Principal Neurons and Small Intensely Fluorescent (SIF) Cells in the Rat Superior Cervical Ganglion Have Distinct Developmental Histories

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Sympathetic ganglia contain 2 adrenergic derivatives of the neural crest: principal neurons and small, intensely fluorescent (SIF) cells. The developmental mechanisms responsible for the generation of these 2 cell classes *in vivo* are not well understood. To examine the possible developmental and lineage relationships between differentiating principal neurons and SIF cells, a fluorescence microscopic study utilizing antibodies against tyrosine hydroxylase (TH) and catecholamine histofluorescence has been combined with the ultrastructural examination of embryonic and postnatal rat superior cervical ganglia (SCG). On embryonic day 12.5, before neuroblasts had become postmitotic, the cells in the SCG possessed intense TH immunoreactivity and had weak to bright catecholamine histofluorescence, but no cells displayed the fine structure of mature SIF cells or neurons. At embryonic days 16.5 and 18.5, postmitotic principal neurons expressed more moderate levels of TH and catecholamines characteristic of the late embryonic and postnatal SCG. By contrast, a small number of cells containing intense TH or catecholamine fluorescence were present in embryonic day 16.5 and older ganglia. Almost all of the intensely fluorescent cells observed were found apposed to capillaries within the ganglion. These embryonic intensely fluorescent cells were larger than SIF cells seen postnatally. Ultrastructural examination of developing ganglia confirmed that cells containing numerous large, dense-cored vesicles (LDCVs) were a prominent feature of ganglia that also contained intensely fluorescent cells. In addition, some embryonic cells containing LDCVs were mitotic. From these and other studies, it seems likely that during development, neuron precursors, in response to differentiation factors such as fibroblast growth factor (FGF) and/or NGF, acquire overt neuronal traits and become postmitotic. Subsequently, cells resembling mature SIF cells appear next to blood vessels, where they may have received other instructional signals such as glucocorticoids. This developmental scheme suggests that the differentiation of principal neurons and SIF cells is independently regulated, and that the ability of SIF cells to convert into principal neurons observed *in vitro* cannot account for the generation of neurons *in vivo*.

The neural crest gives rise to several kinds of adrenergic cell types (Le Douarin, 1976; Landis and Patterson, 1981), including principal neurons and small, intensely fluorescent (SIF) cells found in the sympathetic ganglia, as well as glomus cells of the carotid and aortic bodies, and adrenal chromaffin cells. Although all of these cells contain catecholamines, they differ in their use of particular biogenic amines and/or in their anatomical locations. In the rat, the mature superior cervical ganglion (SCG) contains approximately 30,000 noradrenergic principal neurons and 600 dopaminergic SIF cells (Björklund et al., 1970; Davies, 1978; Eränkö and Soinila, 1981; Smolen et al., 1983). Some SIF cells receive preganglionic innervation, have occasional short cell processes, and have been suggested to act as interneurons (Eccles and Libet, 1961). The carotid and aortic bodies, which are invested with blood vessels, contain cells that resemble SIF cells, but lack short, axonlike processes and contain predominantly dopamine and some norepinephrine (Christie and Hansen, 1983; Chen et al., 1985). Chromaffin cells in the adrenal gland contain noradrenaline and many also contain adrenaline. Ultrastructurally, these adrenergic derivatives each contain osmiophilic membrane-bound vesicles whose size and character are quite distinctive. SIF cells and carotid body cells contain large, dense-cored vesicles (LDCVs) with a diameter of approximately 100 nm in their cytoplasm (Eränkö and Härkönen, 1963, 1965; Grillo, 1966; Norberg et al., 1966; Siegrist et al., 1968; Matthews and Raisman, 1969; Williams and Palay, 1969; McDonald and Blewett, 1981), while sympathetic principal neurons contain a small number of 50-nm granular vesicles (Matthews and Raisman, 1969; Williams and Palay, 1969). Adrenal chromaffin cells contain the largest and most conspicuous granular vesicles, with an average diameter of 200 nm (Coup-land, 1965).

Sympathetic neurons, SIF cells, and chromaffin cells have been suggested to develop from similar embryonic precursors, which reside in ganglionic and adrenal anlagen (Landis and Patterson, 1981; Anderson and Axel, 1986; Anderson, 1989) and whose fates are determined by environmental cues. Cell culture studies have shown that chromaffin cells require glucocorticoids to maintain their phenotype, and that, without glucocorticoids, some chromaffin cells may transdifferentiate into neurons under the influence of fibroblast growth factor (FGF) or NGF (Ogawa et al., 1984; Doupe et al., 1985a; Unsicker et al., 1985; Stemple et al., 1988). During that conversion, chromaffin cells assume SIF cell phenotype. The SIF phenotype appears to be induced or maintained by glucocorticoids both *in vitro* and *in vivo* (Eränkö, 1976; Doupe et al., 1985b), while principal neurons require NGF for their survival (Levi-Mon-

