The Journal of Neuroscience, November 1987, 7(11): 3739–3748

Relationship Between Differentiation and Terminal Mitosis: Chick Sensory and Ciliary Neurons Differentiate After Terminal Mitosis of Precursor Cells, Whereas Sympathetic Neurons Continue to Divide After Differentiation

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A population of undifferentiated cells has been characterized during the early development of nodose and ciliary ganglia. This population is defined by the absence of surface markers specific for neurons (tetanus toxin receptor, Q211 antigen) and for glial cells (O4 antigen). These undifferentiated cell populations were isolated from the ganglia and were shown to contain neuronal precursor cells that were able to differentiate *in vitro* into neurons, as characterized by morphology and surface antigens. Undifferentiated cells were detected during the period of neuronal birth, indicating that dividing neuronal precursor cells do not express neuron-specific surface markers. This was directly shown by ^{3}H-thymidine-labeling studies using nodose ganglia, ciliary ganglia, and dorsal root ganglia.

In sympathetic ganglia, however, no undifferentiated neuronal precursor cells were detectable at developmental stages when sympathetic neurons are born. ^{3}H-Thymidine injected during that stage at E7 was incorporated into cells expressing the neuronal markers tetanus toxin receptor and Q211 antigen. Quantitative fluorimetric determination of the DNA content of dissociated sympathetic ganglion cells demonstrated the presence of a population of Q211-positive sympathetic ganglion cells in the G2 phase of the cell cycle. E7 sympathetic ganglion cells expressing neuronal surface markers were also shown to be able to divide *in vitro*.

We have concluded that the relationship between terminal mitosis and the onset of differentiation differs between ganglia of the chick peripheral nervous system: Sympathetic ganglion cells continue to divide after the acquisition of neuronal properties, whereas neuronal precursor cells from other autonomic and sensory ganglia start to differentiate after a terminal mitosis.

The neurons and glial cells of the peripheral nervous system differentiate from precursor cells of the neural crest and ectodermal placodes (for a review, see LeDouarin, 1982). Analysis of these differentiation pathways requires markers that distinguish between the immature cell types of different cell lineages. We have previously analyzed the differentiation of neurons and glial cells in developing chick and quail dorsal root ganglia (Rohrer et al., 1985, 1986) using cell surface markers specific for neurons, tetanus toxin (Dimpfel et al., 1975; Mirsky et al., 1978) and Q211 antibody (Henke-Fahle, 1983a, b); and for glial cells, O4 antibody (Sommer and Schachner, 1981; Rohrer and Sommer, 1983; Rohrer, 1985). Those studies have led to the identification of a population of undifferentiated cells that is devoid of any of these markers. This cell population, which is present only during early development, was isolated from dorsal root ganglia and was demonstrated to contain neuronal progenitor cells (Rohrer et al., 1985, 1986).

An important event in the development of a neuron is the terminal mitosis, the withdrawal of dividing neuronal precursor cells from the mitotic cycle. The appearance of neurons as postmitotic cells was determined by ^{3}H-thymidine labeling in various parts of the nervous system in different species (Sidman et al., 1959; Fujita, 1964; Carr and Simpson, 1978; Levitt and Rakic, 1982). There is general agreement that in the central nervous system, neuron-specific properties such as neurotransmitters (Olson and Seiger, 1972; Lander and Bloom, 1974), neurotransmitter-synthesizing enzymes (Rothman et al., 1980), but also other neuronal markers (Schmechel et al., 1980; Koulakoff et al., 1983), are not expressed in dividing neuronal precursor cells. In contrast, in the peripheral nervous system, dividing cells that express neuron-specific properties have been demonstrated in chick sympathetic ganglia (Cohen, 1974; Rothman et al., 1978) and it was implied that these findings were characteristic for the whole peripheral nervous system.

The undifferentiated cell populations identified in chick and quail dorsal root ganglia were detected only during the periods of neuronal birth, when neuronal precursor cells withdraw from the mitotic cycle, indicating that dividing neuronal precursor cells do not express neuron-specific surface markers in these systems. Thus, it was of interest to analyze other sensory and autonomic ganglia for the presence of undifferentiated neuronal precursor cells during early stages of their development, and to establish the relationship between terminal mitosis and the expression of neuron-specific cell surface markers in these ganglia, in comparison with sympathetic chain ganglia. A preliminary report of this work has appeared previously (Rohrer and Thoenen, 1986).

Received Feb. 24, 1987; revised May 26, 1987; accepted May 28, 1987.

We wish to thank A. Kleinschmidt, J. Müller, H. Haltmeier, A. Stei, and M. Schörnig, who were involved in the initial phase of this study. We also thank C. Weijer and C. David for their help in the microphotometric measurements. The excellent technical assistance of L. Stegmann is gratefully acknowledged.

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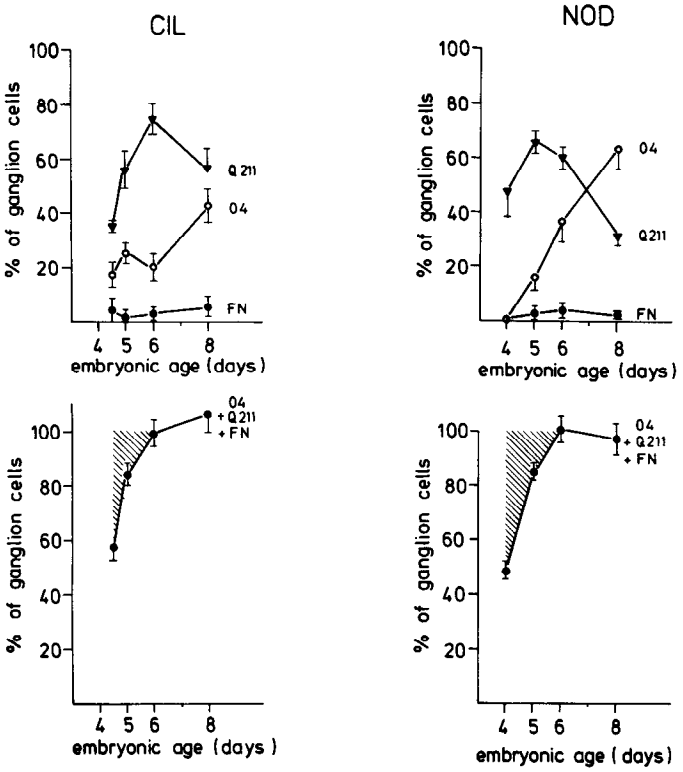

Figure 1. Cellular composition of ciliary and nodose ganglia during development. Ganglia were dissected at the embryonic ages indicated, dissociated to single cells, and brought into culture. Neurons, glial cells, and fibroblasts were identified using the neuronal markers Q211 and tetanus toxin, the glia marker O4, and FN as the marker for fibroblasts (see Materials and Methods). Neurons and glial cells were identified in 3 hr cultures, fibroblasts in 24 hr cultures. The proportion of cells expressing a certain marker was determined at different developmental stages (*upper graph*). The different populations are summated for each age in the *lower graph*. Note that there is a population of cells that is devoid of any of the markers used (*hatched area*).

