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Embryologic development of a mouse sympathetic ganglion *in vivo* and *in vitro*

(embryology/nerve growth factor/tissue culture/tyrosine 3-monooxygenase)

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ABSTRACT **The morphologic and biochemical development of the embryonic mouse superior cervical ganglion was characterized *in vivo* and in tissue culture. From 13 days of gestation, when the superior cervical ganglion was first visible, to birth at 19 days, tyrosine hydroxylase [tyrosine 3-monooxygenase; L-tyrosine,tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2] activity increased 100-fold *in vivo*. Explants of ganglia from 14-day embryos exhibited abundant neurite outgrowth in basal medium without added nerve growth factor (NGF), and increases in tyrosine hydroxylase activity paralleled that observed *in vivo*. Ganglia from 14-day embryos elaborated neurites and exhibited 3-fold increases in enzyme activity *in vitro* in the presence of antiserum to NGF (anti-NGF) or NGF + anti-NGF. In direct contrast, ganglia from 18-day fetuses failed to grow without added NGF or in medium containing anti-NGF or NGF + anti-NGF: virtually no axon outgrowth occurred and tyrosine hydroxylase activity decreased by half. These observations suggest that developmental regulatory mechanisms change radically during embryologic and fetal life of mammalian superior cervical ganglion.**

Autonomic neurons have been used extensively as models of neuronal development. However, mechanisms regulating *embryonic* growth and development of mammalian autonomic ganglia are largely unknown. Recently, the prenatal ontogeny of the parasympathetic innervation of mouse submandibular gland has been described (1, 2). The pattern of axon outgrowth from submandibular ganglion to gland parallels the morphogenesis of the glandular epithelium in time and space, and development *in vitro* reproduces that *in vivo*. Moreover, neurite extension from the ganglion requires the presence of target epithelium.

The embryonic development of mammalian sympathetic ganglia remains to be defined. A great deal of attention has focused on the *perinatal* maturation of the mouse, rat, and guinea pig sympathetic superior cervical ganglion (SCG) *in vivo* (3–10) and in culture (11–16). The SCG is anatomically discrete, allowing various manipulations *in vivo*, and possesses specific markers by which presynaptic and postsynaptic neural development may be monitored. For example, the activity of tyrosine hydroxylase (Tyr-OH) [tyrosine 3-monooxygenase; L-tyrosine, tetrahydropterine:oxygen oxidoreductase(3-hydroxylating); EC 1.14.16.2], the rate-limiting enzyme in catecholamine biosynthesis (17), is highly localized to adrenergic neurons in the SCG (18) and may be used as an index of maturation of these cells (3). Considerable evidence indicates that the normal postnatal maturation of adrenergic neurons is dependent on anterograde transsynaptic regulation (3, 5–7) and on target organ influences (9, 19). In turn, target organs may regulate adrenergic development through the mediation of nerve growth factor (NGF; 19, 20), a protein fraction obtained from snake venom, mesenchymal tumors, and postpuberal male mouse salivary glands (20, 21). Injection of neonates with NGF results in hypertrophy and increased numbers of adrenergic neurons throughout the body (20, 22), while treatment with antiserum to NGF (anti-NGF) is associated with almost total sympathectomy (20, 22). Injection of pregnant mice with anti-NGF late in gestation (15–17 days) leads to partial immunosympathectomy of the offspring, while earlier injections (7–12 days) only affect heart and spleen innervation (23, 24). In tissue culture of postnatal ganglia, added NGF (20), or a related factor produced by supporting cells (25), is required for survival and neurite outgrowth. The regulation of development of the mammalian *embryonic* SCG remains to be defined.

Since the SCG constitutes an important model of mammalian neuronal growth, we have extended our studies to the embryonic ganglion. The SCG has been isolated from mouse embryos, and its morphological and biochemical ontogeny has been defined *in vivo* and *in vitro* from 13 days of gestation to birth. We report that growth and differentiation in culture parallels that *in utero* over a defined period of time, validating use of the cultured ganglion as a model for SCG development. Moreover, growth requirements, and hence regulatory developmental mechanisms, differ markedly between embryonic and late fetal ganglia.