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talchini and Angeletti, 1963; Chun and Patterson, 1977; Greene, 1977; Coughlin and Collins, 1985). These studies have documented the interconversion of sympathoadrenal cell classes and have identified candidate molecules that influence cell determination in this lineage in culture. While the medullary environment is clearly distinct from that in the sympathetic ganglion, SIF cells and principal neurons differentiate alongside each other within the SCG, and it is less obvious what factors are responsible for inducing and/or maintaining either SIF or principal neuron differentiation *in vivo*. While both of these cells have their origins in the neural crest, it is less clear exactly when principal neurons and SIF cells arise during development, and the phenotypes of their cellular precursors have proven elusive.

The present study was undertaken to examine the pre- and postnatal genesis of SIF cells, to understand its relation to principal neuron maturation, and to examine the hypothesis that the neuronal precursor *in vivo* resembles a mature SIF cell (Doupe et al., 1985b). To assay the phenotype of neuronal precursors, it was important to examine ganglia before postmitotic neurons were found. In studies using tritiated thymidine autoradiography, a peak in neuronal birthdates in the rat SCG was observed on embryonic day 14 (E14); few neurons, in fact, have their final cell division before E13, and virtually all neurons have been generated before birth (Hendry, 1977; Landis and Damboise, 1986). Therefore, we examined SCG containing neuronal precursors (E12.5), ganglia containing some young neurons (E14.5, E16.5), and postmitotic neurons of various maturational states [E18.5, postnatal day 0 (PO), P7, and adult]. To examine precursor cells, as well as developing principal neurons and SIF cells, catecholamine histofluorescence, tyrosine hydroxylase (TH) immunoreactivity, and electron microscopy were applied to the developing rat SCG. Our findings suggest that, while principal neurons and SIF cells derive from similar precursors, these precursors do not resemble a mature SIF cell. In addition, mature SIF-like cells are first apparent in the SCG after most neurons have become postmitotic, suggesting that the phenotypic development of SIF cells and principal neurons is regulated by different developmental cues within the ganglion.

Materials and Methods

Timed pregnant Sprague-Dawley rats were obtained from Zivic Miller Co. (Zelienople, PA). The day after evening mating was considered E0.5. Embryos of appropriate developmental ages were staged according to Christie (1962).

Glyoxylic acid histochemistry. Catecholamine histofluorescence was demonstrated using the glyoxylic acid method of de la Torre (1980). Ganglia from embryos at E14.5 or older were quickly dissected and frozen in OCT embedding medium (Miles Laboratories). At E12.5, whole embryos were used and sectioned in the sagittal plane. In all cases, ganglia and embryos were oriented to produce sections in the long axis. Ten-micron cryostat sections were collected on slides and immediately dipped 3 times in the glyoxylic acid solution. Excess glyoxylic acid was removed, and the slides were dried under a cool stream of air for 20–30 min. Several drops of light mineral oil were applied to the sections, and the slides were reacted at 96°C for 3 min. The slides were mounted in mineral oil for examination by fluorescence microscopy.

Tyrosine hydroxylase immunocytochemistry. To localize TH, the rate-limiting enzyme for catecholamine synthesis, immunohistochemistry using a monoclonal antibody (the kind gift of A. Acheson, University of Alberta, Edmonton, CAN; Rohrer et al., 1986) or polyclonal antiserum (the kind gift of J. Thibault, College de France (Paris, France); Thibault et al., 1981) was employed. Animals older than E18.5 were fixed by perfusion with freshly prepared 4% paraformaldehyde/0.1 M phosphate buffer (pH, 7.4). Tissue wedges containing the ganglia of younger embryos were fixed by immersion in the same fixative. In each

case, tissue or ganglia were fixed at room temperature for 1–2 hr. Ganglia were then dissected free (except for E12.5), rinsed in 0.1 M phosphate buffer (pH, 7.4), and placed in 30% sucrose/0.1 M phosphate buffer (pH, 7.4) overnight at 4°C to cryoprotect the tissue. Excess sucrose solution was removed, the tissue was frozen in OCT medium, and 10- μ m sections were collected on gelatin-subbed slides. Tissue sections were then incubated overnight at room temperature with anti-TH antibodies at 1:2000 in dilution buffer (containing 3% BSA, 0.1% sodium azide, 0.3% Triton X-100, and 0.5 M NaCl in 0.01 M phosphate buffer, pH 7.3) with 10% rat serum added to block nonspecific binding. Slides were rinsed and incubated for 2 hr at room temperature with the appropriate goat secondary antibody at 1:200 (Cappel, Organon-Teknika) with 20% goat serum added. Slides were then washed and mounted in 2% *n*-propyl gallate (Sigma) in PBS/glycerol to reduce fluorescence bleaching (Giloh and Sedat, 1982). For immunocytochemical studies, multiple sections in the longitudinal plane from ganglia of at least 3 different animals were examined.