Materials and Methods

Cell culture. Ganglia were dissected from chick embryos at various ages, cleaned carefully of surrounding tissue, and dissociated after trypsinization, as described previously (Barde et al., 1980). Dorsal root ganglia and sympathetic chain ganglia were dissected from the lumbosacral region. Cells were plated at a density of 80,000 cells/35 mm culture dish. For short-term (3 hr) cultures, cells were kept on a polyornithine-coated dish in F14 medium supplemented with 10% (vol/vol) heat-inactivated horse serum and 5% (vol/vol) heat-inactivated fetal calf serum. Twenty-four hour cultures were kept on laminin–polyornithine-coated dishes, which were prepared by sequentially coating them with polyornithine, 0.5 mg/ml (Sigma), and laminin, 10 μg/ml (BRL) by the methods of Collins (1978) and Edgar et al. (1984), respectively. The plating efficiency was very high in all experiments, i.e., virtually all cells had attached to the culture substrates after 3 hr. The medium was further supplemented for 24 hr cultures with neurotrophic factors, according to the type of ganglion: ciliary neurotrophic factor (CNTF; 10 ng/ml) (Manthorpe et al., 1986) was added to ciliary ganglion cultures, NGF (20 ng/ml) to sympathetic ganglion cultures, brain-derived neurotrophic factor (BDNF; 200 ng/ml) (Barde et al., 1982) to nodose ganglion cultures, and both NGF and BDNF to dorsal root ganglion cultures. BDNF was purified as described by Barde et al. (1982) with modifications to be published elsewhere, and was generously provided by Y.-A. Barde; CNTF was a gift of M. Sendtner. NGF was purified from adult mouse salivary glands according to the method of Boccini and Angeletti (1969), as modified by Suda et al. (1978).

Immunofluorescent staining of cultures. Cell surface antigens were visualized in cell cultures by indirect immunofluorescent staining of living cultures, as described in detail previously (Rohrer, 1985; Rohrer et al., 1985). The cultures (maintained for either 3 or 24 hr) were first incubated with antibodies against O4 or Q211 antigen, fibronectin, or with tetanus toxin. Cultures that had been labeled with tetanus toxin were subsequently incubated with human anti-tetanus toxin serum. After these incubations with first antibodies, cells were fixed with 4% paraformaldehyde and incubated with FITC-labeled secondary antibodies. Cultures were then washed, mounted in PBS/glycerol (1:1, vol/vol), and viewed with a Leitz Orthoplan microscope using epifluorescence UV illumination. For the DNA staining, the cells were washed after incubation with FITC-labeled secondary antibodies, once with PBS, twice with 70% ethanol, and then once with 96% and 100% ethanol each. The cells were then incubated with 0.5 μg/ml bisbenzimide (Hoechst H33258 Serva, Heidelberg) in PBS for 1 hr at room temperature. After the staining, the cultures were washed and mounted in PBS/glycerol.

Complement-mediated cell lysis using Q211 and O4 antibodies. Cells from dissociated ciliary, nodose, or sympathetic ganglia were incubated for 40 min at 37°C with guinea pig complement (1:6) and with both O4 antibody (1:40) and Q211 antibody (1:1000), as has previously been described in detail (Rohrer et al., 1985). Since the efficiency of complement-mediated cell lysis varies from preparation to preparation of complement, the concentration of complement had to be adjusted to ensure that the specific cell lysis was complete without reaching the range of nonspecific lysis. After incubation, cells were collected by centrifugation through a cushion of 3% bovine serum albumin (U. Ernsberger and H. Rohrer, unpublished observations), washed once in culture medium, and then plated.

^{3}H-thymidine-labeling studies. Eggs were removed at appropriate times from the incubator, and at the blunt end a small window was cut into the shell. The vitelline membrane was removed from over the embryo and methyl-^{3}H-thymidine (Amersham; 40–60 Ci/mmol) was applied (20 μCi in 100 μl PBS) to the chorioallantoic membrane of the embryos. Following the ^{3}H-thymidine injection, the window was tightly sealed with transparent tape and the egg was returned to the incubator. At different time intervals after the injection, ganglia were dissected, dissociated to single cells, and brought into culture. The cells were then stained for the neuron-specific surface markers (tetanus toxin receptor and Q211 antigen). After incubation with fluorescein isothiocyanate (FITC)-labeled secondary antibody, the cells were rapidly dehydrated with ethanol (see above) and covered with photographic emulsion (Kodak NTB 2). After an exposure period of 4–5 d, the specimens were developed using Kodak D19 developer. Silver grains and staining for the surface markers were viewed using bright-field optics and FITC-fluorescence optics, respectively. *In vitro* ^{3}H-thymidine was added at a concentration of 1 μCi/ml to the complete medium.

Determination of DNA content of cultured cells. Cultured cells were stained for the Q211 antigen or for tetanus toxin receptors. After incubation with the FITC-labeled secondary antibody, the cells were washed with PBS, then twice with 70% ethanol, once with 96%, and once with 100% ethanol. They were then incubated for 1 hr with 0.5 μg/ml of the DNA-specific fluorophore bisbenzimide, 4,6-diamidino-2-phenylindole (DAPI) (Leeman and Ruch, 1982) in PBS. After the incubation, the cells were washed with PBS and mounted in PBS/glycerol (1:1, vol/vol). The flourescence of the nuclei was measured as described by Weijer et al. (1984), using a Leitz Dialux fluorescence microscope with filter block A. Cells were examined with a 100× Fluotar oil-immersion objective, and fluorescence intensity was measured with a Leitz MPV microscope photometer equipped with a variable diaphragm. The diaphragm was adjusted to the size of a nucleus. The photometer was connected to a Kontron PSI 80 microcomputer, which performed all data collection and analysis.

Results

Appearance of neuronal and glial characteristics on cells of the ciliary and nodose ganglion

Ciliary and nodose ganglia were dissected at early stages of embryonic development and dissociated into single cells. Immediately after the cells had attached firmly to the polyornithine culture substrate, the cells were analyzed immunohistochemically for the presence of the glia-specific O4 antigen, the neuron-specific Q211 antigen, and tetanus toxin receptors (Rohrer et al., 1985). Tetanus toxin receptor staining and staining for the Q211 antigen were carried out in parallel in all experimental

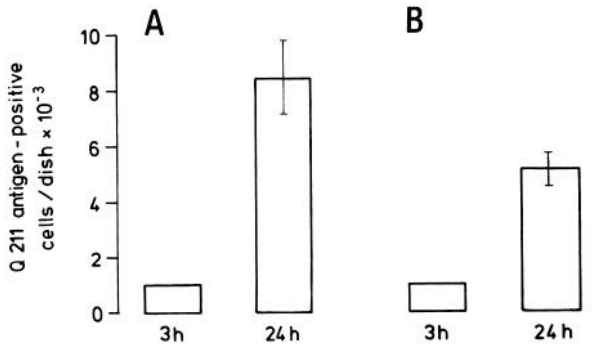

Figure 2. *In vitro* differentiation of neuronal precursor cells from ciliary and nodose ganglia. Undifferentiated cell populations from E4.5 ciliary ganglia or from E4 nodose ganglia were isolated by complement-mediated elimination of O4-positive and Q211-positive cells. The undifferentiated cells from ciliary (*A*) or nodose (*B*) ganglia were plated on laminin–polyornithine-coated dishes in the presence of neurotrophic factors, as described in Materials and Methods. The cultures were analyzed after 3 and 24 hr for the presence of Q211-positive cells. Note the increase in the number of Q211-positive cells within 1 d in culture.

situations initially. Since both markers gave identical results (see also Rohrer et al., 1985), in later experiments only Q211 antibody was used (for reasons of convenience). Fibroblast-like cells were identified in overnight cultures using antibodies against fibronectin. The proportion of neurons, glial cells, and fibroblasts was determined (Fig. 1). The proportion of glial cells increased continuously, whereas the proportion of neurons increased initially, but then decreased.

During early periods of development, a large proportion of the cells was devoid of the cell-type-specific markers. These undifferentiated cells were found both in ciliary ganglia and nodose ganglia up to about E6 (Fig. 1, hatched areas).

Demonstration of neuronal progenitor cells in the undifferentiated cell populations

The undifferentiated cells present in the dissociated E4 nodose ganglion or in E4.5 ciliary ganglia can be obtained as virtually pure glia- and neuron-free cell populations by eliminating the differentiated neurons and glial cells. This elimination is achieved by complement-mediated cell lysis, using the cell surface-specific monoclonal antibodies O4 and Q211 (Rohrer et al., 1985). The undifferentiated cell populations were brought into culture and were shown to contain cells that were able to differentiate into neurons in culture within 24 hr. This differentiation process was characterized by the appearance of neuronal morphology (neurite production) (demonstrated with cells from dorsal root ganglia by Rohrer et al., 1985, 1986), by the appearance of neuron-specific surface antigens (Fig. 2), and by the appearance of voltage-dependent ion channels (K. Gottmann, I. D. Dietzel, H. D. Lnx, and H. Robert, unpublished observations). This neuronal differentiation was observed on a laminin–polyornithine substrate in the presence of neurotrophic factors.

