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Insulin growth factors regulate the mitotic cycle in cultured rat sympathetic neuroblasts

(nerve growth factor/tyrosine hydroxylase)

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ABSTRACT **While neuronal mitosis is uniquely restricted to early development, the underlying regulation remains to be defined. We have now developed a dissociated, embryonic sympathetic neuron culture system that uses fully defined medium in which cells enter the mitotic cycle. The cultured cells expressed two neuronal traits, tyrosine hydroxylase [L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2] and the neuron-specific 160-kDa neurofilament subunit protein, but were devoid of glial fibrillary acidic protein, a marker for non-myelin-forming Schwann cells in ganglia. Approximately one-third of the tyrosine hydroxylase-positive cells synthesized DNA in culture, specifically incorporating [^{3}H]thymidine into their nuclei. We used this system to define factors regulating the mitotic cycle in sympathetic neuroblasts. Members of the insulin family of growth factors, including insulin and insulin-like growth factors I and II, regulated DNA synthesis in the presumptive neuroblasts. Insulin more than doubled the proportion of tyrosine hydroxylase-positive cells entering the mitotic cycle, as indicated by autoradiography of [^{3}H]thymidine incorporation into nuclei. Scintillation spectrometry was an even more sensitive index of DNA synthesis, revealing a 4-fold insulin stimulation with an ED_{50} of 100 ng/ml. Insulin-like growth factor I was 100-fold more potent than insulin, whereas insulin-like growth factor II was less potent, suggesting that insulin growth factor type I receptors mediated the mitogenic responses. In contrast, the trophic protein nerve growth factor exhibited no mitogenic effect, suggesting that the mitogenic action of insulin growth factors is highly specific. Our observations are discussed in the context of the detection of insulin growth factors and receptors in the developing brain.**

One remarkable aspect of the nervous system is that its primary functional elements, the neurons, are not produced during maturity (1, 2). Consequently, the organization of the brain is critically dependent on the initial ontogenetic generation of neuronal populations. This realization has led to extensive empirical documentation of the chronology and geometry of developmental proliferation (3–8). Although these studies suggest that neuronal mitosis is subject to precise regulatory constraints (3–8), underlying mechanisms remain to be defined.

Investigations have been almost exclusively descriptive (9, 10). Experiments performed *in vivo* have been difficult to interpret due to the complexities of the fetal microenvironment and maternal–fetal relations (11, 12). On the other hand, studies employing isolated neural tissue *in vitro* have been confounded by the use of undefined serum-containing medium and by the presence of heterogeneous populations of neural and nonneural cells (9, 10, 13–17). To overcome these limitations, we have developed a defined cell culture system in which presumptive neuroblasts enter the mitotic cycle in serum-free medium. Dissociated sympathetic superior cervical ganglia from rat embryos at gestational day 15.5 were used.

We have begun to characterize mechanisms governing neuronal proliferation to gain insight into unique temporal restrictions on mitosis in the nervous system. Our studies indicate that the insulin family of growth factors, including insulin and the insulin-like growth factors I and II (IGF-I and IGF-II, respectively), regulate entry of neurons into S phase of the cell cycle. In contrast, the trophic protein nerve growth factor (NGF), which is necessary for survival and differentiation of the neurons under study, exhibited no mitogenic effect. Consequently, insulin growth factors (IGFs) are apparently highly specific mitogens for these cultured neurons.

MATERIALS AND METHODS

Experimental Animals and Culture Techniques. Time-mated pregnant Sprague–Dawley rats were obtained from Hilltop Labs (Philadelphia). The day following a late-evening mating was designated gestational day 0.5. Fifty-five to seventy superior cervical ganglia from 15.5-day embryos were dissected as described (18, 19) and incubated in Ca^{2+}/Mg^{2+}-free Puck's saline G for 5 min at 21°C and then in solutions of the same buffer containing trypsin (0.1 mg/ml) for 20 min at 37°C and trypsin inhibitor (0.2 mg/ml) for 5 min at 21°C. Ganglia were dissociated in appropriate control medium lacking bovine serum albumin (see below) by trituration with a 6-inch Pasteur pipette, 75 strokes over 5 min. Aliquots (100–200 μl) of dissociated cells were added to 35-mm culture dishes coated with poly(D-lysine) (0.1 mg/ml) containing defined medium and fibronectin (5 μg/ml) (20). Defined medium, derived from N2 medium of Bottenstein and Sato (21), consisted of a 1:1 (vol/vol) mixture of Ham's F-12 and Dulbecco's modified Eagle's medium containing penicillin (50 units/ml) and streptomycin (50 μg/ml) and supplemented with insulin (10 μg/ml), transferrin (100 μg/ml), putrescine (100 μM), progesterone (20 nM), selenium (30 nM), glutamine (2 mM), glucose (6 mg/ml), guanosine (200 μM), and bovine serum albumin (10 mg/ml). Cultures were maintained for 48 hr in a CO_2 incubator as described (18, 19).