Electron microscopy. For ultrastructural analysis, tissue was fixed in 4% paraformaldehyde and 3% glutaraldehyde in 0.12 M phosphate buffer (pH, 7.35) to preserve ultrastructural characteristics of the SCG and to demonstrate large, dense-cored vesicles (LDCVs). With embryonic ganglia, tissue blocks containing the SCG (E12.5–14.5) were immersed in fixative at room temperature for 2 hr, then the wedge (E12.5) or ganglion (E14.5) was dissected free and placed in the same fixative overnight at 4°C. Intracardiac perfusion was performed on animals at E18.5 and older using the above fixative before removing the ganglia for postfixation overnight at 4°C. Tissue was then washed in 0.12 M phosphate buffer and placed in 1% osmium/0.12 M phosphate buffer for 1 hr on ice. After washing, tissue was stained en bloc using 2% aqueous uranyl acetate overnight at 4°C. The tissue was then dehydrated in ethanol and embedded in Polybed 812 (Polysciences). Semithin (0.5 μ m) sections were stained with toluidine blue to reveal overall ganglion morphology. Thin sections were collected on 0.5% Formvar-coated 1 $\times$ 2 mm slot grids and poststained with uranyl acetate and lead citrate. Photomontages of the ganglia were prepared at approximately 2000 $\times$ negative magnification. In addition, individual cells were photographed at higher magnifications (5000 $\times$ and 10,000 $\times$) in order to demonstrate structural details, including the presence of LDCVs. To analyze the number of LDCVs per ganglionic cell, only cell profiles with a nucleus were included, such that a cross-section of that cell could reasonably be assumed. To estimate the number of LDCV-containing cells found at E12.5 and E14.5, the total number of individual nucleated cells observed to contain LDCVs was compared with the total number of nucleated cells observed. These estimates were made at E18.5 by comparing the number of LDCV-containing cells in a section with the total number of nucleated cells (including non-neuronal cells) in the previous section, stained with toluidine blue. For these LDCV counts, an individual longitudinal section from a whole ganglion from at least 2 different animals was examined.

Results

Mature SIF cell characteristics

In the adult rat, the SCG is comprised of noradrenergic principal neurons containing catecholamines, which may be demonstrated by glyoxylic acid histochemistry, as well as immunoreactivity for the catecholamine synthetic enzyme TH (Eränkő and Härkönen, 1963). In addition, the SCG contained SIF cells that had a smaller diameter (10–15 μ m, compared with 20–40 μ m) and stained more intensely for glyoxylic acid-induced catecholamines as well as TH immunoreactivity than did principal neurons (Fig. 1). SIF cells were generally found in clusters of 2–10 cells, and the number of TH-immunoreactive SIF cells was quite small, such that a typical 10- μ m section through the length of the ganglion would contain from 0 to 2–3 clusters. The clusters of SIF cells were commonly associated with ganglionic capillaries. In addition, mature SIF cells have a characteristic ultrastructural morphology, with abundant polyribosomes and mitochondria, and most notably containing numerous LDCVs, which are evident using conventional aldehyde fixation (Fig. 2). Mature SIF cells in the rat SCG contain over 100 LDCVs in a

Figure 1. SIF cells in adult rat SCG had intense staining for TH immunoreactivity and catecholamines. The adult rat SCG contained clusters of SIF cells that were much smaller (*top*) than the surrounding principal ganglion cells, which were also TH immunoreactive. The TH staining in SIF cells (*bottom left*) was much brighter than that seen in principal ganglion cells. Similarly, SIF cells contained higher levels of catecholamines (*bottom right*) than principal ganglion cells. In each case, the figure represents a frozen section of adult rat SCG labeled by indirect immunofluorescence with TH antibodies (*top, bottom left*) or glyoxylic acid histofluorescence (*bottom right*). Scale bars, 20 μ m.

nucleated cell profile (see Matthews and Raisman, 1969), many of which are found at the cell periphery near the plasma membrane. Principal neurons do not contain any LDCVs of this size, though smaller neuronal vesicles, thought to contain neuropeptides and/or catecholamines, are present (Hökfelt, 1969; Hökfelt et al., 1980). It is clear that the same cells in the ganglion account for the intense monoamine fluorescence as well as the large numbers of LDCVs observed ultrastructurally (Grillo et al., 1974). Thus, mature SIF cells are clearly distinguished from principal neurons in the ganglion by their small size, intense

catecholamine histofluorescence, bright TH immunoreactivity, and presence of numerous LDCVs within their cytoplasm.