Relationship between division of neuronal precursor cells and expression of neuronal surface markers Q211 antigen and tetanus toxin receptors

The undifferentiated cell populations that were shown to contain neuronal precursor cells were found during the time periods when neuronal precursor cells were dividing in the ganglia (d'Amico-Martel, 1982), and the time at which undifferentiated cells disappeared correlated with the end of neuronal precursor

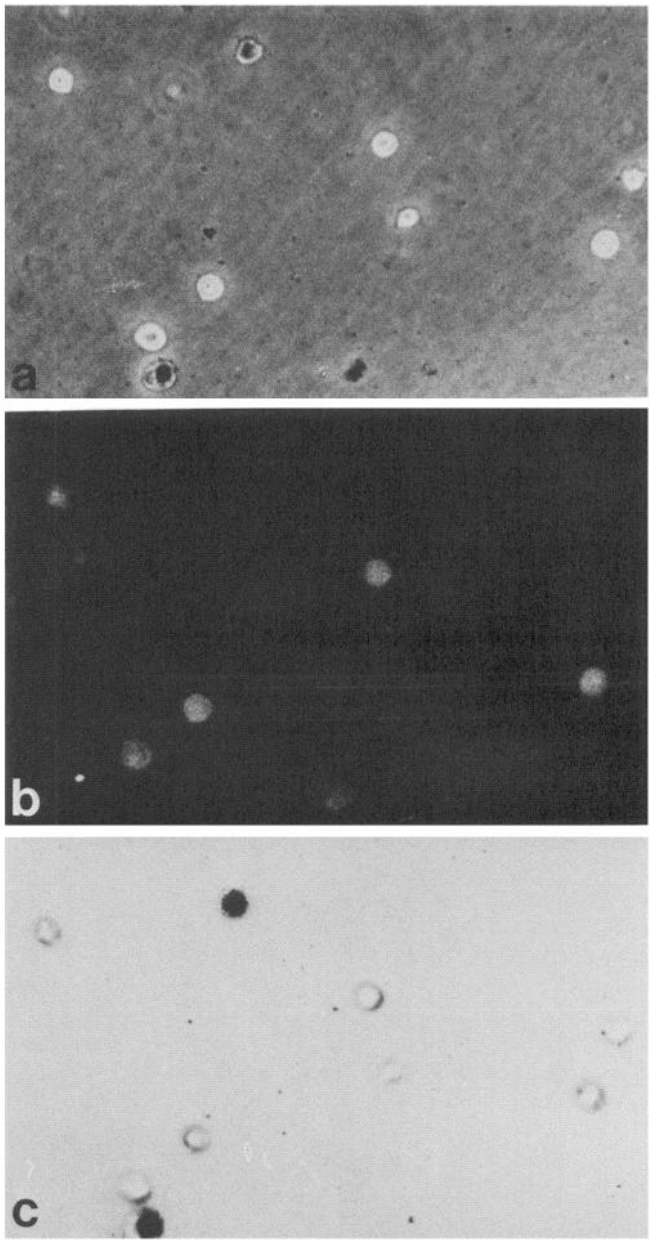

Figure 3. *In vivo* ^{3}H-thymidine-labeling. E5 chick embryos were injected with ^{3}H-thymidine. Two hours after the injection, the dorsal root ganglia were dissected, dissociated, and plated on polyornithine-coated culture dishes. After 2–3 hr, the cells were stained for the Q211-antigen and processed for autoradiography. (*a*) Phase-contrast, (*b*) Q211 staining (FITC fluorescence), (*c*) silver grains (bright-field). Magnification, × 360.

cell division. This correlation suggests that dividing neuronal precursor cells are devoid of neuron-specific surface markers. In order to test this hypothesis, ^{3}H-thymidine was injected *in vivo* at the time of neuronal birth in dorsal root (DRG) (E5), nodose (NOD) (E4), and ciliary (CIL) (E4.5) ganglia. Two hours after the injection, the ganglia were dissected, dissociated to single cells, and brought into culture. Cells were stained for the presence of the neuron-specific surface markers and then processed for autoradiography. Between 9% (E4 NOD) and 25% (E4.5 CIL, E5 DRG) of the total cell population was labeled with ^{3}H-thymidine. None of the cells identified as neurons was labeled by ^{3}H-thymidine (Fig. 3, Table 1). These results indicate

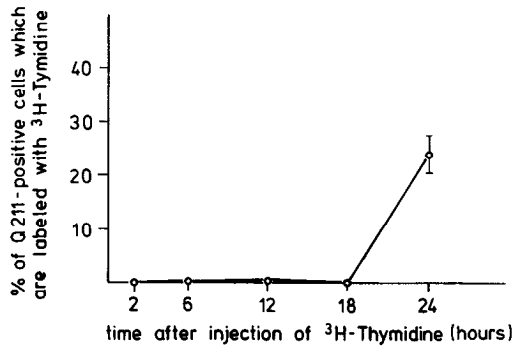

Figure 4. *In vivo* ^{3}H-thymidine labeling. E5 embryos were injected with ^{3}H-thymidine. At 2, 6, 12, 18, and 24 hr following the injection, the dorsal root ganglia were dissected, dissociated, and analyzed after 2–3 hr in culture for Q211 antigen and incorporation of ^{3}H-thymidine, as described in the legend to Figure 3. Data are given as the mean ± SD of triplicate determinations.

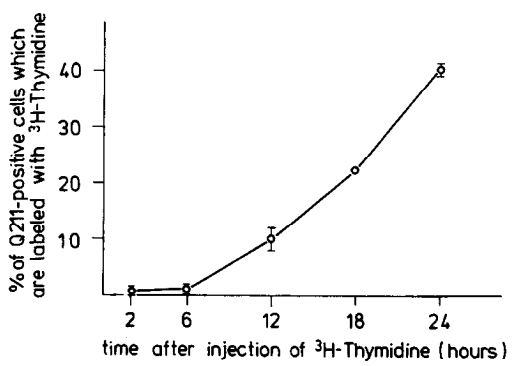

Figure 5. *In vivo* ^{3}H-thymidine labeling. E5 chick embryos were injected with ^{3}H-thymidine. At the *time points* indicated, the dorsal root ganglia were dissected, dissociated, and plated on dishes coated with polyornithine and laminin using F14 medium supplemented with serum, BDNF, and NGF. These conditions allow the differentiation of Q211-negative neuronal precursor cells present in E5 dorsal root ganglia. After 24 hr, the cultures were stained with anti-Q211 antibody and processed for autoradiography. Data are given as the mean ± SD of triplicate determinations.

that dividing neuronal precursor cells in the sensory dorsal root and nodose ganglia, and in the parasympathetic ciliary ganglion, neither express Q211 antigen nor tetanus toxin receptors.

How long is the time interval between the terminal mitosis and the appearance of the Q211 antigen in dorsal root ganglia? Those neuronal precursor cells, which are in the last hour of their terminal S-phase at the time of injection of ^{3}H-thymidine, will be the first cells that express both ^{3}H-thymidine labeling and the Q211 antigen. We thus injected ^{3}H-thymidine *in vivo* during the period of neuronal birth in dorsal root ganglia (E5). At different time intervals after injection, the dorsal root ganglia were dissected, dissociated to single cells, and cultured for 3 hr. The cells were then stained for the Q211 antigen and processed for autoradiography. As demonstrated in Figure 4, none of the cells labeled with ^{3}H-thymidine expressed the Q211 antigen before 18 hr after administration of the thymidine pulse. Cells labeled with both ^{3}H-thymidine and Q211 antibody first appear between 18 anbd 24 hr after thymidine injection. These must have been the cells that were in the last hour of their terminal S-phase during the ^{3}H-thymidine injection. It can thus be concluded that the Q211 antigen appears between 18 and 24 hr after the terminal S-phase of dividing neuronal precursor cells in dorsal root ganglia.

