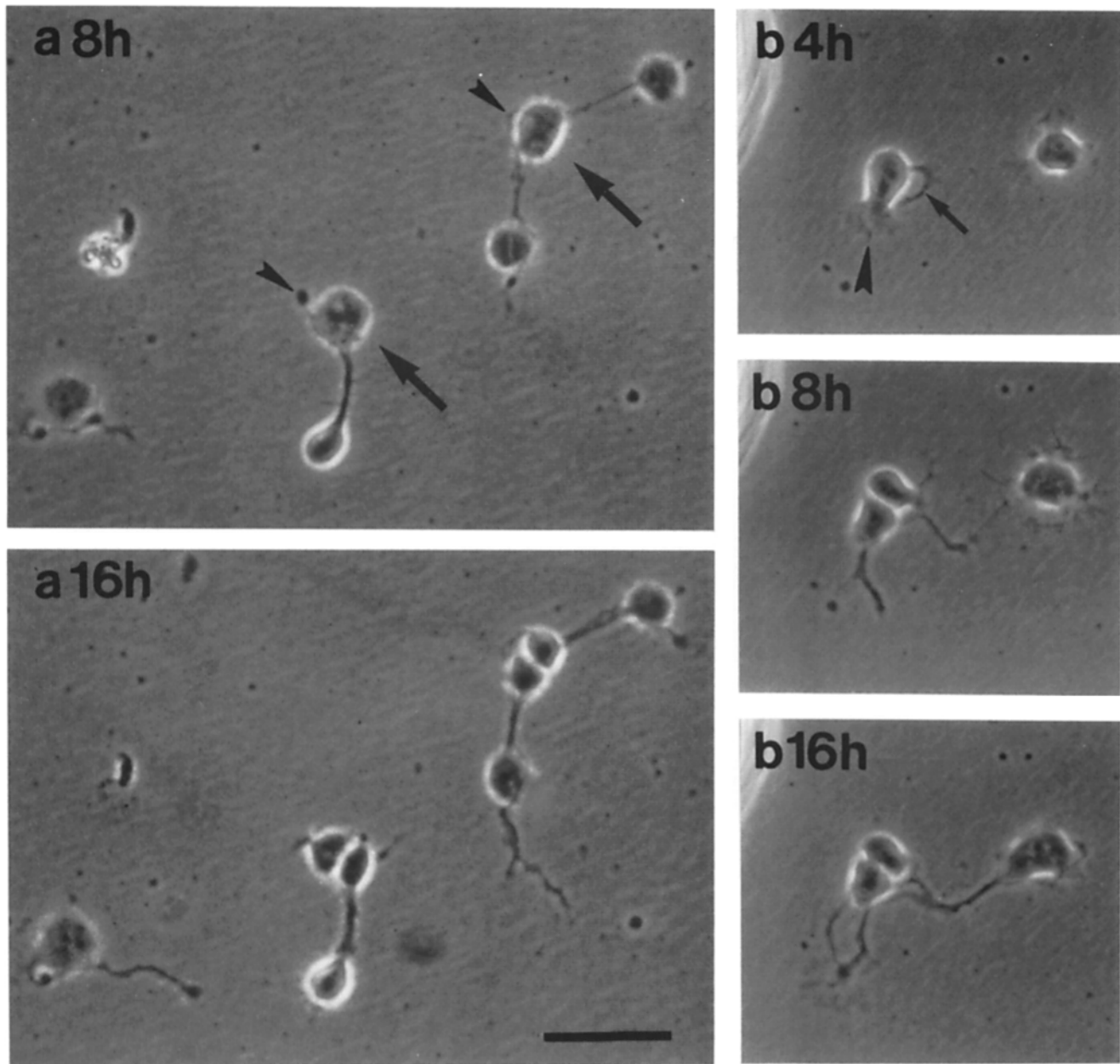


Figure 2. Division of presumptive neuroblasts in culture. Serial time-lapse photographs were taken of randomly selected fields. (a) Two cells observed at 8 h (arrows) have divided by 16 h. Note the presence of small cytoplasmic extensions apparent both before and after division (arrowheads). (b) A cell exhibiting a small process (arrow) and a cytoplasmic lamella (arrowhead) at 4 h has divided to yield two cells bearing processes at similar locations by 8 h. At 16 h, these processes have continued to elongate. A total of 216 cells were observed in three time-lapse experiments as described in Materials and Methods. 50 of these cells or 23% had divided by 42 h. Bar, 50 μ m.

such as neurite elaboration. Initial observations indicated that young neuroblasts extended characteristic varicose processes in culture, increasing in length over time (Fig. 1, *b-d*). Further, there was a progressive increase in the proportion of process-bearing cells during incubation, suggesting that newly generated neuroblasts also elaborated neurites.

To determine whether dividing neuroblasts also extended processes, we examined time-lapse data. Indeed, many of the cells that underwent cytokinesis also exhibited processes (Fig. 2). Further, both cell division and process elaboration

occurred within 4–8 h, indicating that these events were not mutually exclusive, at least within this brief period (Fig. 2). In addition, in some instances, small processes observed before division, were also present after cytokinesis, suggesting that mitosis and neuritogenesis may occur virtually simultaneously in neuroblasts. Do these observations indicate the coincidence of mitosis and differentiation, or simply an aberrant response of cultured cells to an artificial substratum? To address this issue, we examined a range of different neuronal features, including neurotransmitter phenotypic expression.