MATERIALS AND METHODS

Culture Methods. Ganglia were excised from embryos and fetuses of SWV mice, an inbred strain selected for ease of breeding and high litter size. Breeding pairs were time-mated in the evening, and females were examined for vaginal plugs the following morning. Embryonic and fetal age was computed from the day of discovery of the vaginal plug, which was considered day 0. After cervical dislocation of the pregnant mouse, embryos were removed and transferred to Hanks' balanced salt solution, and ganglia were excised under a dissecting microscope. Culture procedures were similar to those described (1). Nutrient Mixture F12 (Kansas Biologicals) was supplemented with 10% fetal calf serum (Grand Island Biological Co.), penicillin (50 units/ml), streptomycin (50 μg/ml), and amphotericin B (0.25 μg/ml) ("basal medium").

NGF was prepared as the 25,000 × *g* supernatant fraction of adult male SWV mouse salivary glands homogenized in 20 volumes of iced distilled water (26). Medium containing a 10^4 dilution of the supernatant elicited maximal outgrowth from newborn mouse ganglia and from 8-day chick embryo spinal ganglia. Anti-NGF was obtained from Burroughs-Wellcome Co. and used at a concentration of 0.5% (vol/vol). The NGF-

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Abbreviations: Tyr-OH, tyrosine hydroxylase; SCG, superior cervical ganglion; NGF, nerve growth factor; anti-NGF, antiserum to nerve growth factor.

elicited outgrowth from newborn SCG could be completely prevented by addition of anti-NGF to a final concentration of 0.05% in the medium.

Isolated ganglia were grown on plastic tissue culture dishes (Falcon Co.). Ganglia from 13-, 14-, and 15-day embryos adhered to the dishes with no difficulty; ganglia from 17- and 18-day fetuses and from neonates did not readily adhere, and were attached using fetal calf serum (27). Ganglia from fetuses aged 17 days and older were sectioned in two or three pieces for culture. Cultures were maintained at 37° in an atmosphere of 95% air, 5% CO_2, and nearly 100% relative humidity. Medium was changed every 2 days.

Histology. Cultured ganglia were stained supravitally with a 0.05% (wt/vol) solution of Janus Green B (Allied Chemical Co.) in Hanks' solution. Ganglia were rinsed free of medium with Hanks' solution, covered with the staining solution, and maintained at room temperature for 30–90 min. Then they were rinsed twice with Hanks' solution, fixed for 1–16 hr in 8% ammonium molybdate, rinsed thoroughly in distilled water, and mounted in glycerin. This method includes procedures reported for supravital staining with methylene blue much simplified by the use of Janus Green B (28). Nerve outgrowth visualized by the staining procedure was confirmed by examination of cultures with phase microscopy.

Biochemistry. Ganglion pairs were transferred to glass conical tubes using silicone-treated (Siliclad) pipettes, and excess fluid was removed. Cultured ganglia were removed from the plastic dishes with a Tygon policeman. Ganglion pairs in 10 μl of ice-cold distilled water were homogenized by six cycles of freeze-thawing in an alcohol/dry ice bath. Preliminary studies indicated that this procedure resulted in release of maximal Tyr-OH activity and total protein. Tyr-OH activity (29) and total protein (30) were determined as described.

Statistical Analysis. Data were analyzed by Student's *t*-test. Multiple data were analyzed with the one-way analysis of variance and the Newman–Keuls test.

RESULTS

Development *In Vivo*. The mouse SCG was first visible under the dissecting microscope late during the 13th day of gestation, lying medial to the nodose ganglion, which, in turn, lay medial to the otic cyst. In less than 9 hr *in utero* the SCG had coalesced to form a well-defined, ovoid tissue mass.

To define normal development of the SCG *in utero*, we selected litters of different gestational ages and assayed ganglia for Tyr-OH activity and total protein. Tyr-OH activity was readily detectable in ganglia of 13-day embryos, and increased approximately 100-fold between this time and birth (Fig. 1). Enzyme activity rose gradually between days 13 and 16, increased rapidly between days 16 and 17, and rose gradually once again between day 17 and birth at 19 days. During the same period, total ganglion protein rose 4-fold, resulting in a dramatic increase in Tyr-OH specific activity (Fig. 1).