Neuronal progenitor cells in the undifferentiated cell population are postmitotic cells

The neuronal progenitor cells that are present in the undifferentiated cell populations have been defined both by the absence of certain neuronal properties and by their developmental potential to survive and to differentiate into neurons *in vitro*. It was therefore of interest to establish the relationship between these cells, defined by their *in vitro* behavior, and dividing neuronal precursor cells, defined by ^{3}H-thymidine labeling. We expected that dividing neuronal precursor cells would be able to survive and differentiate to Q211-positive neurons. Dividing neuronal precursor cells from E5 dorsal root ganglia were labeled *in vivo* by a 2 hr pulse of ^{3}H-thymidine. After the pulse, dorsal root ganglia were dissociated into single cells and brought into culture under conditions that allowed the differentiation of Q211-negative neuronal precursor cells (laminin-coated substrate and the presence of survival factors). After 24 hr in culture, the cells were analyzed for the presence of Q211 antigen and processed for autoradiography. Surprisingly, none of the Q211-positive cells were labeled by ^{3}H-thymidine (Fig. 5, 2 hr time point), indicating that neuronal precursor cells in the S-phase are not

Table 1. ^{3}H-thymidine labeling *in vivo*

Ganglia labeled *in vivo* for 2 hr	No. of Q211-positive cells analyzed	No. of Q211-positive cells which are thymidine-labeled
E5 DRG	746	0
E4.5 Cil	1181	0
E4 NOD	647	0

^{3}H-thymidine was injected at the developmental time periods indicated. Ganglia were dissected 2 hr after the injection and dissociated to single cells. Cells were stained after 2–3 hr in culture for the presence of neuron-specific surface markers, and then processed for autoradiography. Data from 2–3 independent experiments are combined for each ganglion.

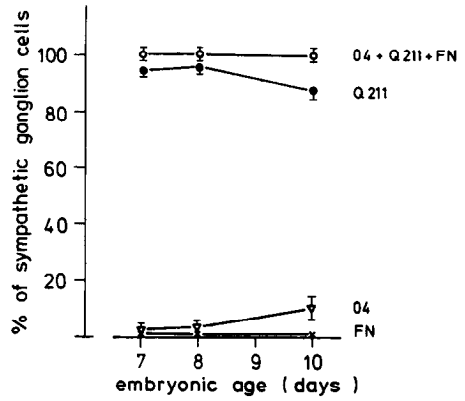

Figure 6. Cellular composition of sympathetic chain ganglia during development. Sympathetic ganglia were dissected at the embryonic ages indicated, dissociated to single cells, and brought into culture. The proportion of cells expressing the neuronal markers (*Q211* antigen, tetanus toxin receptor), the glial marker (O4 antigen), or the marker for fibroblast-like cells (*FN*) were determined (as described in the legend to Fig. 1) at the developmental stages indicated. In the top line (*circles*), the different proportions are summated. Note the absence of undifferentiated cells in comparison to ciliary and nodose ganglia (Fig. 1).

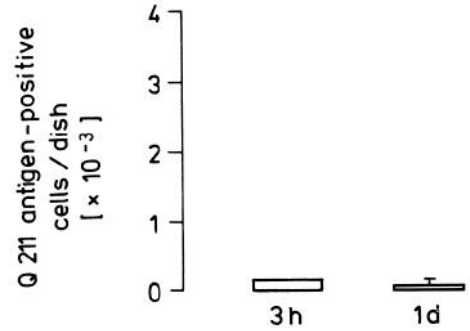

Figure 7. Neuronal precursor cells are not present in E7 sympathetic ganglia. The cell population remaining after complement-mediated elimination of O4-positive and Q211-positive cells was plated on laminin–polyornithine-coated dishes and cultivated in the presence of NGF. The cultures were analyzed after 3 and 24 hr for the presence of Q211-positive cells (compare with Fig. 2).

able to survive and differentiate under these conditions. What is the time interval between terminal mitosis and the ability of neuronal precursor cells to survive and differentiate *in vitro*? At different time intervals after the injection of ^{3}H-thymidine, the ganglia were dissociated and analyzed *in vitro* for the presence of thymidine-labeled cells that had differentiated to Q211-positive neurons within 24 hr *in vitro*. As demonstrated in Figure 5, the ability to survive and differentiate to neurons is acquired between 6 and 12 hr *after* the terminal S-phase. Since dividing neuronal precursor cells can survive and differentiate to neurons under certain culture conditions (high cell density; unpublished observations), it can be concluded that dividing neuronal precursor cells survive the dissociation of the ganglia. The usual culture conditions, however, do not seem to be suitable for survival of these dividing precursor cells. It can also be concluded that the neuronal progenitor cells described in the present and previous studies (Rohrer et al., 1985, 1986) are postmitotic cells that withdrew from the mitotic cycle at least 6 hr previously.

Appearance of neuronal and glial properties in cells of sympathetic chain ganglia

The demonstration that all dividing cells (including neuronal precursors) in dorsal root ganglia, nodose ganglia, and ciliary ganglia lack neuron-specific markers is in contrast to previous findings that in sympathetic ganglia (Rothman et al., 1978) and the enteric nervous system (Teitelman et al., 1981), dividing cells are found that express certain neuron-specific properties (i.e., transmitters and transmitter-synthesizing enzymes). Thus, sympathetic ganglia were analyzed during the period of neuronal birth for the presence of undifferentiated cells. Sympathetic ganglia were shown to contain a very high proportion of cells that express neuronal surface markers between E7 and E10. O4-positive glial cells and fibroblasts account for less than 10% of the total cell population (Fig. 6). Undifferentiated cells (i.e., cells that are devoid of markers specific for neurons, glial cells, or fibroblasts) were not detectable in significant numbers during this time period, which is characterized by a massive proliferation of sympathetic ganglion cells (Cohen, 1974; Rothman et al., 1978). The cell population remaining after elimination of all Q211-positive cells from E7 sympathetic ganglion cells did not contain progenitor cells with the ability to differentiate to neurons *in vitro* (Fig. 7).