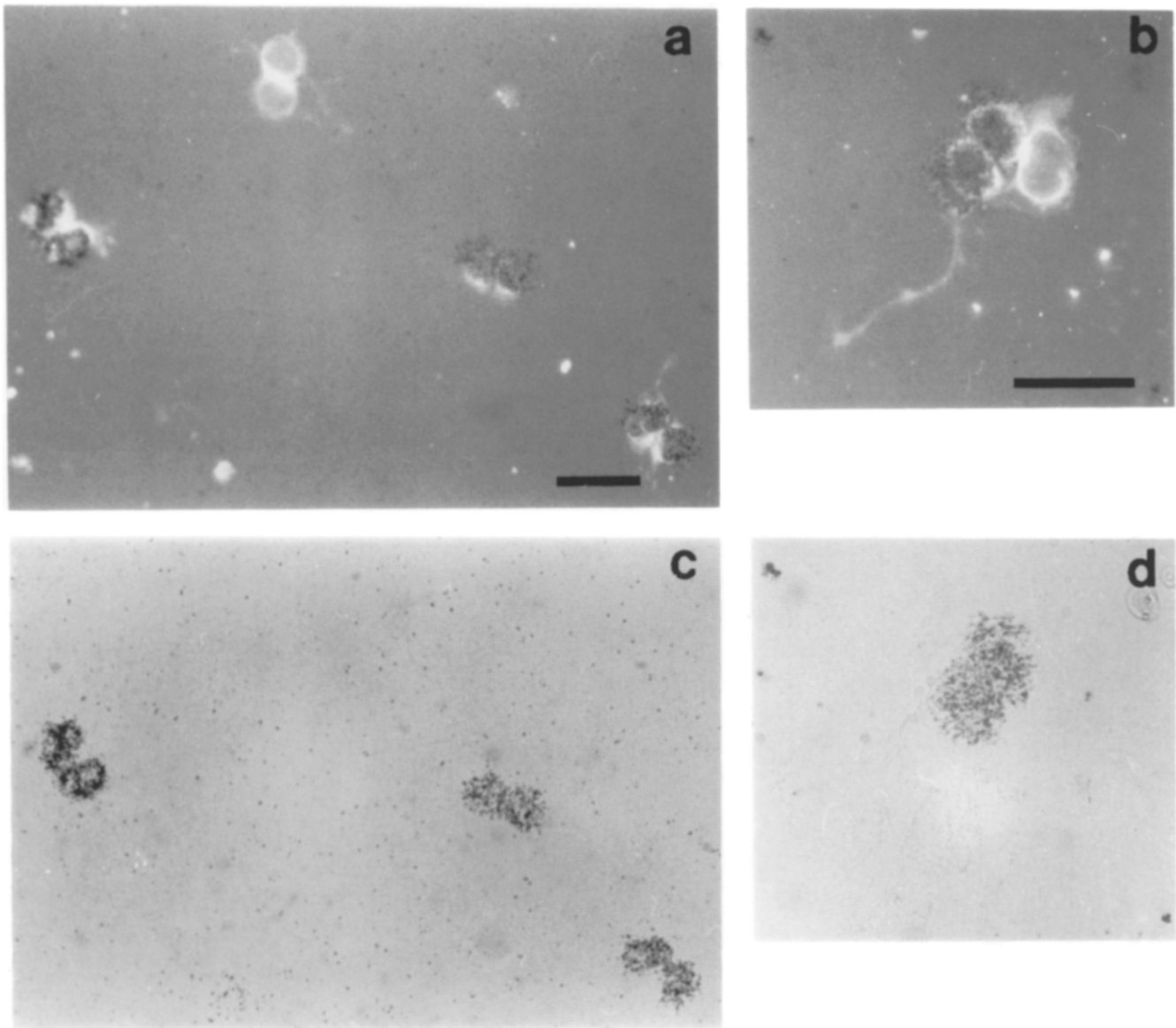


Figure 3. TH-positive neuroblasts incorporate [^3H]thymidine and subsequently divide in culture. Cells were cultured as in Fig. 1. At 4 h of incubation, a pulse of [^3H]thymidine (1 $\mu\text{Ci/ml}$) was added to media for 2 h, after which cultures were washed twice with SalG, and fed fresh medium. After 1- or 2-d incubation, cultures were fixed and processed for combined TH-immunocytochemistry and autoradiography. Cultures were viewed with epifluorescent (a and b) or brightfield (c and d) illumination. At low magnification, four neuroblast doublets exhibiting intense, cytoplasmic TH-immunofluorescence are shown (a). Three of these doublets also exhibit silver grain-labeled nuclei (c). At high magnification, a single TH-positive cell bearing a neuritic process lies next to a doublet, composed of two smaller cells (b) that exhibit nuclear silver grains (d). Production of >75% of doublets in culture was prevented by colchicine (2 $\mu\text{g/ml}$) addition after the [^3H]thymidine pulse. This drug dose completely inhibited neurite outgrowth but had no effect upon [^3H]thymidine incorporation (data not shown). Bar, 25 μm .

Ultrastructural Characteristics of Cultured Neuroblasts

Previous work *in vivo* (47, 48) and *in vitro* (14, 17) indicated that neuronal precursors in the mitotic cycle expressed a neurotransmitter phenotypic character, TH. In combination with the present data on process elaboration, these observations suggest that mitosis and neuronal differentiation were occurring simultaneously. To more precisely define the range of neuronal characteristics expressed by this dividing population, we examined cultures at the ultrastructural level

by electron microscopy. We specifically examined for neuritic processes, growth cones, varicosities and characteristic dense-core vesicles (DCV), known to store the sympathetic catecholamine neurotransmitter (22, 34, 61).

At 24 h, cell profiles exhibited characteristics of immature neuroblasts (Fig. 5) (16, 55, 58). The cytoplasm was filled with abundant free ribosomes and mitochondria as well as glycogen granules, rough endoplasmic reticulum, and a small Golgi apparatus (Fig. 5). A search for DCVs revealed only six vesicular profiles in the 18 cells examined. DCVs contained a centrally located granular density surrounded by

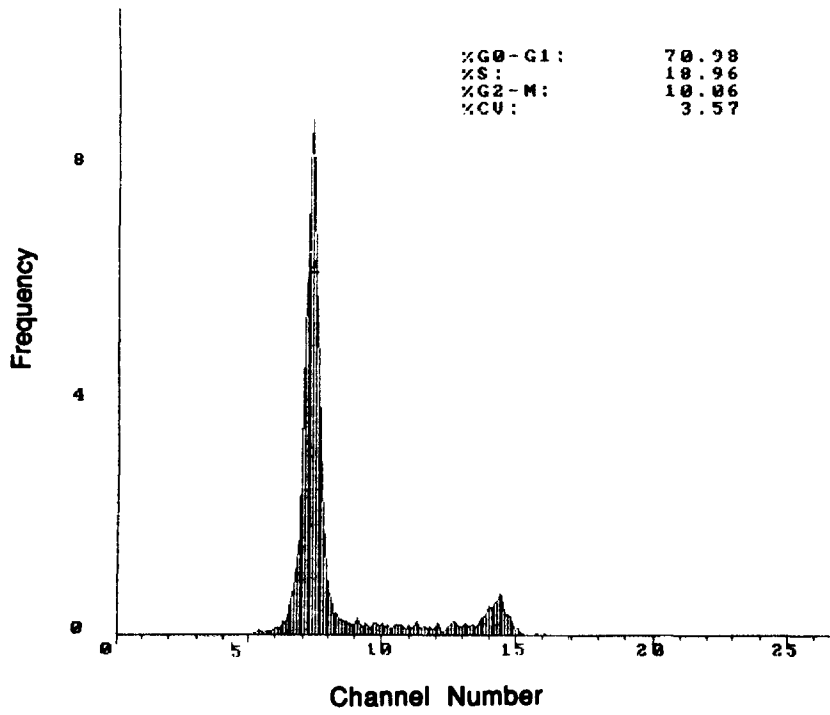


Figure 4. Flow cytometric analysis of freshly dissociated ganglion cells. After propidium iodide staining of cellular DNA, fluorescence intensity (channel number) was plotted against frequency. Approximately 29% of cells have greater than a G₁ quantity of DNA, indicating that they are in the mitotic cycle. Percent CV indicates coefficient of variance.