Development *In Vitro*. Tissue culture studies of the embryonic SCG were undertaken to determine whether embryologic development *in vitro* adequately paralleled that *in vivo*, and whether conditions necessary for ganglion differentiation varied during prenatal growth.

SCG explants from 13-, 14-, and 15-day embryos grown in basal medium *without added NGF* readily adhered to the culture dish surface and exhibited extensive fiber development by 48 hr of incubation (Fig. 2). Axon extension was evident after 24 hr of culture, continued increasing through 48 hr, and, although maintained, exhibited little further development during the third day of incubation. Thereafter, neurites degenerated,

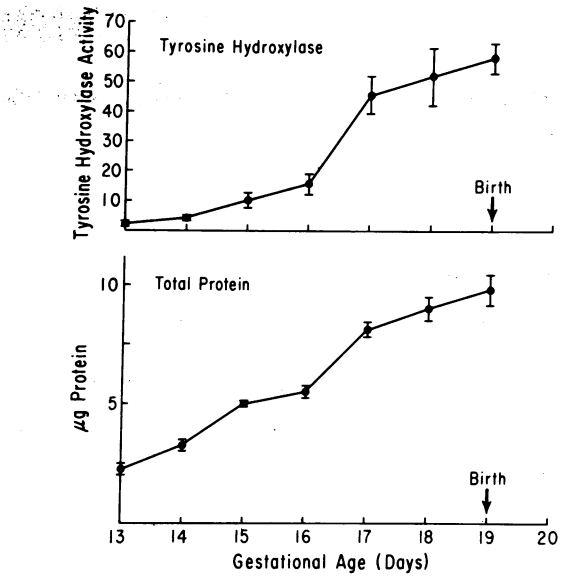

FIG. 1. Developmental increases of Tyr-OH activity and total protein in embryonic ganglia *in vivo*. Groups of 5–11 mice were taken from litters of different gestational ages, and ganglion pairs from each animal were assayed for enzyme activity and total protein (see *Materials and Methods*). Tyr-OH activity is expressed as mean pmol of product per ganglion·hr ± SEM (vertical bars). Total protein is expressed as mean μg per ganglion ± SEM.

while ganglion cells lost cohesiveness and sloughed into the medium.

Cultured ganglia were assayed for Tyr-OH activity to determine whether development *in vitro* paralleled maturation *in vivo*. Explants of 14-day gestation ganglia grown in basal medium without added NGF exhibited a marked increase in Tyr-OH activity during the first 3 days in culture. An initial variable lag period of recovery from the trauma of explantation is common in tissue culture (27, 38) and was reflected in the present studies by a 1-day plateau before the rise in enzyme activity. Thereafter, Tyr-OH activity increased dramatically, resulting in more than an 8-fold total rise (Fig. 3). This increase

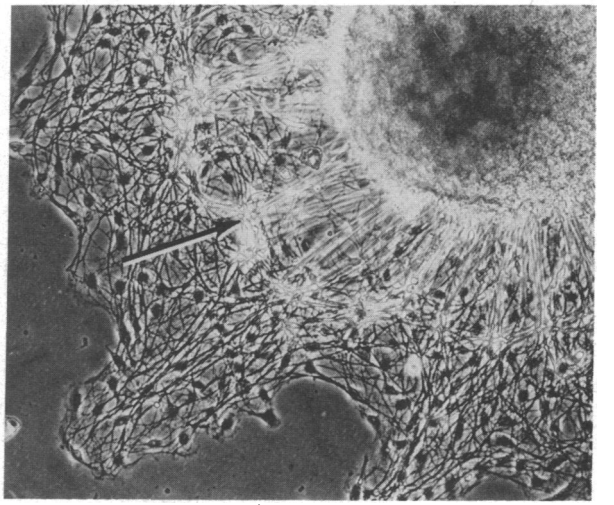

FIG. 2. Phase micrograph of embryonic ganglion in culture. A 14-day gestational age ganglion explant cultured for 48 hr in basal medium without added NGF is shown. Large bundles of fibers (arrow) extend radially from ganglionic cell mass (compare to Fig. 5), while finer bundles form a meshwork over support cells. Under these culture conditions nerve fibers do not extend beyond underlying support cells.