The developing rat SCG

Glyoxylic acid-induced catecholamine histofluorescence

From the time the ganglion anlagen was first apparent during embryogenesis, catecholamines could be demonstrated in the SCG using glyoxylic acid-induced histofluorescence (Fig. 3). The catecholamine content of precursor cells was variable at E12.5 such that the ganglion included both weakly and intensely flu-

Figure 2. SIF cells in adult rat SCG had a characteristic ultrastructure. Mature SIF cells had a small cytoplasm-to-nucleus ratio, with many free ribosomes and rough endoplasmic reticulum, but the most characteristic feature of these cells was the presence of numerous LDCVs found near the plasma membrane (*left*). The LDCVs are shown at higher magnification in the *inset* (*right*). Scale bars: 1 μ m, *left*; 0.3 μ m, *right*.

orescent cells. By E14.5, however, catecholamine histofluorescence was generally less pronounced and appeared homogeneous in all catecholamine-stained ganglion cells. At E16.5, the young neurons and remaining neuron precursors, which together comprised the majority of the cells in the SCG, had moderate levels of catecholamine fluorescence. In addition to these moderately staining cells, individual cells with intense staining for catecholamines were seen at E16.5 and in older ganglia. The intensely stained cells were frequently found near blood vessels at all stages examined. By birth, the intensely fluorescent cells were more numerous, and by P7, many intensely stained cells were found in clusters.

Tyrosine hydroxylase immunoreactivity

TH immunoreactivity by cells in embryonic rat SCG changed during development (Fig. 4). All cells in the rat SCG at E12.5 stained very brightly for TH when assayed by immunocytochemistry. By E14, during the peak of neuronal birthdates, TH staining throughout the ganglion had decreased somewhat in

intensity. At E16.5 and E18.5, while the maturing principal neurons in the ganglion displayed moderate reactivity for TH, intensely TH-reactive cells appeared in the SCG. A 10- μ m cryostat section from an E18.5 ganglion containing approximately 3000 cells could contain as many as 10 such intensely fluorescent cells. The intensely fluorescent cells seen at E16.5 and E18.5 were approximately the same size as the moderately staining neurons in the same section. Beginning with their initial appearance at E16.5/18.5, the brightly stained cells were found adjacent to blood vessels. Three-fourths of the intensely TH-immunoreactive cells at E18.5 were apposed to capillaries visible in a 10- μ m section (80 cells examined). The strongly TH-immunoreactive cells are likely to correspond to the cells that contain large amounts of catecholamines described above, because of their size and location near blood vessels. At birth, maturing principal neurons maintained moderate levels of TH immunofluorescence, and the intensely TH-immunoreactive cells were somewhat more numerous. By P7, the brightly TH-immunofluorescent cells were generally clustered.

Figure 3. Catecholamine histofluorescence in the rat SCG. Glyoxylic acid was used to localize catecholamines in fresh-frozen 10- μ m sections of rat SCG. In the E12.5 SCG anlagen, there were both brightly and weakly fluorescent cells. By contrast, at E14.5, cells in the ganglion exhibited relatively low levels of catecholamine histofluorescence. At E16.5, while most ganglion cells continued to express low levels of catecholamine histofluorescence, individual cells with higher catecholamine levels were seen. At E18.5 and PO, intensely catecholamine-fluorescent cells were more numerous. At P7, cells with high levels of catecholamine were generally found in clusters. The photographs were printed to emphasize the staining in the bright catecholamine cells, such that other ganglion cells actually contain more catecholamine than is apparent. Scale bar, 20 μ m.

The size of both neurons and intensely fluorescent cells changed during development. Principal neurons were larger at birth and in the postnatal animal than their embryonic precursors. Cells in the E12.5, E14.5, E16.5, and E18.5 ganglia were uniformly small when observed by TH immunoreactivity or in semithin plastic sections. From birth onward, the cell diameter of developing principal neurons increased dramatically. NGF is a likely trigger for cell diameter growth in principal neurons of

the embryonic and postnatal SCG. Principal neurons become dependent on NGF at approximately E17/18 (Coughlin and Collins, 1985) and may be responsive to the factor earlier in embryogenesis; therefore, the increase in neuronal size may be due to the cells' response to this target-derived growth factor. By contrast, the intensely fluorescent cells seen at E16.5 and E18.5 were larger than the SIF cells observed 1 week after birth (see Fig. 4).