Immunocytochemistry and Autoradiography. After incubation with [^{3}H]thymidine as below, cultures were rinsed, fixed, and processed for immunocytochemical visualization of tyrosine hydroxylase [TyrOHase; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2] as described (22–24). Alternatively, we used monoclonal antibody from clone NN18 to the neurofilament

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Abbreviations: TyrOHase, tyrosine hydroxylase; IGF, insulin growth factor; IGF-I and -II, insulin-like growth factors I and II, respectively; NGF, nerve growth factor; LI, labeling index; GFAP, glial fibrillary acidic protein.

160-kDa subunit (Boehringer Mannheim) at a 1:5 dilution, visualized with lissamine rhodamine-conjugated secondary antibody at a 1:50 dilution. For control cultures, preimmune serum was substituted for anti-TyrOHase antiserum or buffer for neurofilament antibody. [Some cultures were generously prepared by Cheryl Dreyfus (Cornell), to visualize glial fibrillary acidic protein (GFAP) employing the Vector Laboratories (Burlingame, CA) ABC kit for immunoperoxidase staining, as described (25).] Subsequently, dishes were processed for autoradiography as described (23). Cells were examined and photographed with a Leitz Orthoplan microscope and camera equipped with incident illumination for fluorescence and transmitted light for visualization of silver grains.

Thymidine Incorporation Assays. DNA synthesis was assayed at 48 hr either biochemically by scintillation spectrometry or morphologically by determining the labeling index (LI). Following a 24-hr incubation with [^{3}H]thymidine (1 μCi/ml; 1 Ci = 37 GBq) (10, 15), medium was discarded and cells were scraped from the dish in 80 μl of distilled water, transferred to 2.3-cm filter-paper discs, and batch-processed as described (19). For experimental blanks, cultures were incubated with isotope for 1 min.

The LI was determined by examining cultured cells after processing by combined TyrOHase immunocytochemistry and autoradiography. The LI represents the proportion of the TyrOHase-positive population that incorporated thymidine in the nucleus, reflecting the synthesis of DNA (26). For each experimental group of three culture dishes, 100 TyrOHase-positive cells in three or four random nonoverlapping areas were scored (at $\times$400 magnification) for silver grains on each dish, until 1000 cells were counted.

Cell Counting. Three percent of the cells were counted as described (18).

Statistics. Data were analyzed with the one-way analysis of variance and the Neuman–Keul's test.

Materials. Bovine insulin was obtained from Sigma. Recombinant human IGF-I was obtained from Amgen Biologicals (Thousand Oaks, CA). IGF-II was a generous gift of Michael Czech (Univ. of Massachusetts). NGF was isolated as reported (18). All other materials were obtained commercially.

RESULTS

Identification of Ganglion Cells in Culture. To develop a culture system for neuronal mitosis, sympathetic ganglia from 15.5-day rat embryos were dissociated and grown in fully defined medium. At this early developmental stage, neuronal precursors normally undergo mitosis *in vivo* (27). However, ganglia normally contain neuronal as well as a variety of nonneuronal cell types (28, 29); therefore, before analyzing mitotic potential, it was necessary to identify the cell types that survived and grew *in vitro*. We defined the expression of cell-specific phenotypic characters immunologically.

A large proportion of sympathetic neuronal precursors *in vivo* are identified by catecholaminergic phenotypic traits that first appear in neuroblasts on embryonic day 12.5 (30, 31). To ascertain whether sympathetic neurons, or their precursors, were present in the cultures, we examined cells for expression of TyrOHase, the rate-limiting enzyme in biosynthesis of catecholaminergic transmitters (27, 32). In fact, the vast majority of cells were positive for TyrOHase. Intense immunofluorescence was localized to the cell cytoplasm surrounding unstained nuclei and to long varicose processes that terminated in growth cones (Fig. 1 *A* and *B*), suggesting that the cultures were predominantly neuronal.