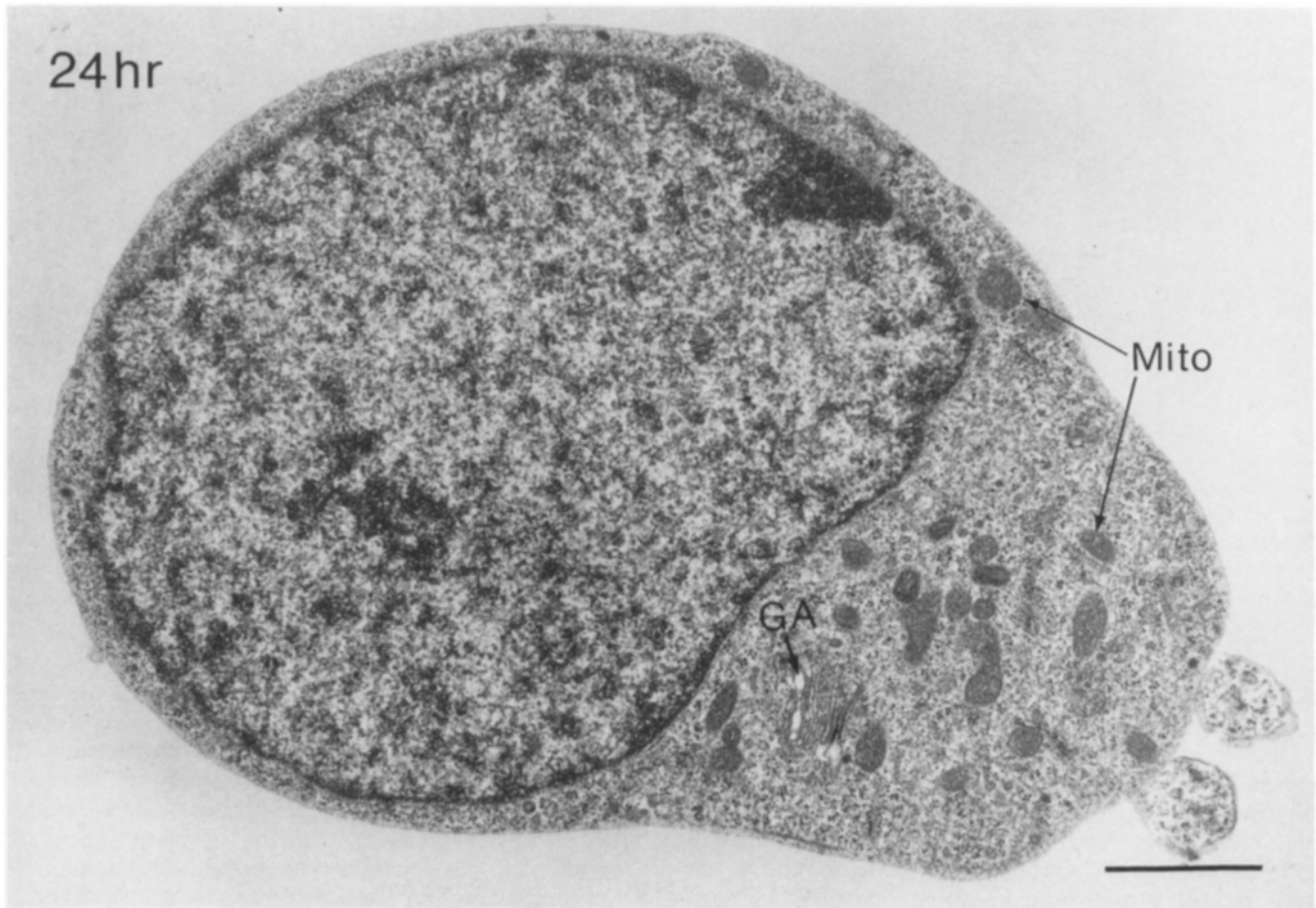


Figure 5. Neuroblast ultrastructure after 24 h in culture. The cytoplasm is filled with free ribosomes and numerous mitochondria (*Mito*); a small Golgi apparatus (*GA*) is also present. Bar, 1 μ m.

a less dense halo (16, 22, 34, 55, 58, 61). Rarely, we observed collections of small clear vesicles (40–60 nm). Thus, after 24 h of incubation, cultures were composed of immature cells that rarely contained specific neuronal vesicles, consistent with descriptions of neuronal precursors of similar age *in vivo* (55, 58).

In marked contrast, by 48 h, the large majority of cells exhibited specific features of neuronal differentiation, including DCVs, varicose neuritic processes and neurotransmitter release sites. Indeed, in 49 cells examined in random thin sections, a full 83% contained DCVs measuring 90–130 nm (Fig. 6). DCVs were found in the peripheral cell cytoplasm, often juxtaposed to the plasmalemma, as well as localized near the Golgi complex, which was now much enlarged (Fig. 6). Moreover, evidence of dense vesicular forms budding from Golgi cisternae suggested that the appearance of DCVs in cultured neuroblasts was due to ongoing vesicular synthesis (Fig. 6).

Further, neuroblasts elaborated highly specialized neuritic processes at 48 h. Processes tended to fasciculate with one another and many were varicose (Fig. 7). Profiles of nonvaricose neurites were filled with microtubules, intermediate filaments, endoplasmic reticulum, and occasional DCVs. Varicosities contained large aggregates of clear and dense-core synaptic vesicles as well as mitochondria and some cytoskeletal elements (Fig. 7), and thus exhibited the characteristic morphology of sympathetic transmitter-release sites documented *in vivo* (22, 34, 61).

Additional striking evidence of neuron-specific development in this mitotic population was apparent. Not only were previously observed growth cones (Figs. 1 and 2) visualized ultrastructurally, but we also found evidence of early synapse formation (Fig. 7). In two process-to-process contacts, we observed regions consisting of closely apposed plasma membranes associated with cytoplasmic densities and asymmetric collections of clear and dense-core vesicles (Fig. 7).

We found no evidence of contaminating small intensely fluorescent cells. These presumptive interneurons (57), characterized by cell bodies containing numerous pleomorphic DCVs of variable diameter (90–300 nm) (16, 53–55, 57, 58), represent a minor subpopulation most abundant in neonatal ganglia (33). Large accumulations of DCVs were not observed in the soma of cultured ganglion cells (Fig. 6). In addition, cultured cells did not exhibit features of fibroblasts or Schwann cells, suggesting that other cell types either did not survive or failed to differentiate under the present culture conditions.