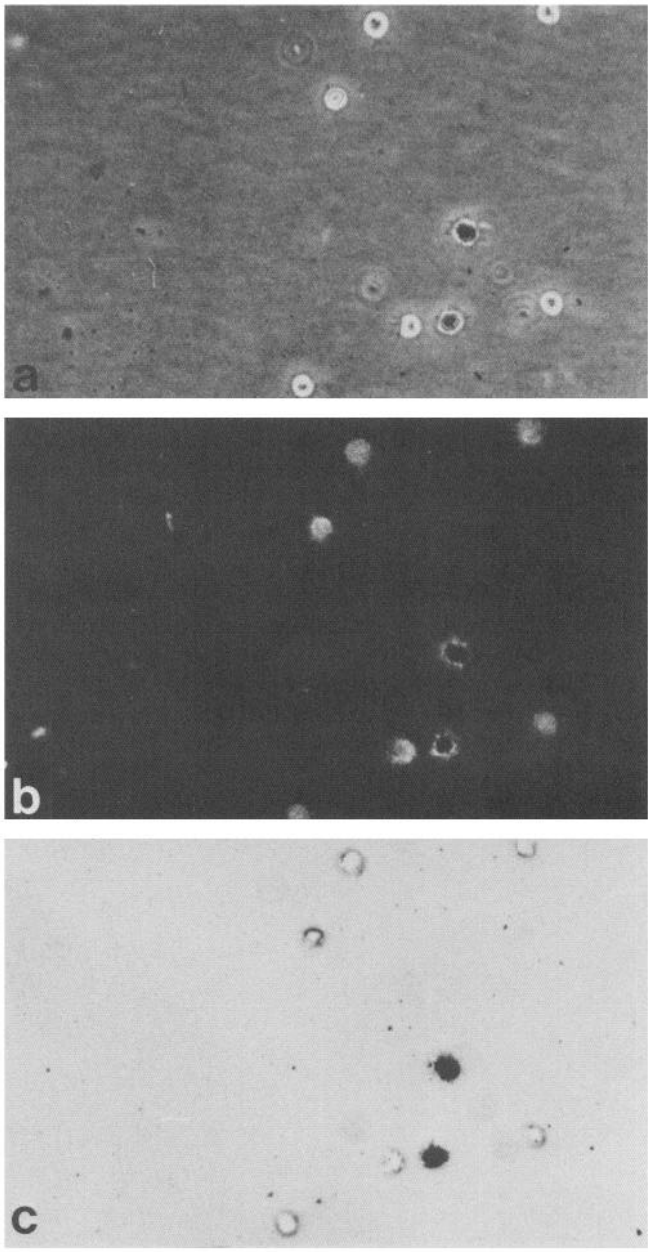

Figure 8. In vivo ^{3}H-thymidine labeling. E7 chick embryos were injected with ^{3}H-thymidine. After 2 hr following the injection, the sympathetic ganglia were dissected, dissociated, and plated on polyornithine-coated culture dishes. After 3 hr, the cells were stained for the Q211 antigen and processed for autoradiography. *a,* Phase-contrast; *b,* Q211 staining (FITC fluorescence); *c,* silver grains (bright-field). Magnification, ×360.

Dividing cells in sympathetic ganglia express neuron-specific surface markers

Dividing cells in E7 sympathetic chain ganglia were labeled *in vivo* by a 2 hr ^{3}H-thymidine pulse. After dissociation of the ganglia, the cells were stained for the presence of neuron-specific surface markers and analyzed for thymidine incorporation by autoradiography. About 11 ± 2% of the Q211-positive cells were thymidine-labeled (Fig. 8). This result was confirmed using quantitative DNA measurements in identified Q211-positive cells (Figs. 9, 10). Sympathetic ganglion cells (E7), after 3 hr in culture, were stained for Q211 antigen and were then perme-

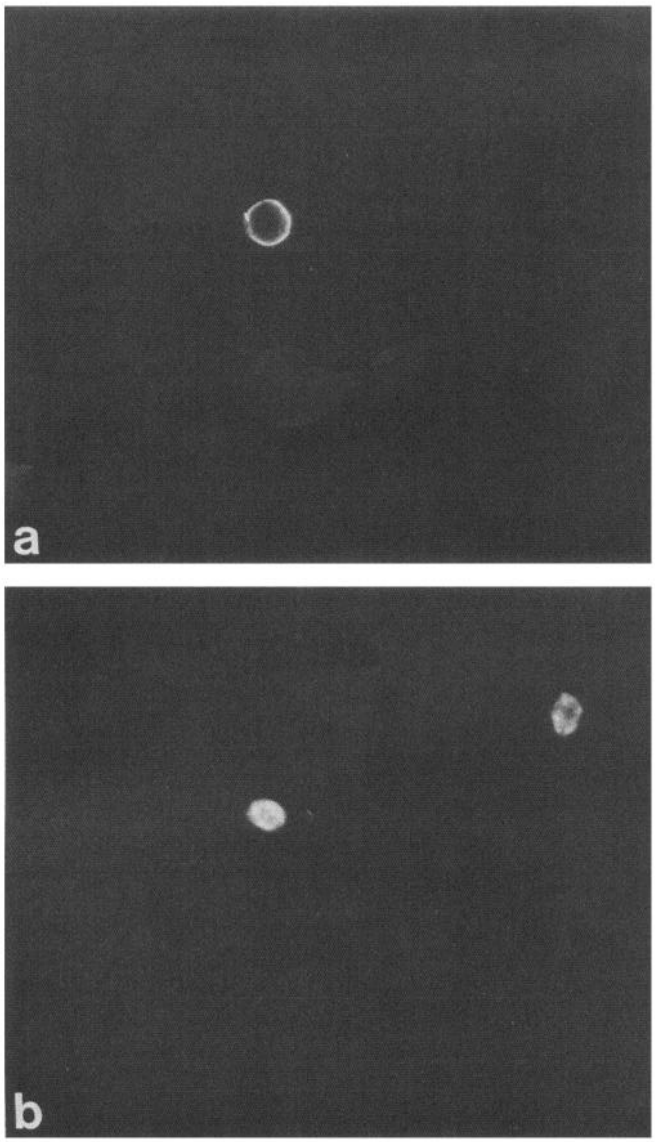

Figure 9. Combined staining of cells for DNA and cell surface antigens. E7 sympathetic ganglion cells after 3 hr in culture were stained for the Q211 antigen, then stained with bisbenzimide (5 μg/ml) for DNA. Fluorescent DNA staining (*b*) and FITC staining (*a*) were visualized using appropriate filter combinations. Magnification, ×750.

abilized and stained with bisbenzimide, a fluorescent DNA-binding dye (Fig. 9). Q211-positive cells were selected, and the amount of bisbenzimide fluorescence, which is proportional to the amount of DNA (Arndt-Jovin and Jovin, 1977), was determined photometrically. The Q211-positive cells in E7 sympathetic ganglia consisted of 2 populations (Fig. 10). The majority of cells contained the amount of DNA characteristic for cells in the G1 phase of the cell cycle. A second population of cells contained twice the amount of DNA, representing cells in the G2 phase. The assignment of DNA content to fluorescence intensity was possible because of the presence of Q211-positive cells with metaphase nuclei (see Fig. 11) that demonstrated high fluorescence levels. The G2 population of Q211-positive cells was detected only in sympathetic ganglia and not in dorsal root ganglia.

Sympathetic ganglion cells with neuronal surface antigens divide in vitro

Dividing sensory neuronal precursor cells did not survive under the culture conditions used in the present study. Since E7 sympathetic chain ganglia contain a large number of dividing cells that express neuronal properties and are thus more differentiated than the sensory precursors, we investigated the ability of these sympathetic ganglion cells to survive and divide in culture. Cells from E7 sympathetic ganglia were cultivated in F14 supplemented with 10% horse serum, 5% fetal calf serum, and 10 ng/ml NGF, using laminin as a substrate. When ^{3}H-thymidine was added during the last 18 hr of a 48 hr culture period, 34 ± 6% (n = 8) of the Q211-positive cells were ^{3}H-thymidine labeled (Fig. 11, *a, b*). Q211-positive cells in different phases of the cell cycle could be observed after DNA staining (Fig. 11, *c–e* shows a cell in the anaphase). Quantitative DNA measurements of identified Q211-positive cells after 1 d in culture demonstrated a population of cells in the G2 phase of the cell cycle (data not shown), as was shown previously for the *in vivo* situation.

Discussion

Our previous analysis of the appearance of neuronal and glial properties in cells of lumbosacral dorsal root ganglia from chick and quail embryos (Rohrer et al., 1985, 1986) demonstrated the presence of an undifferentiated cell population during early development. The disappearance of this cell population correlated with the end of neuronal precursor cell proliferation in chick and quail DRG. The present study demonstrates that an undifferentiated cell population is also present at the periods of neuronal birth in the nodose and ciliary ganglia. Proliferating precursor cells do not express neuron-specific surface markers in these ganglia, one of these markers, the Q211 antigen, being acquired about 18 hr after terminal mitosis. Proliferating neuronal precursor cells acquire the ability for survival and neuronal differentiation *in vitro* about 6 hr after terminal mitosis. In contrast, in lumbosacral sympathetic chain ganglia, no undifferentiated cells were detectable at the time of neuronal birth and evidence is presented that proliferating cells in sympathetic ganglia express neuron-specific surface antigens and are able to survive and divide in culture. These results support and extend previous findings (Cohen, 1974; Rothman et al., 1978; Teitelman et al., 1981; Rohrer et al., 1985, 1986) and demonstrate that the relationship between terminal mitosis and differentiation is dependent on the type of peripheral ganglia.