To further characterize the cultures, we examined cells for the expression of neurofilament protein, one of the earliest

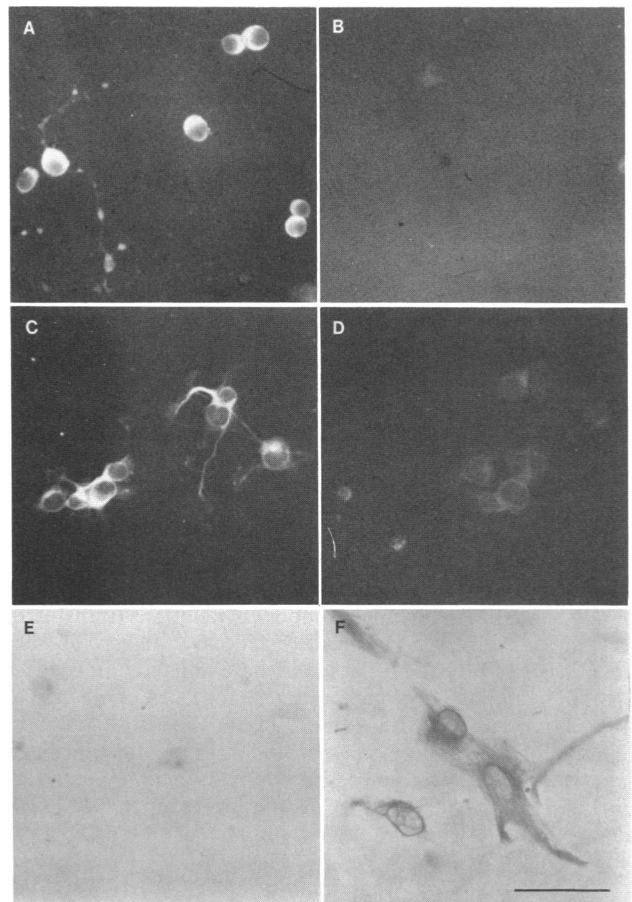

FIG. 1. Immunocytochemical staining of cultured cells from embryonic sympathetic ganglia. Ganglia at embryonic day 15.5 were dissociated and cultured in fully defined serum-free medium. At 48 hr, cultures were fixed and processed for immunocytochemistry. Intense TyrOHase fluorescence is localized to the cell cytoplasm and long varicose processes (*A*). Neurofilament staining is distributed eccentrically in round cells, in punctate fashion, and extends linearly in cells with processes (*C*). Specific primary antibodies were excluded in processing controls for TyrOHase (*B*) and neurofilament (*D*). No specific GFAP peroxidase staining is observed in sympathetic cultures (*E*), whereas non-neuron-like cells from serum-containing cultures of brain at embryonic day 18.5 exhibit intense fibrillary reaction product (*F*). (Bar = 50 μm.)

neuron-specific markers to appear during ontogeny (28, 33, 34). After 48 hr in culture, approximately one-third of the cells exhibited intense immunofluorescence subsequent to labeling with a monoclonal antibody to the 160-kDa neurofilament subunit protein (Fig. 1 *C* and *D*). Staining was distributed eccentrically in the cytoplasm of round cells and extended into cellular processes, when present, in a linear fashion (28). These observations suggest that the vast majority of cultured cells are sympathetic neuronal precursors or perhaps more mature sympathetic neurons.

To detect nonneuronal cells, cultures were examined for the expression of GFAP, a specific marker for non-myelin-forming Schwann cells in peripheral ganglia (29). No cells expressing GFAP were detected, suggesting that the cultures were devoid of nonneuronal cells (Fig. 1 *E* and *F*). These observations conform to reports indicating that Schwann cells survive and proliferate poorly in serum-free medium (35, 36). Consequently, our culture protocol appeared to favor the selective survival of neurons and prevent nonneuronal survival.

Thymidine Incorporation of TyrOHase-Positive Cells. To determine whether TyrOHase-positive cells synthesized DNA, cultures were incubated with [^{3}H]thymidine from hr 24

to 48 and were processed for combined immunocytochemistry and autoradiography. The percentage of TyrOHase-positive cells that simultaneously incorporated thymidine into their nucleus is referred to as the LI, which reflects those cells synthesizing DNA in the culture (26). In fact, the LI was 20–35%, indicating that up to one-third of embryonic TyrOHase-positive cells synthesized DNA *in vitro* (Fig. 2). The specific incorporation of thymidine in the nucleus suggests that a proportion of the TyrOHase-positive cells represent neuron progenitors or presumptive neuroblasts.

Effects of Insulin on DNA Synthesis. Considerable evidence indicates that hormones and growth factors regulate cell division in nonneural systems (37–40). To examine the role of defined factors in the present system, presumptive neuroblasts were cultured in control medium or in medium lacking one of five components. DNA synthesis was estimated quantitatively by scintillation spectrometry of culture acid-precipitates.