Figure 4. TH staining of rat SCG. Ten-micron longitudinal cryostat sections of rat SCG were stained with antibodies to TH. At *E12.5*, virtually all sympathoblasts exhibited very bright TH immunofluorescence restricted to the elongated ring of cytoplasm around the nonstaining nucleus, as well as some staining in processes. The ganglion at this stage had not completely coalesced, but consisted of a long stream of cells along the dorsal aorta. At *E14.5*, ganglion cells appeared rounder and more densely packed, but exhibited bright TH staining. By *E16.5*, most cells in the ganglion exhibited moderate TH staining overall. However, a few intensely TH-immunoreactive cells were seen at this age, which were brighter than the majority of cells at *E14.5* (but similar to cells at *E12.5*). Most cells in the ganglion at *E18.5* and *P0* exhibited TH staining similar to that seen at *E16.5*, except that the individual intensely TH-immunoreactive cells were more numerous. By *P7*, the intensely TH-immunoreactive cells were smaller in size and were more commonly found in clusters. Most TH-immunoreactive cells remained approximately the same small size from *E12.5* to after *E18.5*, when the principal neurons in the SCG increased in diameter. By contrast, the intensely fluorescent cells that appeared at *E16.5/18.5* were about the same size as the moderately staining principal neurons at these ages, but intensely fluorescent cells decreased in diameter after birth. In each case, fields were selected to show intensely fluorescent cells, and therefore, the prevalence of these cells is not representative of their total number. Scale bar, 20 μm .

Ganglion cell ultrastructure

The morphological organization of the SCG changed with development (Fig. 5). At *E12.5*, the SCG primordium appeared as a column of morphologically similar cells found along the dorsal aorta, surrounded by mesenchymal cells. At *E14.5*, the

SCG was a discretely segregated ganglion and contained mitotic cells, but lacked both a capsule and blood vessels. A small number of capillaries were seen in the *E16.5* ganglion, primarily at the perimeter of the ganglion (data not shown). By *E18.5*, several changes in the ganglion were observed. A distinct connective tissue capsule surrounded the ganglion, and numerous

Figure 5. Cellular morphology of rat SCG. Toluidine blue-stained semithin sections were examined for overall ganglion morphology. At *E12.5*, the SCG appeared as a stream of relatively homogeneous cells along the dorsal aorta. At *E14.5*, the ganglion cells were more closely packed, and mitotic figures were seen (arrows). By *E18.5*, several changes have occurred in the ganglion. A distinct capsule (asterisk) not present at *E14.5* had formed around the ganglion. Furthermore, blood vessels (*bv*) were commonly seen in the ganglion at *E18.5*, but were absent at *E14.5*. At birth (*PO*), principal neurons were larger than those seen during embryogenesis. Scale bar, 20 μ m.

capillaries were evident within the ganglion. In addition, cellular heterogeneity within the ganglion was more pronounced at *E18.5*, when maturing neurons were clearly identifiable by morphology and non-neuronal cells became numerous. By birth, the ganglion had assumed a more adult organization, with maturing principal neurons, regions of neuropil, and increasing numbers of non-neuronal satellite cells.

Precursor cells in the ganglion at *E12.5* had the ultrastructural appearance of undifferentiated cells (Fig. 6). They contained numerous polyribosomes, few mitochondria, and little rough endoplasmic reticulum in their cytoplasm. The cells that will make up the SCG are still migrating rostrally at *E12.5*, and an elongated appearance (in the direction of migration) is exhibited by most cells at this stage. All cells in a longitudinal section from 2 different ganglia were assayed for the presence of LDCVs, which occur in large numbers in mature SIF cells. Forty percent

of the nucleated cell profiles in these ganglion sections contained no LDCVs, while cell profiles containing LDCVs contained only a few vesicles (average, 5 per cell profile; 104 nucleated cells counted; Fig. 7). While mature SIF cells exhibit many LDCVs near and associated with their plasma membranes, at *E12.5*, few such vesicles were seen associated with the membrane, but rather were often found associated with the Golgi apparatus. Thus, cells in the *E12.5* ganglion appeared relatively immature and did not have the ultrastructural characteristics of either mature SIF cells or neurons.

At *E14.5*, the peak of neurogenesis, mitotic cells were present in the ganglion, and some cells had begun to acquire overtly neuronal characteristics: the cell bodies were more rounded and contained more organelles. In addition, some cells possessed neurites containing smooth endoplasmic reticulum and microtubules. Most cells in a section of the ganglion did not contain

Figure 6. Cells at E12.5 appeared undifferentiated. *a*, The ganglion anlage at E12.5 consisted of loosely associated and relatively undifferentiated cells. Large spaces were seen between the sympathoblast cells, and many cells had an elongated appearance consistent with migrating cells. Many cells did not contain any LDCVs. Some cells had a few LDCVs in their cytoplasm, and many of these vesicles were found deeper in the cytoplasm, rather than at the plasma membrane (*arrows*). In each case, the cell cytoplasm was relatively abundant, the Golgi apparatus was undeveloped, and few mitochondria were apparent, suggesting that these cells were immature. *b*, Cells containing LDCVs frequently had vesicles near the Golgi apparatus, rather than close to the plasma membrane. *c*, Some LDCVs were also seen in processes in the E12.5 ganglion. Scale bars: 2 μm , *a*; 1 μm , *b* and *c*.