Using cell surface markers specific for neurons, glial cells, and fibroblasts in cell preparations of dissociated ganglia, it is possible to quantify the proportions of the total ganglion cell population belonging to the various cell lineages at different developmental time periods. Double-label experiments have previously established that these markers identify nonoverlapping populations (Rohrer et al., 1985). It should also be mentioned, in this context, that tetanus toxin, which has been demonstrated to bind not only to neurons but also to certain types of glial cells in the mammalian brain (Raff et al., 1983; Miller and Raff, 1984), in our cultures of chick peripheral ganglion cells never stained cells with non-neuronal morphology (Rohrer and Sommer, 1983; Rohrer, 1985). The analysis of the changing cellular composition of the developing nodose and ciliary ganglion showed a result similar to the situation in the chick and quail dorsal root ganglion (Rohrer et al., 1985, 1986): the proportion of glial cells increased throughout development, whereas

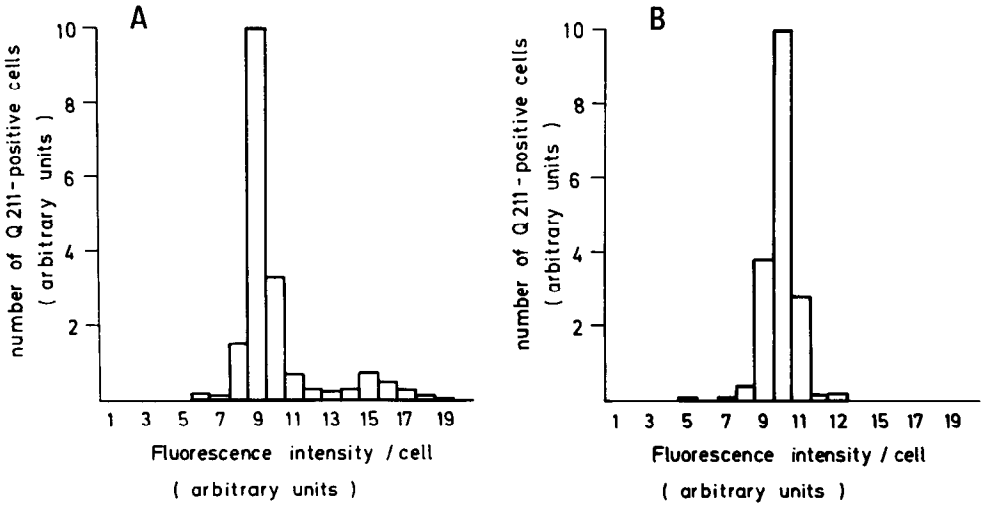

Figure 10. Determination of DNA content of identified Q211-positive cells. Cultures (3 hr) of dissociated E7 sympathetic chain ganglia (*A*) and E6 dorsal root ganglia (*B*) were stained for DNA and the Q211 antigen. The amount of DNA in single Q211-positive cells was determined by measuring the amount of nuclear bisbenzimide fluorescence using a fluorescence microscope equipped with a photometer (see Materials and Methods). Note the presence of 2 populations of cells in sympathetic ganglia. The smaller cell population has approximately twice as much DNA as the major peak.

the neuronal population initially increased but then decreased again. The increase in the proportion of neurons is due to the generation of neurons during the period of neuronal birth (see below). Since the glial cells continue to divide after the period of neuronal birth, the proportion of neurons starts to decrease rapidly.

At early stages of development a large proportion of cells in sensory and parasympathetic ganglia was found to be negative for the markers used and could not be assigned to a cell lineage. For these cells, the term "undifferentiated cell population" is used. The undifferentiated cell population was detected in these ganglia up to E6. The disappearance of undifferentiated cells correlates with the termination of neuronal precursor proliferation in these ganglia (d'Amico-Martel, 1982), suggesting that neuronal precursor cells are present in the undifferentiated cell population. These neuronal precursor cells were identified by their ability to acquire in culture neuronal morphology and neuron-specific surface antigens. However, not only neuronal precursor cells but also glial precursor cells are present in the undifferentiated cell population (H. Haltmeier and H. Rohrer, unpublished observations). Thus, the disappearance of undifferentiated cells during development reflects the differentiation of both glial cells and neurons, and for this reason no strict correlation between periods of neuronal birth and the presence of undifferentiated cells can be expected. It should be pointed out that in all these ganglia (sensory and parasympathetic), the cells of the glial cell lineage have acquired the O4 antigen around the time that all neurons have been generated. This suggests that glial differentiation may not proceed independently, but could be linked to neuronal differentiation. There is considerable evidence for neuron-dependent glial differentiation, not only at the stage of myelination (for a review, see Bunge et al., 1986), but also at earlier stages of glial development (Holton and Weston, 1982).

The present analysis provides evidence that the Q211 antigen is expressed around the time of neuronal birth. To analyze the relationship between terminal mitosis and expression of the neuronal surface markers in more detail, ^{3}H-thymidine-labeling experiments were carried out. It was shown that cells in the S-phase of the cell cycle do not express neuronal surface markers in dorsal root ganglia, nodose ganglia, and ciliary ganglia. In dorsal root ganglia, the expression of the Q211 antigen was found to begin about 18 hr *after* the terminal round of DNA synthesis. In addition, dividing precursor cells identified by thymidine labeling were found to acquire the ability to survive and differentiate into neurons about 6 hr after the terminal S-phase. Thus, it is possible to follow the stepwise maturation of cells of the neuronal lineage from dividing undifferentiated cells to Q211-positive cells. It can also be concluded that the neuronal progenitor cells present in the undifferentiated cell population of peripheral ganglia (Rohrer et al., 1985, 1986, and the present study) are postmitotic and must have withdrawn from the mitotic cycle between 6 and approximately 18 hr previously. In this context, it should be mentioned that the neuronal precursor cells not only lack the neuron-specific cell surface markers but also are devoid of voltage-dependent ion channels, which are acquired during differentiation *in vitro* (Rohrer et al., 1985). The fact that the neuronal precursor cells are born within a narrow time range explains their relatively synchronous differentiation to neurons *in vitro*. It is not known at present if the time interval between terminal mitosis and the appearance of Q211 antigen or tetanus toxin receptor is similar in the ciliary and nodose ganglia.

The observation that neuronal differentiation starts *after* the terminal mitosis of neuronal precursor cells in sensory and parasympathetic ganglia is similar to previous results obtained in the central nervous system (Olson and Seiger, 1972; Rothman et al., 1980; Schmechel et al., 1980; Koulakoff et al., 1983). It has been shown that, in the central nervous system, neuron-specific properties, such as tetanus toxin receptors (Koulakoff et al., 1983) or tyrosine-hydroxylase immunoreactivity (Rothman et al., 1980), are not expressed by dividing neuronal precursor cells. The zone of proliferating precursor cells was found to be devoid of neuronal markers (Cochard and Paulin, 1984).

In contrast, it has been observed that in sympathetic ganglia of the PNS of both chick and rat (Cohen, 1974; Rothman et al., 1978, 1980) and in the gut (Teitelman et al., 1981), thymidine-labeled cells express neuron-specific properties, such as transmitters, transmitter-synthesizing enzymes, transmitter uptake, and storage mechanisms. Dividing cells that express tetanus toxin receptors have also been described in the dorsal root ganglion of the mouse embryo, which seems to differ in this respect from the chick embryo (Koulakoff et al., 1983).