Insulin deletion resulted in a marked 77% decrease in [^{3}H]thymidine incorporation [control, 6915 ± 203 cpm; minus insulin, 1582 ± 185 cpm (mean ± SEM)]. Moreover, the magnitude of the insulin deletion effect differed significantly from that of all other component deletions (transferrin, 21% decrease; progesterone, 22% decrease; putrescine, 40% decrease; selenum, 52% decrease; data not shown), suggesting that insulin was critically required for DNA synthesis. To further characterize the effects of insulin, dose–response relationships were defined. Increasing the insulin concentration produced a 4-fold increase in [^{3}H]thymidine incorporation with a plateau at 5–10 μg/ml, suggesting that insulin acted through a saturable receptor-mediated mechanism (Fig. 3*A*).

The apparent insulin stimulation of DNA synthesis may have been attributable to several processes. Experiments were performed to determine whether insulin simply increased cell survival or, alternatively, increased the proportion of cells undergoing DNA synthesis. Cultures were grown with or without insulin and assessed for survival by morphometry and for DNA synthesis by scintillation spectrometry or by combined TyrOHase immunocytochemistry and autoradiography.

Exposure to insulin did not alter cell numbers after 48 hr, whether counting was performed on living cultures or after fixation [control, 30,500 ± 1150 cells; plus insulin, 32,050 ± 1050 cells (mean ± SEM)], suggesting that insulin did not alter survival. In contrast, insulin increased the LI >2-fold, indicating that the hormone more than doubled the number of cells entering S phase of the cell cycle (Fig. 4). Further, in sister cultures, insulin elicited a 5-fold increase in [^{3}H]thymidine incorporation, assayed by scintillation spectrometry, confirming the LI results and indicating that spectrometry is a sensitive method for screening potential mitogens (Fig. 4).

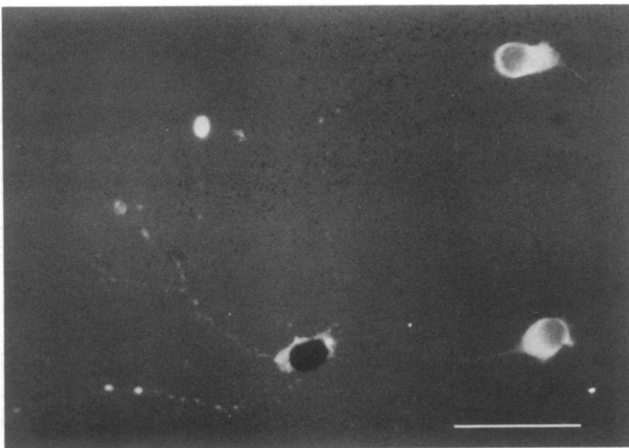

FIG. 2. Combined TyrOHase immunocytochemistry and [^{3}H]thymidine autoradiography of dissociated ganglia cultures. Cells were cultured as in Fig. 1, except for the addition of [^{3}H]thymidine (1 μCi/ml) for the final 24 hr of incubation. At 48 hr, cultures were processed for combined TyrOHase immunocytochemistry and autoradiography and assayed for the LI. Three TyrOHase-positive cells are shown. One cell (lower center) also exhibits dense black silver grains localized to the nucleus. (Bar = 50 μm.)