Figure 7. Histogram of LDCVs in sympathoblast cell profiles at E12.5. All nucleated cells in an individual sagittal section were assayed for the presence of LDCVs in a cell profile in 2 E12.5 ganglia from different animals.

any LDCVs, while a small population had an average of 30 LDCVs per cell profile, and no cell had more than 59 (15 nucleated cells with LDCVs counted). Cells containing LDCVs had more abundant rough endoplasmic reticulum, and more granules appeared associated with the membrane than observed at E12.5, but LDCV-containing cells were otherwise difficult to distinguish from other ganglion cells at E14.5. However, while

granule-containing cells contained more numerous LDCVs than at E12.5, the number of cells with *any* dense-cored vesicles was quite small (approximately 15 per 500 cells).

At E18.5, LDCV-containing cells with an ultrastructural morphology similar to mature SIF cells were seen (Fig. 8). Cells containing LDCVs comprised a very small proportion of the cells in the ganglion (approximately 10 per 3000 cells, including

Figure 8. Cells containing numerous LDCVs were found near blood vessels at E18.5 and resembled mature SIF cells. While LDCV-containing cells were not common, these cells were found located near blood vessels in the ganglion. These cells resembled the mature SIF cells found in the postnatal ganglion in that both organelles and LDCVs were numerous. Golgi apparatus, rough endoplasmic reticulum, and mitochondria were seen in these cells. Cells or processes containing vesicles directly apposed the perivascular space (*inset*). Scale bar, 1 μ m.

Figure 9. Some cells containing LDCVs at E18.5 were mitotic. A cell from E18.5 with numerous LDCVs contained chromosomes (*Ch*) in the anaphase stage of mitosis. The *arrows* indicate some of the LDCVs found at the plasma membrane; many were also seen elsewhere in the cytoplasm. Scale bar, 1 μ m.

non-neuronal cells). These cells, though not numerous, had many LDCVs (average, 62 LDCVs/cell profile; 16 nucleated cells with LDCVs counted) and presumably corresponded to the cells that stained brightly for TH and glyoxylic acid-induced catecholamine histofluorescence. At least 75% of LDCV-containing cells were found apposed to capillaries within the ganglion (in individual sections from 2 ganglia), and extensive areas of the SIF cell surface were separated only by basement membrane from the perivascular spaces of the capillaries. In addition, small cell processes filled with dense-cored vesicles were found close to capillaries (Fig. 8), suggesting that cells contacted blood vessels both by apposition of the cell body and by cell processes. In addition, some of the LDCV-containing cells at E18.5 contained mitotic figures, indicating that embryonic cells with the SIF-like phenotype can divide *in situ* (Fig. 9).

Discussion

The present study indicates that principal neurons and SIF cells appear at different times and in different locations in the embryonic SCG, suggesting that these cell types may be affected by distinct developmental regulatory cues. Neuroblasts, which initially express TH and high levels of catecholamines, do not begin to stop cell division until E14. Ultrastructurally, the neuronal precursor cells present at E12.5 lack the characteristics of mature SIF cells and principal neurons, but by late embryogenesis (E18.5), postmitotic principal neurons are morphologically distinct in the ganglion. By contrast, cells with intense TH immunoreactivity, catecholamine histofluorescence, and numer-

ous LDCVs characteristic of SIF cells appear at E16.5/18.5 near blood vessels in the ganglion. In addition, some of the cells containing LDCVs are mitotic. These embryonic cells are larger than SIF cells observed postnatally and thus may represent SIF cell precursors.

Precursor cells in the SCG acquire either neuronal or SIF features during embryonic development

Sympathoblasts present in the embryonic SCG before any neurons had undergone their final cell division contained catecholamines and the catecholamine synthetic enzyme TH, but ultrastructurally, appeared relatively undifferentiated. Although precursor cells in the E12.5 ganglion displayed many catecholaminergic traits, including bright TH immunoreactivity and variable levels of catecholamines (see also Cochard et al., 1979), they continued to divide despite this phenotypic specialization (Rothman et al., 1978; Rohrer and Thoenen, 1987; DiCicco-Bloom and Black, 1988; DiCicco-Bloom et al., 1990). While the intense catecholamine histofluorescence and TH immunoreactivity seen in many E12.5 cells are 2 properties of SIF cells, ultrastructurally the precursor cells in the rat do not resemble mature SIF cells, as only some cells possess the characteristic LDCVs, and these cells contain only a few. These results confirm and extend the findings of Soinila and Eränkő (1985), which described only "occasional" cells at E12.5 that possessed any LDCVs. Furthermore, in cells that did contain LDCVs at this stage, these vesicles tended to be present near the Golgi apparatus (the characteristic site of neuronal vesicles in the soma)