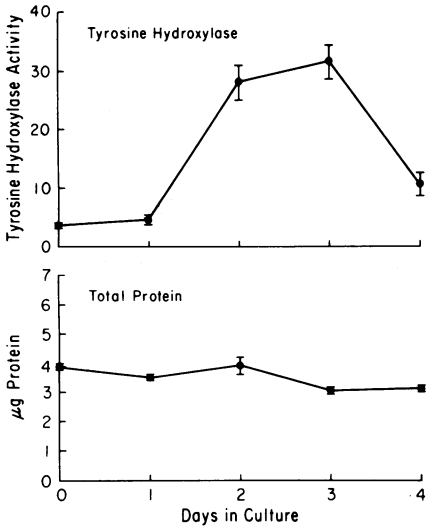

FIG. 3. Development of Tyr-OH activity and total protein in embryonic ganglia in tissue culture. Ganglion pairs were removed from groups of 4–21 mice of gestational age 14 days, and were grown in basal medium for the indicated periods of time. Enzyme activity and total protein are expressed as in Fig. 1. For Tyr-OH activity the 2- and 3-day values differ from all other times at $P < 0.001$. The total protein values do not differ significantly ($P > 0.05$).

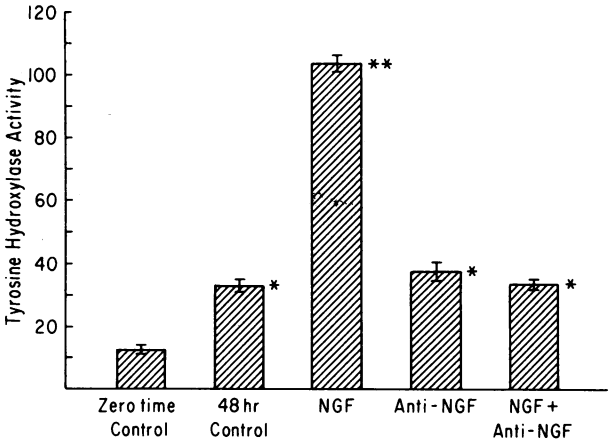

FIG. 4. Development of Tyr-OH activity in 14.5-day embryonic ganglia in culture under different conditions. Ganglion pairs were removed from groups of 5–6 mouse embryos and grown for 48 hr in basal medium (48 hr control), basal medium + NGF (NGF), basal medium + anti-NGF (anti-NGF), or basal medium + NGF + anti-NGF (NGF + anti-NGF). Zero time controls consist of ganglion pairs not incubated in tissue culture. Tyr-OH activity is expressed as in Fig. 1. * Differs from zero time control and NGF groups at $P < 0.01$, and does not differ from other groups with single asterisk ($P > 0.05$). ** Differs from all other groups at $P < 0.01$.

in Tyr-OH activity *in vitro* paralleled that which occurred *in vivo:* after 3 days in culture Tyr-OH activity was the same as that of the 16- to 17-day ganglion *in vivo* (Figs. 1 and 3). After the third day in culture Tyr-OH activity began to decline. Explants of 15-day ganglia responded similarly. Although there was no significant increase of total ganglion protein in culture, the values of 4 μg per ganglion approximated that observed *in vivo,* and resulted in a comparable increase in specific Tyr-OH activity (Figs. 1 and 3).

Regulatory Mechanisms *In Vitro.* To more critically define the relationship of differentiation in culture to the presence or absence of NGF, 14.5-day ganglia were grown for 48 hr in (*a*) basal medium, (*b*) basal medium + NGF, (*c*) basal medium + anti-NGF, or (*d*) basal medium + NGF + anti-NGF, and compared to zero time controls. In the *absence* of added NGF, Tyr-OH activity increased approximately 3-fold (Fig. 4). At the time of explantation, these mice were 12 hr older than those represented in Fig. 3, accounting for the higher zero time value. Moreover, ganglia grown in anti-NGF or in NGF + anti-NGF exhibited the same 3-fold increase (Fig. 4). Consequently, added NGF was not necessary for normal development of ganglion enzyme activity. Although added NGF was apparently not necessary for ganglion development, addition of NGF was associated with stimulation of growth, resulting in an 8.6-fold increase in enzyme activity compared to zero time controls (Fig. 4). Neurite development paralleled biochemical differentiation: extensive fiber growth occurred in basal medium without added NGF. Medium with anti-NGF and with anti-NGF + NGF supported neuritic growth equivalent to that seen in basal medium, whereas addition of NGF elicited a more dense elaboration of fibers (Fig. 5). Although NGF elicited outgrowth from 14-day ganglia, the NGF-independent outgrowth in basal medium or medium with anti-NGF was most striking. Such NGF-independent development was not observed in later fetal ganglia (compare Fig. 7).