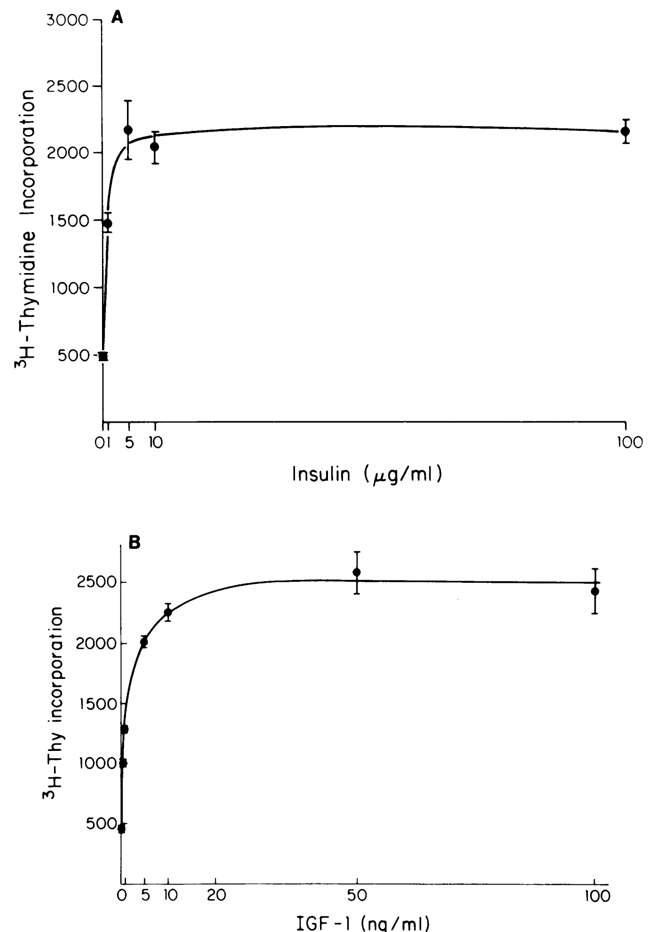

FIG. 3. Effect of IGFs on [^{3}H]thymidine incorporation. Cultures were incubated for 48 hr with various concentrations of insulin (*A*) or IGF-I (*B*). [^{3}H]Thymidine (1 μCi/ml) was added at 24 hr of incubation, and incorporation into culture acid-precipitates was assayed 1 day later. Each experimental value represents the mean incorporation of three culture dishes (calculated after subtracting blank; range = 25–50 cpm) and is expressed as cpm [mean ± SEM (vertical bars)]. Note that insulin was used in concentrations of μg/ml, whereas IGF-I was used in concentrations of ng/ml.

Effect of IGF-I and -II on DNA Synthesis. Insulin is but one member of a family of growth factors that bind homologous and heterologous receptors, serving as autocrine growth regulators during histogenesis (37–39, 41, 42). In a number of nonneural systems, micromolar concentrations of insulin, as we employed, evoke mitogenesis through IGF type I receptors (37–39). To determine whether IGF-I stimulated DNA synthesis in our neuronal system, a dose–response study was performed. In fact, IGF-I elicited a 5-fold increase in [^{3}H]thymidine incorporation, reaching a plateau at 10–50 ng/ml (Fig. 3*B*). IGF-I was more potent than insulin: the ED_{50} for IGF-I was 1 ng/ml, whereas that for insulin was ≈100 ng/ml (Fig. 3), indicating that IGF-I was at least 100 times more potent than insulin. In contrast, incubation of the cultures with IGF-II (100 ng/ml) increased [^{3}H]thymidine incorporation only 53% above control (data not shown). Consequently, although all IGFs stimulated DNA synthesis in presumptive neuroblasts, IGF-I was the most potent,

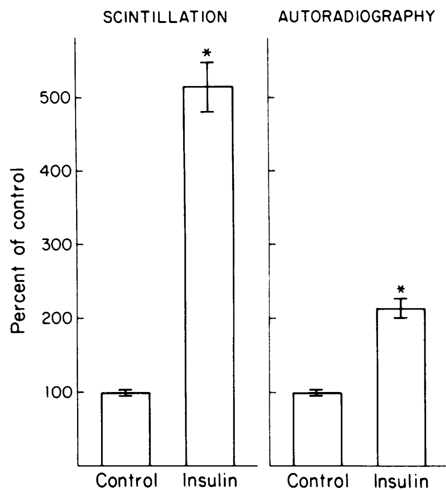

FIG. 4. Assessment of insulin-stimulated [^{3}H]thymidine incorporation by spectrometry and LI. Cultures were incubated without insulin (control) or with insulin (10 μg/ml) for 48 hr. [^{3}H]Thymidine incorporation was assessed in sister cultures either by scintillation spectrometry (as cpm), as in Fig. 3, or by combined TyrOHase immunocytochemistry and autoradiography (LI), as in Fig. 2, and is expressed as percent of control (mean ± SEM). The data were derived from three independent experiments. (*) Differs from control at $P < 0.005$.

suggesting that IGF type I receptors mediated the mitogenic effect.

Effect of NGF on DNA Synthesis. To further define the specificity of IGF action, we examined the effect of NGF, the potent sympathetic trophic protein (43). NGF has multiple effects on sympathetic neurons, including enhanced survival during development (44, 45). To ascertain whether NGF may affect sympathetic mitogenesis or interact with insulin, cultures were exposed to NGF alone or to insulin and NGF.

NGF exposure had no effect on the LI of presumptive neuroblasts compared to controls not cultured with insulin or NGF (Fig. 5*B*). In contrast, insulin more than doubled the LI compared to controls (Fig. 5*B*). Further, even cells stimulated by insulin were unaffected by NGF, suggesting that