Effect of Insulin on Neuroblast DNA Synthesis: Specificity and Selectivity

To ascertain whether epigenetic factors potentially synchronize expression of differentiated neuronal characters and mitosis, several putative agents were studied. Specifically, we examined insulin, a factor known to regulate neuroblast mitosis (14, 65), neuritogenesis (44), and synapse formation (30, 64), comparing the peptide to a number of molecules localized to the nervous system and known to be mitogenic for other cell types (Table I). In fact, the large majority of known mitogens failed to stimulate embryonic neuroblasts (Table I), suggesting that insulin is a highly specific mitogenic signal for neuronal precursors.

Table I. Effects of Mitogens on Neuroblast [³H]Thymidine Incorporation

Agent	Concentration used	Stimulation*
Basic FGF	0.25–5 ng/ml	Yes
EGF	0.1–100 ng/ml	Yes
Transforming growth factor β	0.05–30 ng/ml	No
Platelet-derived growth factor	5–25 ng/ml	No
Endothelial cell growth factor	0.01–10 μ g/ml [‡]	No
Interleukin 2	20 U/ml	No
Interleukin 1	1–1,000 U/ml	No
Substance P	10 ⁻⁹ –10 ⁻⁵ M [§]	No
Bombesin	10 ⁻⁸ –10 ⁻⁵ M [§]	No
Met-enkephalin	10 ⁻⁷ –10 ⁻⁵ M	No
Thyroxine	10 ⁻¹¹ –10 ⁻⁷ M	No
Dexamethasone	10 ⁻⁸ –10 ⁻⁵ M	No
Others		No

* Incorporation of [³H]thymidine was tested in the absence and presence of insulin and was assayed by scintillation spectroscopy.

[‡] Tested in the presence of 1 μ g/ml heparin.

[§] Also tested in the presence of protease inhibitors.

^{||} The following agents, tested at maximal concentrations of 10⁻³–10⁻⁵ M according to availability, were also without effect: cholecystokinin, growth hormone, carbamylcholine, nicotine, isoproterenol, phenylephrine, and metaraminol.

Theoretically, insulin stimulation of DNA synthesis may have reflected a nonspecific increase in overall metabolism. Consequently, we examined the effect of the hormone on another cellular process, bulk protein synthesis. Insulin had no effect on leucine incorporation, suggesting that insulin did not simply stimulate all metabolic processes (Fig. 8). In contrast, nerve growth factor (NGF), the trophic protein critical for survival and differentiation of more mature sympathetic neurons (12, 59), elicited a fivefold increase in leucine incorporation. Thus, neuroblasts remained responsive to metabolic signals in the absence of insulin (Fig. 8). Further, NGF, a molecule known to influence overall metabolism, failed to replicate the mitogenic effects of insulin (14). In sum, these observations suggest that insulin specifically and selectively stimulated DNA synthesis in these cultured neuroblasts.

Effect of EGF And FGF on Neuroblast Mitosis

Regulation of the sympathetic mitotic cycle by insulin indicates that peripheral neuroblast division is responsive to extracellular factors, as recently demonstrated for central precursors (20, 45, 65, 67). In addition to insulin, two other epigenetic signals known to influence neuronal development, EGF (37) and FGF (25, 38, 62, 63), also appear to play a role (Table I). EGF elicited approximately an 80% rise in [³H]thymidine incorporation (Fig. 9). EGF was highly potent, with an EC₅₀ of 0.5 ng/ml, and a plateau at 5–10 ng/ml (data not shown), consistent with actions via its own receptor (6).

To define the mechanism by which EGF stimulated DNA synthesis, we determined the LI. EGF treatment increased the LI by 37%, indicating that the factor increased the proportion of neuroblasts entering the S phase (Fig. 10). Although EGF and insulin synergistically stimulate nonneuronal mitosis (6), maximal effects were not additive for neuroblasts (Fig. 11), suggesting that the factors share some common cellular mechanism(s).