To determine whether growth requirements, and responses, were similar later in gestation, ganglia were removed from 18-day fetuses and grown in the different media described above. Tyr-OH activity not only failed to increase, but decreased by half in the absence of added NGF (Fig. 6). Similar decreases occurred in media containing anti-NGF and NGF + anti-NGF (Fig. 6). On the other hand, addition of NGF to the medium resulted in over a 3-fold increase in Tyr-OH activity.

Neurite development and biochemical differentiation were again comparable. Virtually no axon outgrowth occurred in the *absence* of added NGF or in media containing anti-NGF or NGF + anti-NGF (Fig. 7). By contrast, addition of NGF resulted in a dense halo of neurites after 48 hr in culture (Fig. 7).

DISCUSSION

Although the postnatal development of the mammalian SCG has been studied extensively, regulation of its embryologic development is essentially unknown. The present investigations were undertaken to systematically analyze the morphological and biochemical ontogeny of embryologic mammalian sympathetic neurons *in vivo* and in culture.

The ganglion undergoes profound developmental changes well before birth. Tyr-OH activity increases 100-fold from the time the ganglion condenses as a discrete mass at 13 days of gestation to birth at 19 days. Since total ganglion protein rises 4-fold during this period, there is approximately a 25-fold increase in Tyr-OH specific activity. By comparison, Tyr-OH activity in mouse SCG increases only 6- to 8-fold during all of postnatal development (3, 7). It is of additional interest that substantial Tyr-OH activity is present when the ganglion is first recognizable as a discrete structure. It may be inferred that initial expression of this information encoded within the developing neuron occurs when or before the neurons arrive at their definitive site(s). The early development of Tyr-OH activity in these mammalian neurons is consistent with reports which indicate that the neurotransmitter fate of chick sympathoblasts is determined very early in embryologic life (31, 32).

Tissue culture techniques were used to perform environmental manipulations impossible *in vivo.* Initial studies indicated that during a specific segment of embryologic growth,