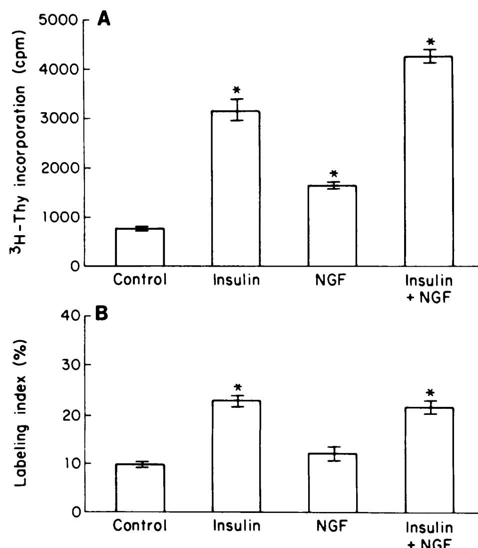

FIG. 5. Effect of NGF on [^{3}H]thymidine incorporation. Cultures were incubated in control medium or in medium containing insulin (10 μg/ml), NGF (10 units/ml), or insulin plus NGF for 48 hr. [^{3}H]Thymidine (^{3}H-Thy) incorporation was assessed in sister cultures by scintillation spectrometry (*A*), as in Fig. 3, or by determination of the LI (*B*), as in Fig. 2. Results are expressed as mean ± SEM. (*) Differs from control at $P < 0.005$.

NGF exerts neither positive nor negative effects on mitogenesis (Fig. 5*B*).

While NGF did not alter the fraction of cells synthesizing DNA, some presumptive neuroblasts were, indeed, sensitive to NGF. NGF increased neurite complexity and TyrOHase staining intensity in responsive neurons (data not shown), well-delineated effects of NGF, and even had a small, but reproducible, effect on [^{3}H]thymidine incorporation by spectrometry (Fig. 5*A*). However, NGF did not change the LI of this presumptive neuroblast population (Fig. 5*B*).

DISCUSSION

Mechanisms regulating neuronal mitosis have been virtually unexplored, heretofore, due to the lack of suitable *in vitro* model systems (9–17). We have developed a defined neuronal system that largely circumvents previous difficulties, permitting the examination of mitotic regulation. Our observations indicate that presumptive sympathetic neuroblasts synthesized DNA in fully defined medium and that IGFs influenced entry into the mitotic cycle.

To define a system for the study of neuronal mitosis, we focused on an *in vitro* system to avoid the undefined complexities of the embryonic microenvironment and the maternal–fetal unit (11, 12). We used only serum-free conditions, which allowed us to examine the effects of specific growth factors (13, 20, 21, 37–40). A dissociated-cell culture system was developed that consisted of specifically identified neurons in the virtual absence of nonneuronal cells to eliminate the confounding interactions of various cell types (9, 10, 13–17, 46). Finally, specific phenotypic markers were employed to ensure that neuronal mitogenesis itself was being examined.

In fact, the vast majority of cells in the cultures expressed TyrOHase, indicating that the cells were catecholaminergic. Moreover, the cells also exhibited the 160-kDa neurofilament subunit, a neuron-specific protein expressed during or after the final mitotic cycle (28, 33, 34). Conversely, the cultures were virtually devoid of nonneuronal cells, since no cells were positive for GFAP, a specific marker for non-myelin-forming Schwann cells in ganglia (29). Our observations suggest that the serum-free dissociated-cell cultures were composed almost exclusively of sympathetic neurons or their precursors.

The nuclei of as many as one-third of the TyrOHase-positive embryonic cells incorporated [^{3}H]thymidine, suggesting that these cells were in S phase of the cell cycle in culture. The ongoing synthesis of DNA suggests that the cells are sympathetic progenitors or presumptive neuroblasts (7, 9, 10, 26, 27). Our observations *in vitro* are entirely consistent with studies *in vivo* (27), indicating the occurrence of mitosis in sympathetic neurons expressing catecholaminergic traits at this stage. Nevertheless, several alternative explanations must be considered. For example, [^{3}H]thymidine incorporation may conceivably have represented mitochondrial DNA synthesis or entry into cytoplasmic precursor pools. However, the discrete distribution of grains over nuclei is inconsistent with localization to mitochondrial or cytoplasmic precursor pools. Similarly, insulin did not appear to simply increase [^{3}H]thymidine uptake since (*i*) labeling of a putative cytoplasmic pool was undetectable, (*ii*) extensive study has failed to indicate an effect of the hormone on thymidine transport (37, 38), and (*iii*) conversely, NGF, which is known to increase nucleoside uptake (43–45), did not alter the LI in our experiments. Alternatively, [^{3}H]thymidine incorporation may have reflected DNA repair. However, since potential DNA injury was most likely to have occurred during ganglion dissociation, all cultures should have exhibited incorporation that was not specifically responsive to insulin regulation. Finally, although the production of polyploidy or binucleate

cells might have increased incorporation (14), <1% of the neurons in our cultures were binucleate. Consequently, we conclude that embryonic sympathetic neurons synthesized DNA in fully defined medium in culture, signifying entry into S phase of the cell cycle. Since regulation of mitosis tends to occur prior to entry into S phase (47), we were in a position to examine regulatory factors in the present neuronal system.