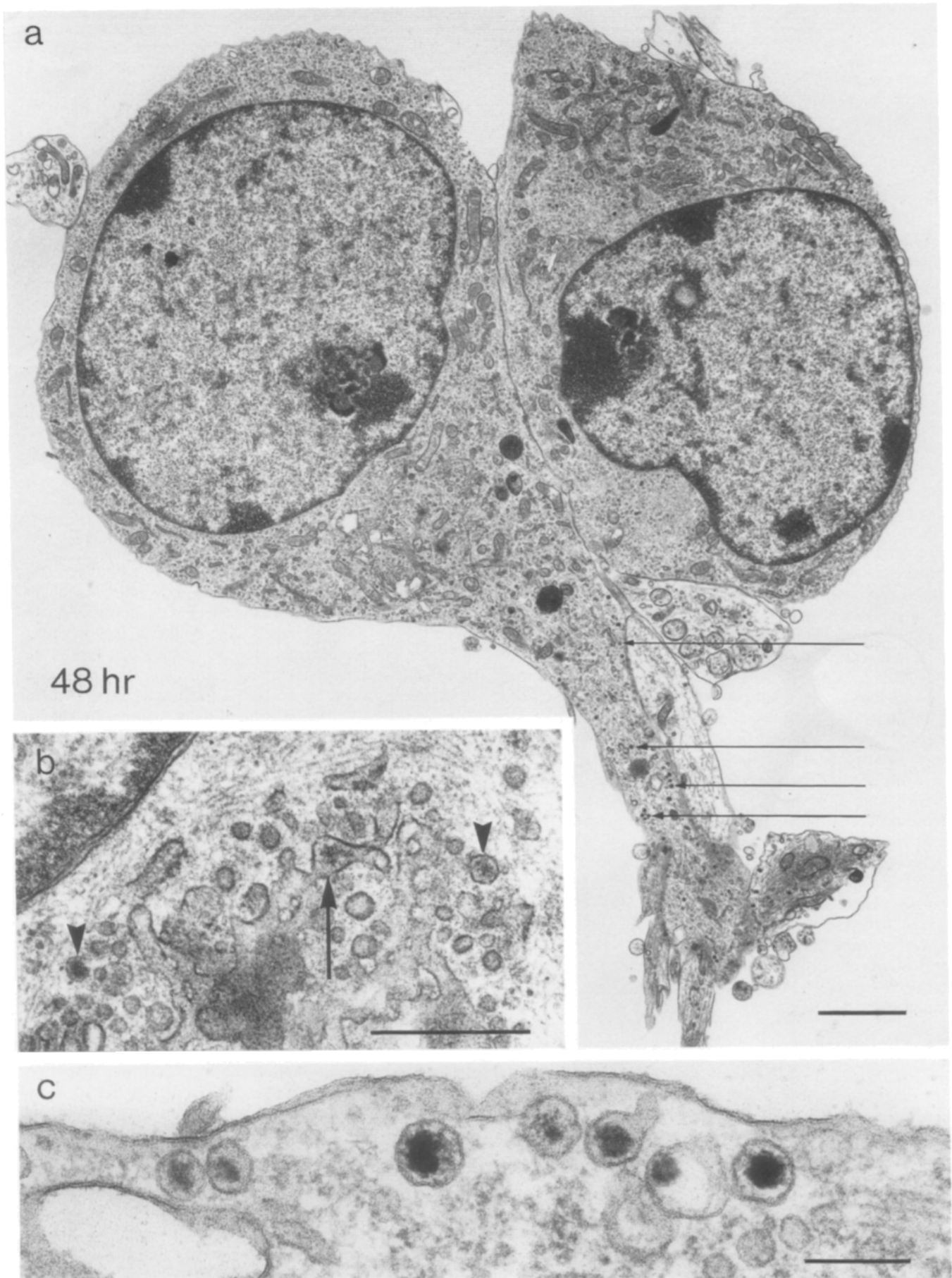


Figure 6. Neuroblast ultrastructure after 48 h in culture. (a) Both cells of this doublet have a cytoplasm rich in ribosomes and mitochondria; in addition, the cells have extended processes. Small numbers of DCVs are observed in the cell somas. In one process, numerous DCVs are present (arrows). Aldehyde fixation. (b) DCVs are also found near the Golgi apparatus (arrowheads). The accumulation of dense granular material in an expanded Golgi cisterna indicates the formation of a DCV (arrow). Aldehyde fixation. (c) DCVs measure 90–130 nm in diameter. Note that the size of the dense core is quite variable. Here the vesicles accumulate along the plasma membrane of a cell soma. Chromate fixation, used here to optimize preservation of DCVs (37), did not alter the quantity or quality of vesicles visualized. Bars: (a) 2 μm ; (b) 0.5 μm ; (c) 200 nm.

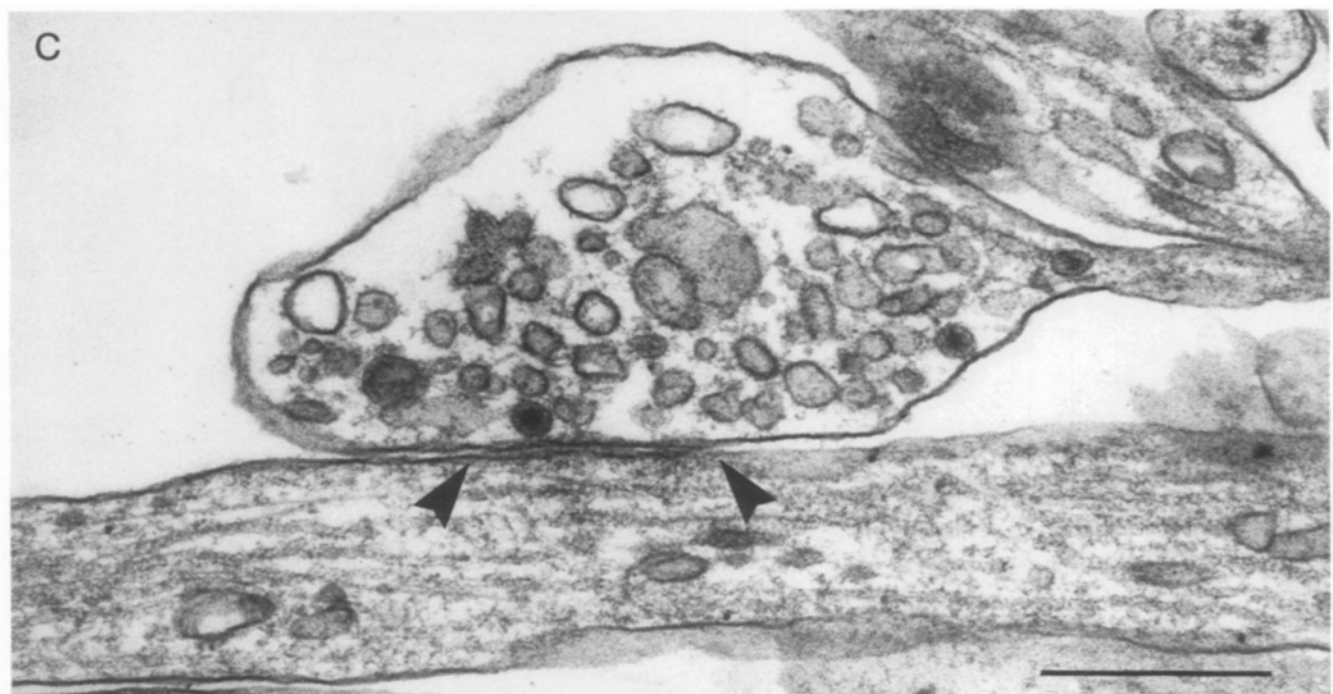
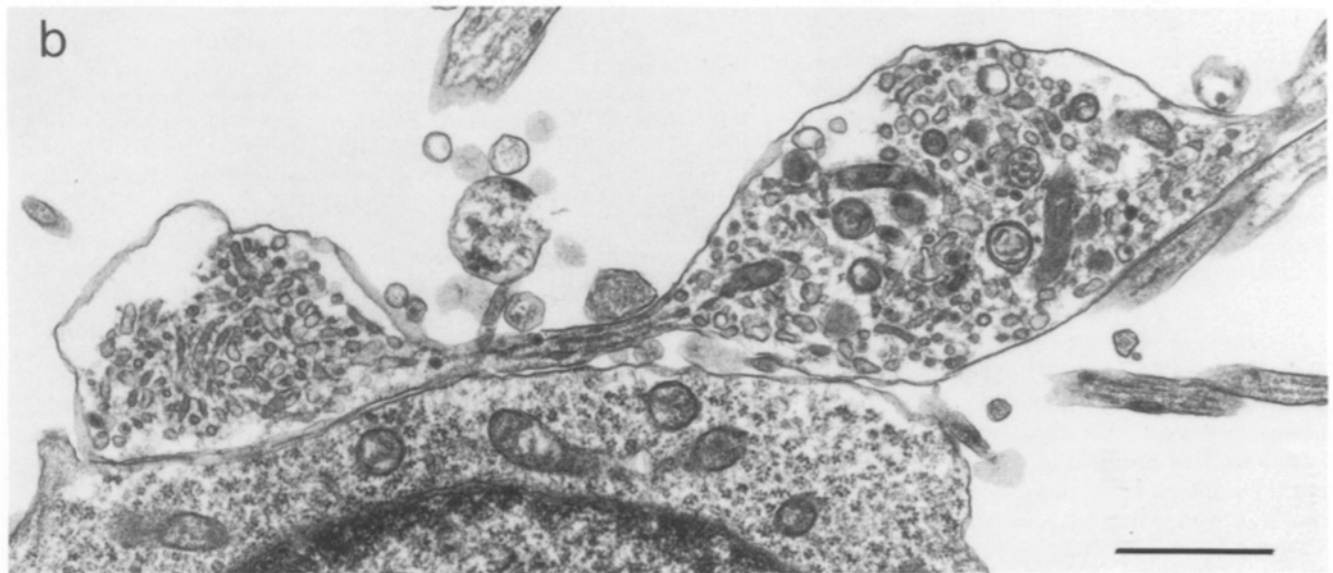
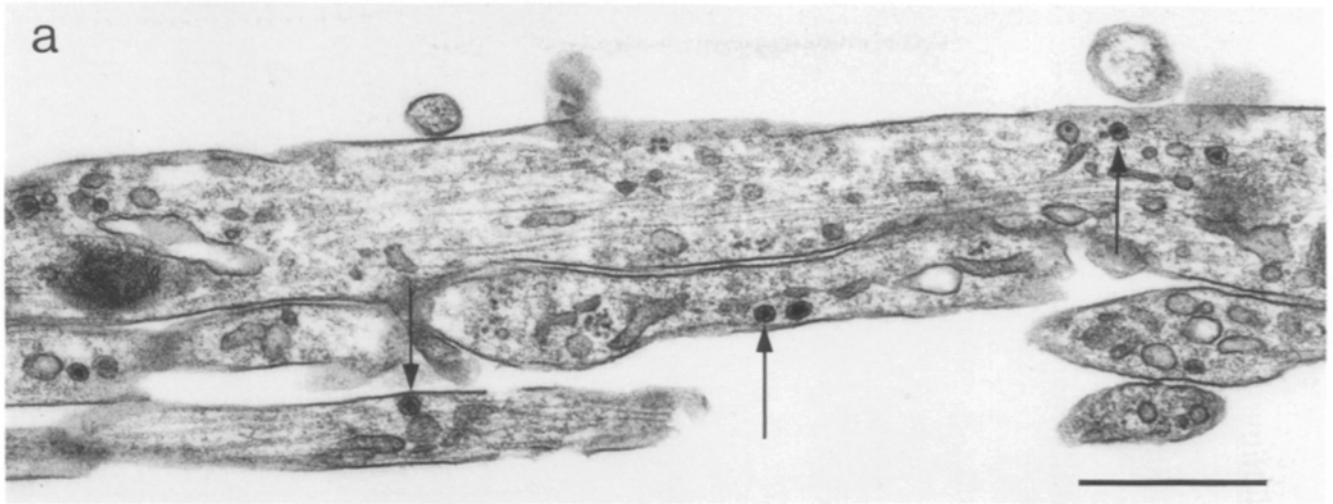