and not at the plasma membrane. In agreement with the present findings, a recent report on E15.5 rat SCG precursor cells placed in tissue culture for 1 d indicated that the neuroblasts appeared ultrastructurally immature, rarely contained LDCVs, and did not acquire large numbers of these vesicles for several days in culture (DiCicco-Bloom et al., 1990). Furthermore, in the neonatal rat SCG, cells that may be experimentally induced *in vitro* to become either neurons or SIF cells (thereby having the properties of precursor cells) also initially appear ultrastructurally immature (Doupe et al., 1985b). Thus, the precursors that give rise to differentiated principal neurons and SIF cells in the SCG exhibit catecholaminergic properties, but do not display the ultrastructure characteristic of mature SIF cells found at later times in the ganglion. Indeed, the appearance of mature SIF-like cells in the developing SCG does not correspond temporally or numerically with the final divisions of precursors to produce postmitotic neurons. During the peak period of neuronal birth-dates (E14.5), catecholamine and TH staining intensity have already begun to diminish in the ganglion, but very few cells containing even small numbers of dense-cored vesicles are seen. Not enough LDCV-containing cells are present at E14.5 to account for the large number of neurons born during this period (Hendry, 1977; Landis and Damboise, 1986). Furthermore, LDCV-containing cells also cannot represent an intermediate developmental form in the ganglion between a precursor cell and a neuron, because they are not sufficiently numerous. The percentage of ganglion cells that contained any LDCVs decreased between E12.5 and E14.5, perhaps due to the continued proliferation of precursor cells lacking any vesicles. Thus, it is possible that the few cells at E14.5 containing LDCVs (but lacking intense TH and catecholamine expression) represent the earliest SIF precursors, whose full complement of SIF properties is not acquired until late embryogenesis. What remains unclear is whether cells of the SIF and neuronal lineages become committed at the same time, or whether SIF cells are generated later, from a residual precursor population that has not yet committed to the neuronal pathway.

Cells that had high levels of catecholamine histofluorescence, intense TH immunoreactivity, and an ultrastructure resembling that of mature SIF cells were first seen between E16.5 and E18.5 in cells found apposed to ganglionic capillaries. The E14.5 rat SCG was not invested with capillaries and did not contain cells with intense catecholamine fluorescence. The E16.5 ganglion appeared to have a very small number of capillaries, primarily found at the perimeter of the ganglion, and from TH-immunoreactivity studies, the intensely fluorescent cells were also apposed to capillaries at this stage. The E18.5 ganglion contained numerous blood vessel profiles, and $\frac{3}{4}$ of the intensely TH-immunoreactive (and LDCV-containing) cells were found adjacent to blood vessels. Thus, not only are intensely fluorescent LDCV-containing cells found adjacent to capillaries in the adult SCG, but these cells appear in the same location during development. The apposition of SIF cells to fenestrated capillaries in the SCG (Siegrist et al., 1968; Matthews and Raisman, 1969; Eränkö, 1976) permitting the ready exchange of substances between the blood and ganglionic parenchyma may reflect the ability of glucocorticoids to induce/maintain the SIF phenotype. Both *in vivo* (Eränkö and Eränkö, 1972) and *in vitro* (Eränkö et al., 1972a,b; Doupe et al., 1985b), treatment of SCG cells with glucocorticoids increases the number of detectable SIF cells. This change is probably due to the induction of the SIF phenotype in a previously indifferent cell. It is of interest

that the levels of plasma glucocorticoids have been reported to increase dramatically between E16.5 and E17.5 (Siedl and Unsicker, 1989), consistent with a glucocorticoid signal becoming available to cells in the SCG during this period. In addition to glucocorticoids, some other characteristic of blood vessels, such as their basal lamina, may provide an important additional signal for SIF differentiation. The capillaries in embryonic SCG have intense reactivity with antibodies to laminin (A. K. Hall and S. C. Landis, unpublished observations) and may express other cues that stabilize SIF cell differentiation. It is also possible that SIF cells are generated spontaneously in the ganglion, but that the SIF phenotype is stabilized by the cells' location near capillaries. In this case, the detection of SIF cells near capillaries could be a consequence, rather than a cause, of their differentiation.

The intensely fluorescent cells seen in the embryo were larger than the SIF cells seen 1 week after birth and in the adult, suggesting that the embryonic intensely TH-immunoreactive cells represent the precursors of SIF cells seen in mature ganglia. The intensely TH-fluorescent cells that initially appear at E16.5/18.5 are the same size as the young principal neurons in the ganglion, suggesting that both neurons and SIF cells are derived from a similarly sized cell, whose postmitotic neuronal progeny grow larger, and whose SIF progeny divide to become smaller. In support of these findings, using TH immunofluorescence, Soinila (1984a) has reported the presence of large, intensely fluorescent cells in the embryonic SCG that decreased in size by $\frac{1}{4}$ at birth. It is possible that the cell diameter decrease is due to division of the larger intensely fluorescent cells in the embryo to produce smaller, clustered SIF cells (Soinila, 1984b) in the postnatal animal. We have found that some of the LDCV-containing cells at E18.5 contain mitotic figures by ultrastructural examination, indicating that cells with an SIF-like phenotype divide *in situ*. In addition, using TH immunoreactivity combined with thymidine autoradiography, Bohn (1987) has described the postnatal division of SIF cells during the first postnatal week. Taken together, these results are consistent with a large, individually occurring SIF precursor cell dividing in late embryogenesis or at birth to give rise to clusters of smaller mature SIF cells seen postnatally.