Our studies indicate that the IGF family specifically regulates the entry of cultured presumptive neuroblasts into the mitotic cycle. Insulin more than doubled the proportion of neurons initiating DNA synthesis. Insulin stimulation of neuroblasts was not accompanied by increased cell numbers at 48 hr. The production and detection of new cells, however, is a function of the cell cycle length, including the time required for actual cell division after DNA synthesis. Cycle length is known to vary from 24 to 72 hr or more, depending on cell type and culture conditions (47). In the present studies we explicitly chose conditions under which the cell number was constant, to avoid confounding survival with DNA synthesis and the LI.

While insulin regulated neuroblast mitosis in culture, other members of the IGF family were also active. Indeed, IGF-I was at least 100 times more potent in stimulating DNA synthesis. Based on work in nonneural systems, our observations suggest that IGF effects were mediated by binding to IGF type I receptors (37–39). This hypothesis is consistent with the finding that IGF-II stimulated DNA synthesis by only 50% at concentrations higher than those required for peak IGF-I effects.

The effects of NGF were examined to determine whether mitogenic stimulation was relatively specific to the IGF family of molecules. While neurons exhibited characteristic developmental responses to NGF in culture, the proportion of cells entering the mitotic cycle was not altered by NGF. We conclude that IGFs are specific neuronal mitogens in our culture system.

Our observations in culture naturally raise questions regarding the role of IGFs in neuronal mitogenesis *in vivo*. In fact, IGFs appear to serve as autocrine growth factors during histogenesis of many tissues, apparently regulating proliferation (37, 41). While little is known regarding regulation of neurogenesis, studies indicate that IGFs are synthesized locally in the central nervous system during ontogeny (41, 48, 49). Further, IGF receptors appear in the brain at the onset of neurogenesis and decrease with the cessation of neuronal proliferation (15, 42, 50–52). Finally, IGFs are detectable in cultured embryonic brain that, in turn, responds to the agents (13, 15, 48, 53). Our fully defined dissociated neuronal culture system may aid in the investigation of the regulation of neuronal mitosis.