Figure 7. Ultrastructure of neuritic processes after 48 h in culture. (a) Neuritic processes fasciculate in culture and contain both microtubules and intermediate filaments. Smooth-surfaced cisternae as well as clear and dense-core vesicles (arrows) are present. (b) Two varicosities are filled with clear and dense-core vesicles and other irregular membrane profiles but few cytoskeletal elements; one terminal also contains some mitochondria. (c) A synaptic contact has formed between two processes (arrowheads). The presynaptic terminal contains both clear and dense-core vesicles. The postsynaptic membrane has a diffuse density along its cytoplasmic surface. Bars: (a and b) 1 μm ; (c) 0.5 μm .

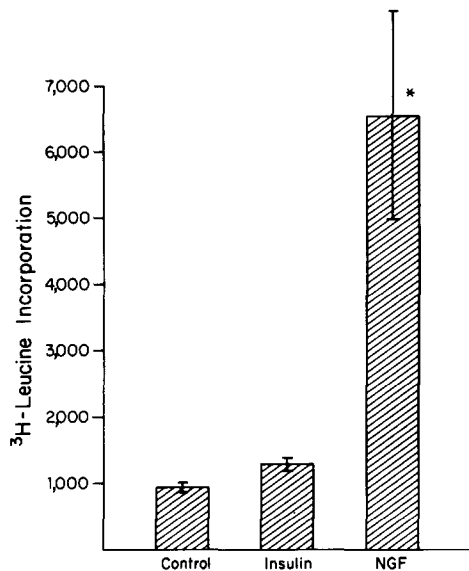


Figure 8. Insulin does not stimulate protein synthesis. Cells were cultured without insulin (control), or with insulin (10 $\mu\text{g/ml}$) or NGF (10 biological U/ml) for 48 h. Procedures for assaying incorporation of [^3H]leucine were the same as for [^3H]thymidine (Materials and Methods), except that isotope addition at 24 h was 5 $\mu\text{Ci/ml}$ of [^3H]leucine. Each experimental value represents the mean incorporation of three culture dishes (calculated after subtracting blank, range :25–50) and is expressed as cpm \pm SEM (vertical bar). (*) Differs from control at $P < 0.005$.

The effects of FGF differed markedly from those of EGF. FGF stimulated neuroblast [^3H]thymidine incorporation more than twofold (Fig. 12) with an EC_{50} of 0.25 ng/ml (data not shown). Although FGF stimulated DNA synthesis, the factor did not alter the LI (Fig. 12), suggesting that the factor served a trophic but not mitogenic role for neuroblasts. Regardless of underlying mechanisms, however, these observations suggest that of many potential agents (Table I), only specific epigenetic signals interact to regulate neuroblast mitosis.

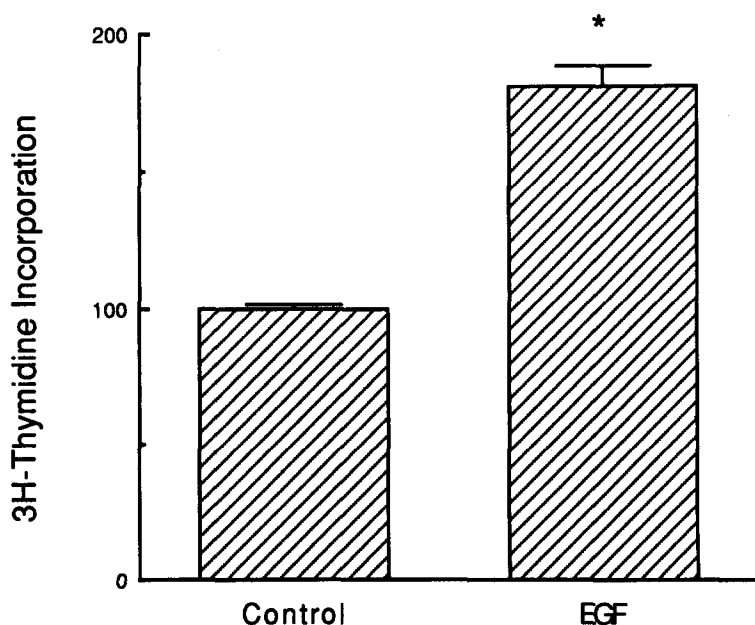


Figure 9. EGF stimulates DNA synthesis. Cells were cultured without insulin (control) or with EGF (20 ng/ml) and assayed for [^3H]thymidine incorporation. Data are the sum of two experiments and are expressed as percent of control, mean \pm SEM (vertical bar). (*) Differs from control at $P < 0.0005$.