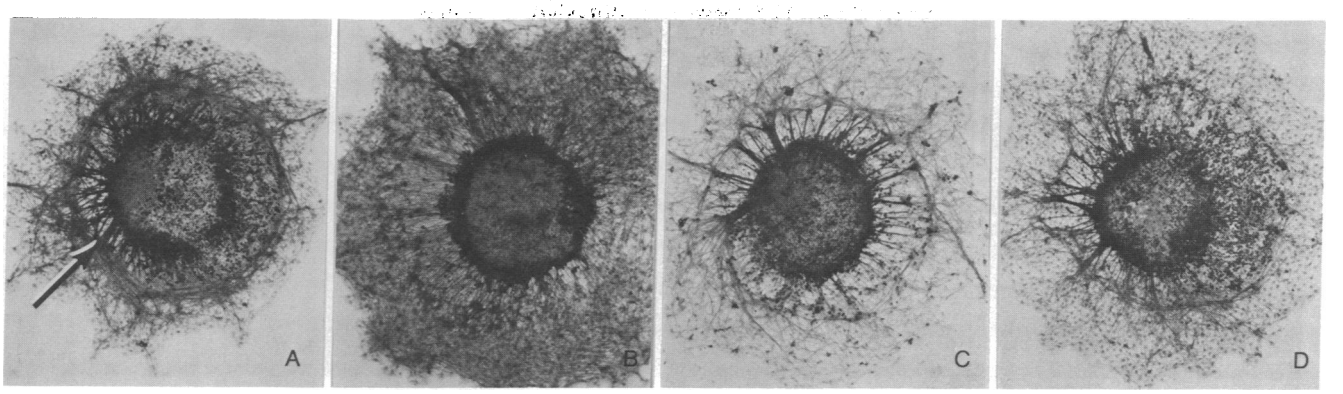

FIG. 5. Embryonic ganglia cultured under different conditions and stained supravitally with Janus Green B. Fourteen-day gestational age ganglion explants were cultured for 48 hr under the conditions described in Fig. 4. (*A*) SCG grown in basal medium without added NGF. Thick bundles of fibers radiate from ganglionic cell mass and terminate in circular neurite meshwork (compare with Fig. 1). (*B*) SCG in basal medium + NGF. Dense outgrowth of fibers extends from cell mass. Neuritic production is much greater than in basal medium alone. (*C*) SCG in basal medium + anti-NGF. The extent and pattern of neurite outgrowth is essentially the same as in basal medium alone. (*D*) SCG in basal medium + NGF + anti-NGF. The extent and pattern of neurite development is basically the same as in basal medium alone. (×33.)

development *in vitro* corresponded to that *in vivo*. Over a 3-day period the ontogenetic increase of Tyr-OH activity in cultured 14-day ganglia paralleled that *in vivo*. After 3 days in basal medium the embryonic ganglia began degenerating and Tyr-OH activity decreased. In contrast, 18-day ganglia degenerated from culture day zero in basal medium, suggesting that 14-day ganglia cultured for 3 days are comparable to 18-day ganglia *in vivo*, and that growth requirements may change with age. Having concluded that cultured ganglia adequately reflect development *in vivo*, experiments were performed to define regulatory mechanisms.

Requirements for growth and development changed radically during embryologic and fetal life as indicated above. Ganglia from 14-day embryos exhibited abundant neurite outgrowth and a 3-fold increase in Tyr-OH activity in the absence of added NGF, or in the presence of anti-NGF concentrations that entirely inhibited outgrowth from neonatal ganglia. In direct contrast, ganglia from 18-day fetuses exhibited a 50% *decrease* in Tyr-OH activity and virtually no axon elaboration in the absence of added NGF, or in the presence of anti-NGF, in agreement with previous results obtained with neonatal ganglia (31). However, in both the 14- and 18-day ganglia, addition of NGF to the medium resulted in augmented neurite extension and enzyme activity.

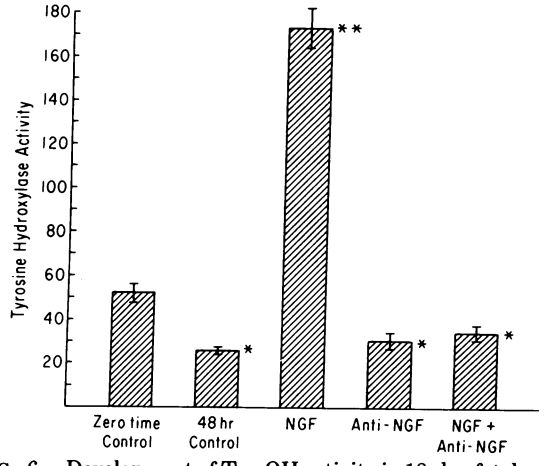

FIG. 6. Development of Tyr-OH activity in 18-day fetal ganglia in culture under different conditions. Ganglion pairs were removed from groups of 4–5 mice at 18 days of gestation and were grown for 48 hr as described in Fig. 4. * Differs from zero time control at $P < 0.02$. Does not differ from other groups with single asterisk ($P > 0.05$). ** Differs from all other groups at $P < 0.001$.

These observations suggest that there are fundamental ontogenetic differences between embryologic and late fetal ganglia. Our results may indicate that NGF is not an absolute requirement for differentiation of the 14-day ganglion. However, other explanations may be considered. It is possible that in 14-day, but not 18-day, ganglia, support cells produce NGF or an NGF-like substance. This appears unlikely, since the neurons developed even in the presence of anti-NGF, and since in previous reports anti-NGF has attenuated the putative influence of support cells (25). Nevertheless, it is conceivable in our studies that support cells directly transferred NGF to neurons in an antibody-resistant form in 14-day, but not 18-day, ganglion cultures. Alternatively, different subpopulations of neurons may develop at different times in the ganglion. Thus, the 14-day ganglion may contain a subpopulation that differentiates independent of NGF, as well as one that requires NGF. By 18 days of gestation, the entire ganglion population may require NGF. Such a postulate would imply, however, either disappearance of the NGF-independent population by 18 days of gestation or the development of an NGF requirement in this population. We are presently attempting to distinguish among the foregoing alternatives. Moreover, previous work has indicated that treatment of pregnant mice with anti-NGF effects the SCG of the progeny only after the critical 16th day of gestation (23, 24). Consequently, we would tentatively favor the view that initial biochemical and morphologic development does not require NGF.