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1. Jacobson, M. (1978) *Developmental Neurobiology* (Plenum, New York).
2. Schacher, S. (1985) in *Principles of Neural Science*, eds. Kandel, E. R. & Schwartz, J. H. (Elsevier/North-Holland, New York), pp. 729–742.
3. Hamburger, V. & Levi-Montalcini, R. (1949) *J. Exp. Zool.* **111**, 457–502.
4. Carr, V. M. & Simpson, S. B. (1978) *J. Comp. Neurol.* **182**, 727–740.
5. Landmesser, L. & Pilar, G. (1974) *J. Physiol.* **241**, 715–736.
6. Angevine, J. R. (1970) *J. Comp. Neurol.* **139**, 129–188.
7. Rakic, P. (1981/82) *Neurosciences Res. Prog. Bull.* **20**, 439–451.
8. Cowan, W. M. (1973) in *Development and Aging in the Nervous System*, ed. Rockstein, M. (Academic, New York), pp. 19–41.
9. Kriegstein, A. & Dichter, M. A. (1984) *Brain Res.* **295**, 184–189.
10. Sensenbrenner, M., Wittendorp, E., Barakat, I. & Rechenmann, R. V. (1980) *Dev. Biol.* **75**, 268–277.
11. LeGrand, J. (1977) in *Brain: Fetal and Infant*, ed. Berenberg, S. R. (Nijhoff Medical Division, The Hague), pp. 137–164.
12. Lauder, J. M. (1983) *Psychoneuroendocrinology* **8**, 121–155.
13. Lenoir, D. & Honegger, P. (1983) *Dev. Brain Res.* **7**, 205–213.
14. Cone, C. D., Jr., & Cone, C. M. (1976) *Science* **192**, 155–158.
15. Raizada, M. K., Yang, J. W. & Fellows, R. E. (1980) *Brain Res.* **200**, 389–400.
16. Pettmann, B., Louis, J. C. & Sensenbrenner, M. (1979) *Nature (London)* **281**, 378–380.
17. Barakat, I., Courageot, J., Devilliers, G. & Sensenbrenner, M. (1984) *Neurochem. Res.* **9**, 263–271.
18. Coughlin, M. D., Bloom, E. M. & Black, I. B. (1981) *Dev. Biol.* **82**, 56–68.
19. Bloom, E. M. & Black, I. B. (1979) *Dev. Biol.* **68**, 568–578.
20. Bottenstein, J. E. & Sato, G. H. (1980) *Exp. Cell Res.* **129**, 361–366.
21. Bottenstein, J. E. & Sato, G. H. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 514–517.
22. Dreyfus, C. F., Markey, K. A., Goldstein, M. & Black, I. B. (1983) *Dev. Biol.* **97**, 48–58.
23. Jonakait, G. M., Markey, K. A., Goldstein, M., Dreyfus, C. F. & Black, I. B. (1985) *Dev. Biol.* **108**, 6–17.
24. Markey, K. A., Kondo, L., Shenkman, L. & Goldstein, M. (1980) *Mol. Pharmacol.* **17**, 79–85.
25. Hsu, S. M., Raine, L. & Fanger, H. (1981) *J. Histochem. Cytochem.* **29**, 577–580.
26. Sidman, R. L. (1970) in *Contemporary Research Techniques of Neuroanatomy*, eds., Ebbesson S. O. E. & Nauta, W. J. (Springer, New York), pp. 252–274.
27. Rothman, T. P., Specht, L. A., Gershon, M. D., Joh, T. H., Teitelman, G., Pickel, V. M. & Reis, D. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6221–6225.
28. Cochard, P. & Paulin, D. (1984) *J. Neurosci.* **4**, 2080–2094.
29. Mirsky, R. & Jessen, K. R. (1986) in *Neurofibromatosis*, eds. Rubenstein, A. E., Bunge, R. P. & Housman, D. E. (N.Y. Acad. Sci., New York), Vol. 486, pp. 132–146.
30. Cochard, P., Goldstein, M. & Black, I. B. (1979) *Dev. Biol.* **71**, 100–114.
31. Teitelman, G., Baker, H., Joh, T. H. & Reis, D. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 509–513.
32. Levitt, M., Spector, S., Sjoerdsma, A. & Udenfriend, S. (1965) *J. Pharmacol. Exp. Ther.* **148**, 1–8.
33. Tapscott, S. J., Bennett, G. S., Toyama, Y., Kleinbart, F. & Holtzer, H. (1981) *Dev. Biol.* **86**, 40–54.
34. Raju, T., Bignami, A. & Dahl, D. (1981) *Dev. Biol.* **85**, 344–357.
35. Cassel, D., Wood, P. M., Bunge, R. P. & Glaser, L. (1982) *J. Cell. Biochem.* **18**, 433–445.
36. Sobue, G., Kreider, B., Asbury, A. & Pleasure, D. (1983) *Brain Res.* **280**, 263–275.
37. Moses, A. C. & Pilistine, S. J. (1985) in *Control of Animal Cell Proliferation*, eds. Boynton, A. L. & Leffert, H. L. (Academic, New York), pp. 91–119.
38. King, G. L. & Kahn, C. R. (1985) in *Control of Animal Cell Proliferation*, eds. Boynton, A. L. & Leffert, H. L. (Academic, New York), pp. 201–249.
39. Goustin, A. S., Leof, E. B., Shipley, G. D. & Moses, H. L. (1985) *Cancer Res.* **46**, 1015–1029.
40. Rozengurt, E. (1986) *Science* **234**, 161–166.
41. D'Ercole, A. J., Stiles, A. D. & Underwood, L. E. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 935–939.
42. Sara, V. R., Hall, K. Misaki, M., Fryklund, L., Christensen, N. & Wetterberg, L. (1983) *J. Clin. Invest.* **71**, 1084–1094.
43. Levi-Montalcini, R. & Angeletti, P. U. (1968) *Physiol. Rev.* **48**, 534–569.
44. Black, I. B. (1978) *Annu. Rev. Neurosci.* **1**, 183–214.
45. Thoenen, H. & Barde, Y. A. (1980) *Physiol. Rev.* **60**, 1284–1334.
46. Patterson, P. H. & Chun, L. L. Y. (1977) *Dev. Biol.* **56**, 263–280.
47. Baserga, R. (1985) *The Biology of Cell Reproduction* (Harvard Univ., Cambridge, MA).
48. D'Ercole, J. A., Applewhite, G. T. & Underwood, L. E. (1980) *Dev. Biol.* **75**, 315–328.
49. Soares, M. B., Ishii, D. & Efstratiadis, A. (1985) *Nucleic Acids Res.* **13**, 1119–1134.
50. Bassas, L. DePablo, F., Lesniak, M. A. & Roth, J. (1985) *Endocrinology* **117**, 2321–2329.
51. Young, W. S., III, Kuhar, M. J., Roth, J. & Brownstein, M. J. (1980) *Neuropeptides* **1**, 15–22.
52. Van Schravendijk, C. F. H., Hooghe-Peters, E. L., Van den Brande, J. L. & Pipeleers, D. G. (1986) *Biochem. Biophys. Res. Commun.* **135**, 228–238.
53. Enberg, G., Tham, A. & Sara, V. R. (1985) *Acta Physiol. Scand.* **125**, 305–308.