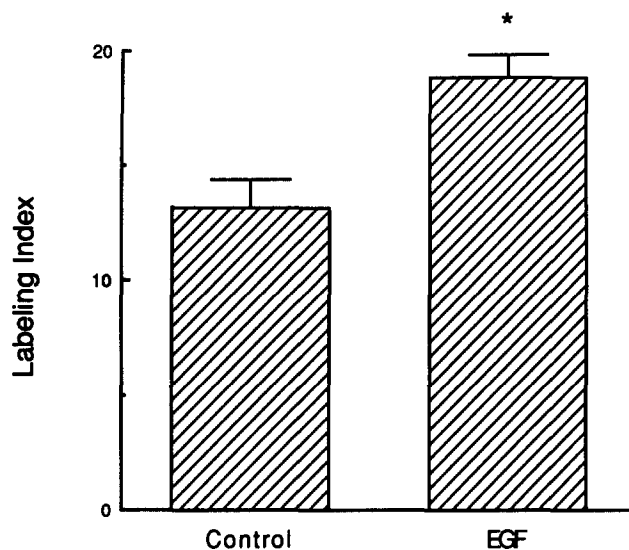


Figure 10. EGF stimulates the LI in cultured neuroblasts. Cells plated as sister cultures to those described in Fig. 9 were processed for combined TH-immunocytochemistry and autoradiography as in Fig. 3. The percentage of TH-positive cells incorporating [^3H]thymidine in the nucleus (LI) was assessed. The data are the sum of two experiments and are expressed as mean percent \pm SEM. (*) Differs from control at $P < 0.025$.

Discussion

Although neuron generation is precisely regulated during ontogeny, little is known about underlying mechanisms (7, 13, 17, 19, 20, 23, 24, 31, 45, 65, 67). In addition, relationships between precursor proliferation and other developmental phenomena, such as process elaboration, transmitter expression, and even synaptogenesis, remain unknown. We have used a fully defined neuronal cell culture system to examine sympathetic neuroblast mitosis. Our observations indicate that neuroblasts not only synthesized DNA, but also underwent cytokinesis in culture, allowing analysis of mi-

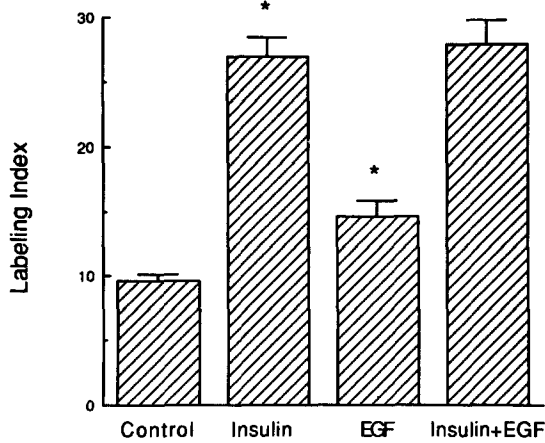


Figure 11. Effects of combined mitogens on neuroblast LI. Cells were cultured without insulin (control) or in medium containing insulin and EGF individually or in combination. Cultures were assayed and data are expressed as in Fig. 10 (*) Differs from control at $P < 0.025$.

otic regulation and developmental relationships. The population expressed a wide array of neuron-specific characteristics, including extension of neuritic processes with growth cones, synthesis and transport of transmitter vesicles, and organization of transmitter release sites. These data suggest that neuroblasts in the cell cycle may simultaneously differentiate. How, then, are diverse developmental events coordinated? Our observations indicate that neuroblast mitosis is regulated by a small number of epigenetic factors, including insulin and EGF. Since these signals also influence other processes in developing neurons, epigenetic regulation normally may synchronize diverse ontogenetic events.

Neuroblast Mitosis in Culture

Our results indicate that sympathetic neuroblasts underwent cell division in fully defined culture conditions. Several lines

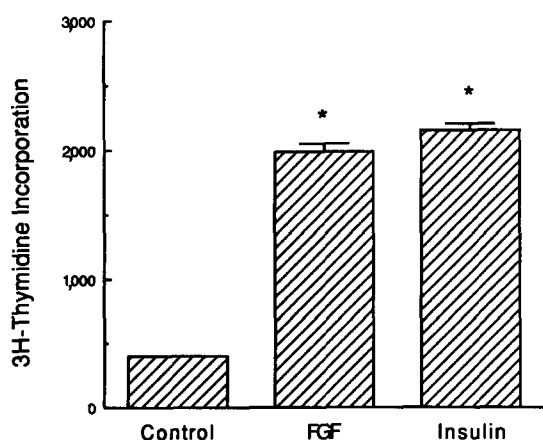


Figure 12. FGF stimulates DNA synthesis. Cells were cultured without insulin (control) or with insulin or FGF (1 ng/ml) and incorporation was assayed. Data are expressed as in Fig. 8. (*) Differs from control at $P < 0.005$. Examination of the LI in sister cultures indicated that FGF did not alter the percent of neuroblasts in the mitotic cycle (control = 6 ± 0.73 ; insulin = 16 ± 1.07 ; FGF = 6.2 ± 1.2 ; expressed as mean percent \pm SEM).

of evidence support this conclusion. First, we observed cells in various stages of nuclear mitosis. Second, time-lapse analysis demonstrated ongoing cytokinesis. Finally, neuroblast behavior was not simply restricted to a single mitotic process in culture, such as DNA synthesis. Rather, neuroblasts that synthesized DNA *in vitro* subsequently underwent cell division as well, as indicated by [3 H]thymidine pulse autoradiography.

Although our data indicate ongoing neuroblast division, we do not detect increased cell numbers in culture (14; unpublished observations). During this developmental period, neurogenesis in the ganglion is accompanied by naturally-occurring neuronal death (12, 13, 26, 40). "Trophic" agents, such as NGF, may alter the course of postnatal neuronal death *in vivo* and *in vitro*, though the embryonic population of neurons is only variably responsive to this factor (3, 12, 26, 59). In recent preliminary experiments, addition of trophic molecules to cultures prevents neuronal death, allowing detection of increased cell numbers.

Relationship of Neuroblast Mitosis *In Vitro* to Division *In Vivo*

Neuroblast division in culture accurately reflected developmental events in the animal: flow cytometry indicated that freshly dissociated cells were in different stages of the mitotic cycle. Nevertheless, mitosis *in vitro* did not merely represent continuation of events initiated *in vivo*. For example, although 10% of cells *in vivo* had completed chromosome replication (G_2), twice that number divided in culture. Further, [3 H]thymidine pulse-labeling indicated that some neuroblasts traversed the entire cell cycle *in vitro*. Finally, additional neuroblasts were recruited into the mitotic cycle on day 2 of culture consequent to growth factor treatment. Thus, use of this model system may allow identification of neuroblast mitotic signals and developmental relationships.