In the present study it was demonstrated that, in addition to

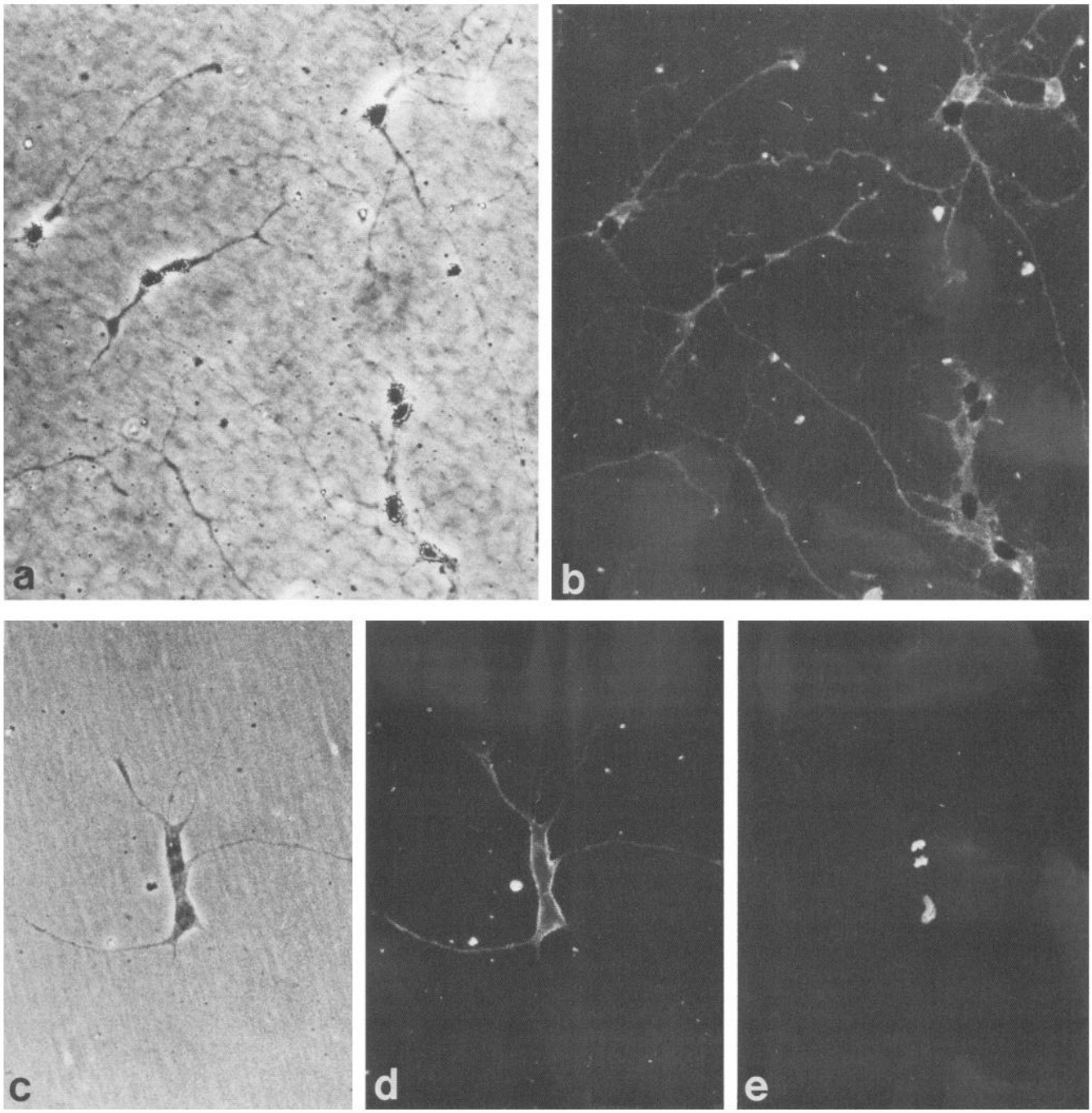

Figure 11. Cells with neuron-specific surface markers from E7 sympathetic chain ganglia divide *in vitro.* Cultures from E7 sympathetic ganglion cells after 1 d in culture were labeled with ^{3}H-thymidine for 18 hr, stained for Q211 antigen, and processed for autoradiography. *a,* Phase-contrast; *b,* Q211 antigen. Cultures from E7 sympathetic ganglion cells after 1 d in culture were stained simultaneously for the Q211 antigen and for DNA (using bisbenzimide). *c,* Phase-contrast; *d,* Q211 antigen; *e,* DNA staining. Magnification, ×315.

adrenergic properties, dividing cells in E7 chick sympathetic ganglia express neuron-specific surface antigens. DNA synthesis of Q211-positive cells in E7 sympathetic ganglia was demonstrated by ^{3}H-thymidine incorporation. The demonstration of Q211-antigen-positive cells that were in the G2 phase of the cell cycle and of mitotic figures *in vitro* supports the conclusion that ^{3}H-thymidine incorporation reflected DNA replication rather than DNA repair. At present, the place of these cells in the sympathetic cell lineage is unclear. In the chick, in addition to adrenergic sympathetic neurons and small, intensively fluorescent (SIF) cells (Luckenbill-Edds and van Horn, 1980), cholinergic sympathetic neurons are also present in considerable numbers in lumbosacral sympathetic ganglia (Edgar et al., 1981; Rohrer et al., 1983; Hayashi et al., 1985; New and Mudge, 1986). It is not known if these cells arise from common or different precursors. It has been demonstrated that SIF cells of the rat sympathetic nervous system can be precursors of adrenergic neurons (Doupe et al., 1985) that may subsequently develop cholinergic properties (Patterson and Chun, 1977; Landis and Keefe, 1983). For the chick there is evidence that adrenergic

and cholinergic lineages are separated early during development (Edgar et al., 1981; Rohrer et al., 1983; see also Zurn and Mudry, 1986). The present finding that sympathetic ganglion cells expressing neuronal properties continue to divide in culture opens the possibility of analyzing in more detail the sympathetic cell lineage *in vitro*. In addition, it will allow the study of factors involved in neuronal proliferation.

The analysis of the cellular composition of sympathetic ganglia demonstrated that all cells that incorporate ^{3}H-thymidine between E7 and E10 already express cell type-specific surface markers. Even at E7, the earliest time point studied, no undifferentiated cells have been detected. Thus, it must be concluded that the hyperblastic growth of sympathetic ganglia during that time period is entirely due to cell division of differentiated cells rather than to proliferation and differentiation of undifferentiated cells.

Taking adrenergic cells from developing central and peripheral nervous systems as models, it was previously concluded that central and peripheral neurons differ with respect to the relationship between mitotic arrest and early phenotypic differentiation (Rothman et al., 1980). The present results demonstrate, however, that the adrenergic cells from sympathetic ganglia are more of an exception than the rule in the PNS as well: there appears to be no common principle linking the arrest of cell division to the acquisition of the differentiated neuronal phenotype.

References

Arndt-Jovin, D. J., and T. M. Jovin (1977) Analysis and sorting of living cells according to deoxyribonucleic acid content. J. Histochem. Cytochem. *25:* 585–589.

Barde, Y.-A., D. Edgar, and H. Thoenen (1980) Sensory neurons in culture: Changing requirements for survival factors during embryonic development. Proc. Natl. Acad. Sci. USA *77:* 1199–1203.

Barde, Y.-A., D. Edgar, and H. Thoenen (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J. *1:* 549–553.

Bocchini, V., and P. U. Angeletti (1969) The nerve growth factor. Purification as a 30,000-molecular weight protein. Proc. Natl. Acad. Sci. USA *64:* 787–794.

Bunge, R. P., M. B. Bunge, and C. F. Eldridge (1986) Linkage between axonal ensheathment and basal lamina production by Schwann cells. Annu. Rev. Neurosci. *9:* 305–328.

Carr, V. Mc., and S. B. Simpson (1978) Proliferative and degenerative events in the early development of chick dorsal root ganglia. J. Comp. Neurol. *182:* 727–740.

Cochard, P., and D. Paulin (1984) Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo *in vivo*. J. Neurosci. *4:* 2080–2093.

Cohen, A. M. (1974) DNA synthesis and cell division in differentiating avian adrenergic neuroblasts. In *Wenner-Gren Center International Symposium Series: Dynamics of Degeneration and Growth in Neurons.* K. Fuxe, L. Olson, and Y. Zotterman, eds., pp. 359–370, Pergamon, Oxford, UK.