Sympathetic ganglia and adrenal glands are populated by a similar precursor

Sympathetic ganglia and adrenal glands are initially populated by very similar precursor cells, and these similarities have led to the notion of a common sympathoadrenal precursor. Both the sympathetic ganglia and adrenal glands are invested by precursors that initially express some neuronal features, including the neuronal cell adhesion molecule NCAM (Henion and Landis, 1989) and a growth-associated neural specific marker, SCG-10 (Anderson and Axel, 1986). Furthermore, the molecule 1A11, which is found on adult chromaffin cells but not adult SCG neurons (thereby operationally functioning as a chromaffin marker), appears on sympathoadrenal precursors during early development (Carnahan and Patterson, 1988). Examination of the developing sympathetic ganglion and adrenal glands discloses some ultrastructural similarities in their precursor cells. Like developing SIF cells in the SCG, chromaffin cells also acquire cytoplasmic membrane-bound vesicles during embryogenesis. Some adrenal precursor cells in the sympathomedullary region show signs of granule production in the Golgi area at E12.5, while other cells with similar morphology lack granules

in their cytoplasm (Elfvin, 1967). At E14.5/15.5, medullary cells contain membrane-limited granules about 62–140 nm in diameter scattered in the cytoplasm (Daikoku et al., 1977), and by the 18th d of gestation, 100–270-nm membrane-limited granules are numerous, though granular exocytosis is not evident until birth (Daikoku et al., 1977). Thus, like SCG precursors, adrenal precursor cells also initially appear immature and do not resemble mature SIF cells. Chromaffin cells acquire membrane-limited granules in their cytoplasm during late embryogenesis, similar to the period in which LDCVs appear in a small number of SIF-like cells in the SCG.

Experimental studies on chromaffin cell plasticity led to the hypothesis that the sympathoadrenal precursor has chromaffin-like or SIF-like features. In tissue culture, chromaffin cells can either retain chromaffin features or assume those of sympathetic neurons, depending on their environmental conditions. Glucocorticoids are required for chromaffin cells to survive and maintain their phenotype in culture, and without this factor most chromaffin cells die (Doupe et al., 1985a). However, some neonatal chromaffin cells can be rescued by the addition of NGF and mature into neurons, indicating that chromaffin cells can transdifferentiate into neurons in culture (Ogawa et al., 1984; Doupe et al., 1985a; Unsicker et al., 1985). During the conversion of adrenal chromaffin cells to neurons *in vitro* under the influence of NGF, an intermediate SIF cell morphology was observed. The observation that SIF cells could have an intermediate position in the differentiative pathways of both adrenal chromaffin and sympathetic neuron interconversions *in vitro* led to the suggestion that the neuronal precursor in the SCG *in vivo* might have characteristics of mature SIF cells found in the adult ganglion (Doupe et al., 1985b). However, in the present study, SCG precursors in the E12.5 ganglion *in vivo* were shown to have catecholamine histofluorescence and intense TH immunoreactivity, but no cells were seen that resembled mature SIF cells, suggesting that the SIF cell-to-neuron transdifferentiation observed in culture is not representative of the developmental origins of these cells *in vivo*.

To explain how the development of SIF cells and principal neurons in the SCG may be differentially regulated *in vivo*, parallels may be drawn with studies on adrenal chromaffin cells. One of the factors that may act to initiate commitment to the principal neuron phenotype in the sympathetic ganglia *in situ* is one that sustains neuronal differentiation from chromaffin cells *in vitro*. During their conversion to the neuronal phenotype in culture, chromaffin cells respond to FGF initially and subsequently become NGF-dependent neurons (Stemple et al., 1988). If sympathetic ganglion precursors have similar characteristics, it may be that the majority of cells respond early in development to FGF for division and process elongation and later to target-derived NGF for sustained sympathetic neuron survival as well as growth. From the present data, we would suggest that cells in the SCG that escape a strong neuronal influence (FGF and NGF), and which also receive an SIF signal (glucocorticoids), may instead develop an SIF phenotype. It is clear from experiments on neonatal SCG that there are cells that have escaped *both* the neuronal and the SIF differentiative cues during embryonic development, which appear undifferentiated, and which may be manipulated to give rise to either principal neurons or SIF cells (Doupe et al., 1985b). It remains of interest to determine whether these "latent precursors" have the antigenic properties of precursors seen in the young ganglion, and whether the application of neuronal differentiative factors

to these cells results in the inability of these cells to acquire an SIF phenotype when subsequently placed in glucocorticoid-containing medium.

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