Mitotic Neuroblasts Simultaneously Express Neuron-specific Characteristics

Traditional anatomic studies in the developing embryo have stressed the sequential nature of neural ontogeny. After precursor proliferation, newly born neurons migrate to distal sites where the cells extend processes, elaborate neurotransmitter systems and establish synaptic contacts (28, 40, 41, 43, 52). The occurrence of neuroblast mitosis in culture afforded the opportunity to study relationships among these ontogenetic events in sympathetic precursors. Remarkably, populations containing neuroblasts in the mitotic cycle expressed a wide range of neuron-specific characteristics. For example, mitotic neuroblasts simultaneously exhibited features of a neurotransmitter phenotype: the catecholamine synthetic enzyme, TH, was present during both S phase and cytokinesis (14). Moreover, neuroblasts possessed the complex machinery required for transmitter storage and release, as evidenced by typical DCVs in 83% of cells (16, 22, 34, 55, 58, 61). This figure underestimates the DCV-containing population, however, as only a single ultrastructural profile was examined per cell. Since $\sim 30\%$ of this population was engaged in DNA synthesis, multiple neuronal gene products were expressed in mitotic neuroblasts. In turn, these data suggest that mitotic neuroblasts may simultaneously synthesize, store, and release catecholamine neurotransmitter, as

concluded previously in studies performed in vivo (11, 47, 48, 58).

The present study, however, identifies an extended repertoire of characteristics expressed by mitotic neuroblasts. Time-lapse data indicated that neuritic processes were elaborated either during or very soon after neuroblast division. Processes appeared to be neuron specific, since neuroblasts synthesizing DNA elaborated characteristic varicose neurites. These data suggest that specialized neuritic processes may be present throughout the cell cycle. Indeed, ultrastructural analysis supported this contention: processes contained microtubules, intermediate filaments, and varicosities packed with vesicles. Further, since DCVs were apparently synthesized by mitotic neuroblasts in vitro, the localization of DCVs to varicosities suggests ongoing transport and appropriate targeting of transmitter vesicles as well. In aggregate, these observations indicate that sympathetic neuroblast cell division may be accompanied not only by expression of transmitter systems, but also by elaboration of highly specialized neuritic processes. The presence of differentiated neurites, and even synapses, raises the possibility that transsynaptic signals may influence neuroblast mitosis.

While neuronal ontogeny is apparently sequential in the central nervous system, this schema may not apply universally to all neurons. Studies of neural crest-derived neurons, including sensory, enteric, and autonomic systems, indicate that peripheral precursors may exhibit greater plasticity than their central counterparts (3, 11, 17, 48, 66). While sensory neurons apparently differentiate following cell cycle withdrawal (46, 66), sympathetic precursors exhibit transmitter systems during neurogenesis (11, 47, 48, 66) and potentially neuritic processes as well, as indicated by the present observations. However, differences between peripheral and central neuronal precursors may be more apparent than real. For example, precursors and migratory neurons of central origin may exhibit a number of differentiated, neuronal characteristics, including 150-kD neurofilaments (10), neurotransmitter phenotypic traits (48, 50), and axonal processes (50). These data suggest that central neuronal ontogeny may be more plastic than previously conceived, an issue we are actively investigating.

Extracellular Signals Regulate Neuroblast Mitosis

The present results indicate that multiple ontogenetic processes may occur simultaneously in developing neuroblasts. In turn, our observations suggest that neuronal ontogeny is not constrained by an immutable, intrinsic cellular sequence. What mechanism(s), then, serves to synchronize diverse developmental events? Potentially, environmental signals may support and/or select ongoing ontogenetic processes. Indeed, our observations indicate that epigenetic factors regulate the neuroblast mitotic cycle: insulin and EGF stimulated neuroblast DNA synthesis. For example, insulin specifically and selectively increased the proportion of neuroblasts entering S phase (and did not simply serve as a generalized metabolic stimulus). EGF similarly increased the LI, though the effect was less than that of insulin. Numerous nonneuronal mitogens, however, failed to affect neuroblast DNA synthesis. Consequently, the insulin growth factor family (IGFs) and EGF are apparently highly specific signals for mitotic neuronal precursors. In sum, these results indicate that neuroblast mitosis is subject to specific epigenetic regulation.

In contrast to the IGFs and EGF, distinctly different effects were elicited by FGF. Although FGF stimulated DNA synthesis, the factor did not alter the proportion of cells entering the cycle. One possible explanation would be that the factor increased overall cell survival, including those synthesizing DNA. Interestingly, similar stimulatory effects were found with the sympathetic neurotrophic factor, NGF, which also failed to alter the LI (14). Moreover, FGF and NGF share a number of similar actions in two related populations, neural crest-derived adrenal chromaffin cells (8, 56) and PC12 cells (49, 60). These observations suggest that epigenetic signals may also influence neuroblasts by altering survival. Indeed, of several possibilities, trophic effects may account for the observed NGF stimulation of neuroblast protein synthesis.

Do epigenetic signals, then, serve to coordinate component processes in neuronal development? While specific answers regarding sympathetic neuroblasts must await further analysis, some clues may be obtained from recent studies. Evidence suggests that each of these factors serves a "neurotrophic" role during development, maintaining cell viability and stimulating neuritogenesis and even synaptogenesis (1, 25, 30, 37, 38, 44, 62–64). For example, IGFs influence all three of these processes in sympathetic neurons (30, 44, 64). Similarly, in adrenal chromaffin cells (8, 56) and PC12 cells (49, 60), FGF stimulates survival, metabolism, and neuritogenesis (8, 49, 56, 60), as well as cell division (8, 56). Moreover, FGF enhances survival, process outgrowth (25, 38, 62, 63), and perhaps even proliferation (20) of central neuronal precursors. In sum, these observations suggest that epigenetic signals may influence multiple ongoing neuronal processes, apparently as a function of cellular developmental state.

Our study, along with others (11, 14, 46–48, 50, 58), suggests that a number of developmental options may be expressed coincidentally in a cell. Consequently, the apparent sequence of ontogenetic processes is not an immutable, intrinsic neuronal program. Though multiple factors may influence any single neuronal process, orderly sequence may depend upon the proper timing and cellular locus of factor presentation, as well as the appropriate environmental context (3, 40). As a function of epigenetic regulation, different sequences of ontogenetic processes may be selected from the cellular menu, satisfying subsystem requirements. Continued study of the present model system will hopefully elucidate some of these issues.

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