Collins, F. (1978) Axon initiation by ciliary neurons in culture. Dev. Biol. *65:* 50–57.

d'Amico-Martel, A. (1982) Temporal patterns of neurogenesis in avian cranial sensory and autonomic ganglia. Am. J. Anat. *163:* 351–372.

Dimpfel, W., J. H. Neale, and E. Habermann (1975) ^{125}I-labelled tetanus toxin as a neuronal marker on tissue cultures derived from embryonic CNS. Naunyn-Schmiedebergs Arch. Pathol. *290:* 329–333.

Doupe, A. J., P. H. Patterson, and S. C. Landis (1985) Small intensely fluorescent cells in culture: Role of glucocorticoids and growth factors in their development and interconversions with other neural crest derivatives. J. Neurosci. *5:* 2143–2160.

Edgar, D., Y.-A. Barde, and H. Thoenen (1981) Subpopulations of cultured sympathetic neurons differ in their requirements for survival factors. Nature *289:* 294–295.

Edgar, D., R. Timpl, and H. Thoenen (1984) The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. EMBO J. *3:* 1463–1468.

Fujita, S. (1964) Analysis of neuron differentiation in the central nervous system by tritiated thymidine autoradiography. J. Comp. Neurol. *122:* 311–327.

Hayashi, M., D. Edgar, and H. Thoenen (1985) Nerve growth factor changes the relative levels of neuropeptides in developing sensory and sympathetic ganglia of the chick embryo. Dev. Biol. *108:* 49–55.

Henke-Fahle, S. (1983a) Dissertation, Medical Faculty, Tübingen.

Henke-Fahle, S. (1983b) Monoclonal antibodies recognize gangliosides in the chick brain. Neurosci. Lett. *14:* 160.

Holton, B., and J. A. Weston (1982) Analysis of glial cell differentiation in peripheral nervous tissue. II. Neurons promote S100 synthesis by purified glial precursor cell populations. Dev. Biol. *89:* 72–81.

Koulakoff, A., B. Bizzini, and Y. Berwald-Netter (1983) Neuronal acquisition of tetanus toxin binding sites: Relationship with the last mitotic cycle. Dev. Biol. *100:* 350–357.

Lander, J. M., and F. E. Bloom (1974) Ontogeny of monoamine neurons in the locus coeruleus, raphe nuclei and substantia nigra of the cat. I. Cell differentiation. J. Comp. Neurol. *155:* 469–482.

Landis, S. C., and D. Keefe (1983) Evidence for neurotransmitter plasticity *in vivo*: Developmental changes in properties of cholinergic sympathetic neurons. Dev. Biol. *98:* 349–372.

Le Douarin, N. M. (1982) *The Neural Crest,* Cambridge U. P., Cambridge, UK.

Leeman, U., and F. Ruch (1982) Cytofluorometric determination of DNA base content in plant nuclei and chromosomes by the fluorochromes DAPI and chromomycin A3. Exp. Cell Res. *140:* 275–282.

Levitt, P., and P. Rakic (1982) The time of genesis, embryonic origin and differentiation of the brain stem monoamine neurons in the rhesus monkey. Dev. Brain Res. *4:* 35–57.

Luckenbill-Edds, L., and C. van Horn (1980) Development of chick paravertebral sympathetic ganglia. I. Fine structure and correlative histofluorescence of catecholaminergic cells. J. Comp. Neurol. *191:* 65–76.

Manthorpe, M., S. D. Skaper, L. R. Williams, and S. Varon (1986) Purification of adult rat sciatic nerve ciliary neuronotrophic factor. Brain Res. *367:* 282–286.

Miller, R. H., and M. C. Raff (1984) Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. J. Neurosci. *4:* 585–592.

Mirsky, R., L. M. B. Wendon, P. Black, C. Stolkin, and D. Bray (1978) Tetanus toxin: A cell surface marker for neurons in culture. Brain Res. *148:* 251–259.

New, H. V., and A. W. Mudge (1986) Distribution and ontogeny of SP, CGRP, SOM, and VIP in chick sensory and sympathetic ganglia. Dev. Biol. *116:* 337–346.

Olson, L., and A. Seiger (1972) Early prenatal ontogeny of central monoamine neurons in the rat: Fluorescence histochemical observation. Z. Anat. Entwicklungs. *137:* 301–316.

Patterson, P. H., and L. L. Y. Chun (1977) The induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neurons. I. Effect of conditioned medium. Dev. Biol. *56:* 263–280.

Raff, M. C., E. R. Abney, J. Cohen, R. Lindsay, and M. Noble (1983) Two types of astrocytes in cultures of developing rat white matter: Differences in morphology, surface gangliosides and growth characteristics. J. Neurosci. *3:* 1289–1300.

Rohrer, H., (1985) Nonneuronal cells from chick sympathetic and dorsal root sensory ganglia express catecholamine uptake and receptors for nerve growth factor during development. Dev. Biol. *111:* 95–107.

Rohrer, H., and I. Sommer (1983) Simultaneous expression of neuronal and glial properties by chick ciliary ganglion cells during development. J. Neurosci. *3:* 1683–1693.

Rohrer, H., and H. Thoenen (1986) Relationship between differentiation and terminal mitosis of neuronal precursor cells in the chick peripheral nervous system. Soc. Neurosci. Abstr. *12:* 1121.

Rohrer, H., H. Thoenen, and D. Edgar (1983) Presence of nerve growth factor receptors and catecholamine uptake in subpopulations of chick sympathetic neurons: Correlation with survival factor requirements in culture. Dev. Biol. *99:* 34–40.

Rohrer, H., S. Henke-Fahle, T. El-Sharkawy, H. D. Lux, and H. Thoenen (1985) Progenitor cells from embryonic chick dorsal root ganglia differentiate *in vitro* to neurons: Biochemical and electrophysiological evidence. EMBO J. *4:* 1709–1714.

Rohrer, H., A. L. Acheson, J. Thibault, and H. Thoenen (1986) Developmental potential of quail dorsal root ganglion cells analyzed *in vitro* and *in vivo*. J. Neurosci. *6:* 2616–2624.

Rothman, T. P., M. D. Gershon, and H. Holtzer (1978) The relationship of cell division to the acquisition of adrenergic characteristics by developing sympathetic ganglion cell precursors. Dev. Biol. *65:* 322–341.

Rothman, T. P., L. A. Specht, M. D. Gershon, T. H. John, G. Teitelman, V. M. Pickel, and D. J. Reis (1980) Catecholamine biosynthetic enzymes are expressed in replicating cells of the peripheral but not the central nervous system. Proc. Natl. Acad. Sci. USA *77:* 6221–6225.

Schmechel, D. E., M. W. Brightman, and P. J. Marangos (1980) Neurons switch from non-neuronal enolase to neuron-specific enolase during differentiation. Brain Res. *190:* 195–214.

Sidman, R. L., I. L. Miale, and N. Feder (1959) Cell proliferation and migration in the primitive ependymal zone, an autoradiographic study of histogenesis in the nervous system. Exp. Neurol. *1:* 322–333.

Sommer, J., and M. S. Schachner (1981) Monoclonal antibodies (01 to 04) to oligodendrocyte cell surfaces: An immunocytological study in the central nervous system. Dev. Biol. *83:* 311–327.

Suda, K., Y.-A. Barde, and H. Thoenen (1978) Nerve growth factor in mouse and rat serum: Correlation between bio-assay and radioimmunoassay determinations. Proc. Natl. Acad. Sci. USA *75:* 4042–4046.

Teitelman, G., M. D. Gershon, J. P. Rothman, T. H. Joh, and D. J. Reis (1981) Proliferation and distribution of cells that transiently express a catecholaminergic phenotype during development in mice and rats. Dev. Biol. *86:* 348–355.

Weijer, C. J., G. Duschl, and C. N. David (1984) A revision of the dictyostelium discoideum cell cycle. J. Cell Sci. *70:* 111–131.

Zurn, A. D., and F. Mudry (1986) Conditions increasing the adrenergic properties of dissociated chick superior cervical ganglion neurons grown in long-term culture. Dev. Biol. *117:* 365–379.