Regardless of the mechanisms that ultimately prove to be involved, our data suggest that regulatory influences differ markedly in embryologic and late fetal ganglia. Such differences during prenatal development have been defined in nonmammalian systems. Early studies revealed that *chick* sympathetic and sensory ganglia respond to NGF-producing tumors only after the seventh day *in ovo* (34). Existence of a critical period of sensitivity during development has also been documented by experiments *in vitro*. Sympathetic ganglion explants from chick embryos do not respond to NGF at 8 days, become minimally responsive at 9–10 days, and exhibit maximum sensitivity at 13–14 days (35). Similar development of sensitivity occurs in chick sensory ganglia: NGF effects are first present at 6 days (36, 37) and become maximal at 7–9 days (34, 36). However, very early (4-day) spinal ganglion neurons do not require added NGF for development in culture (37, 38).

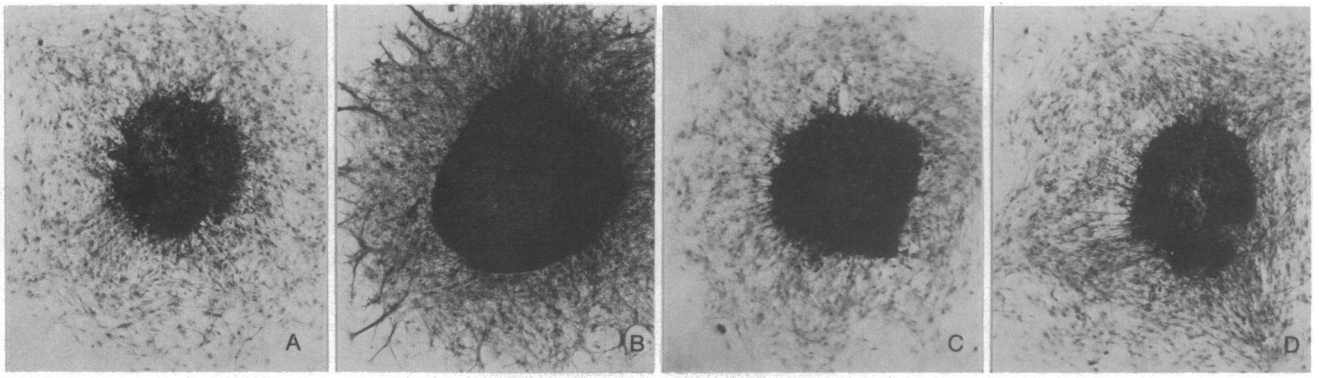

FIG. 7. Late fetal ganglia in culture stained supravitally with Janus Green B. Ganglia were removed from mice on the 18th day of gestation and cultured for 48 hr as described in Fig. 6. (*A*) SCG grown in basal medium without added NGF. A few neurites extend from the cell mass, but axon production is minimal compared to *B*. (*B*) SCG grown in basal medium + NGF. Typical dense halo of neurites surrounds cell mass. (*C*) SCG cultured in basal medium + anti-NGF. (*D*) SCG cultured in basal medium + NGF + anti-NGF. Both *C* and *D* exhibit practically no neurite production and are similar to the SCG grown in basal medium alone. Compare to the relatively extensive outgrowth from 14-day gestational age explants in corresponding media (Fig. 5). (×33.)

Lastly, our observations suggest that *abnormally* extensive biochemical and morphological growth may occur in culture in the presence of NGF. Consequently, the injudicious use of such growth-promoting factors *in vitro* may lead to results not reflective of normal growth and development *in vivo*.

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