An autoradiographic analysis of the development of the chick trigeminal ganglion

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SUMMARY

The avian trigeminal ganglion, which is embryonically derived from the neural crest and epidermal placodes, consists of two topographically segregated classes of immature neurons, large and small, during the second week of incubation, and two neuronal cell types, dark and light, interspersed throughout the mature ganglion. In order to establish the times of terminal mitosis of trigeminal sensory neurons, embryos were treated with [³H]thymidine during the first week of incubation and their ganglia fixed on embryonic day 11. The embryonically large, distal, placodal-derived neurons are generated between days 2 and 5, while the small, proximal, neural crest-derived neurons are formed mostly between days 4 and 7.

By comparing the locations of labeled cells in ganglia treated with isotope but fixed on day 18 of incubation with their 11-day counterparts, we have proved that there are no morphogenetic rearrangements of neurons during the final week of incubation. Thus, no unique relationship exists between the two neuron types in the mature ganglion and the two cell classes in the immature trigeminal. Therefore, both the light and the dark neurons in the mature trigeminal ganglion arise from neural crest as well as placodal primordia.

INTRODUCTION

The avian trigeminal ganglion, like other cranial sensory ganglia, has a dual embryonic origin, being formed by cells derived from the neural crest and the epidermal placodes. These two embryonic primordia initially form the small and large immature neurons, respectively, which are found in separate regions of the ganglion during the second week of incubation (Hamburger, 1961; Noden, 1978a). However, the stages at which these cells withdraw from the mitotic population are not known. Elucidation of these neuronal birthdates is the first objective of this study.

In the mature (18-day to adult) ganglion there also exist two cytologically distinct neuronal cell types, referred to as dark and light neurons. These two populations are not segregated but rather are interspersed throughout the ganglion (Gaik & Farbman, 1973a, b; Noden, 1978c). The second goal of this research is to investigate the relationship between the two cell classes in the

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immature ganglion and the two populations of neurons in the mature ganglion.

The trigeminal ganglion is located ventro-lateral to the metencephalon and is morphologically divided into two lobes (see Fig. 1). The ophthalmic lobe, which mediates sensory input from the naso-superior and upper beak regions, is situated median and anterior to the maxillo-mandibular lobe. The maxillary and mandibular rami emerge from the maxillo-mandibular lobe and transmit both exteroceptive and proprioceptive sensory input from the upper and lower jaw regions as well as temporal and inferior periocular tissues. The ventral motor root passes through the ganglion adjacent to the maxillo-mandibular lobe and joins the mandibular ramus.

In the chick embryo, cells from the metencephalic and posterior mesencephalic neural crest and the adjoining epidermal placodes aggregate late on the second day to form the trigeminal anlage (Johnston, 1966; Noden, 1975). Between the sixth and eleventh days of incubation, two morphologically distinguishable cell types are present within the ganglion (Gaik & Farbman, 1973b; Hamburger, 1961). Large, argyrophilic neuroblasts are found in the distal part of both lobes, while in the proximal (core) region the neuroblasts are smaller, more densely packed, have poor affinity for silver and appear immature. Although these two populations are recognizable, some of the cells in the ganglion, especially those located between these two populations, do not fit this strict dichotomy with respect to their size or silver-staining properties.

Based on extirpation experiments Hamburger (1961) concluded that the small, proximal cells originate from the neural crest and the large distal cells from the placodal ectoderm. This pattern of cell derivation has been confirmed by experiments in which the normal complement of neural crest or placodal cells was removed from a chick embryo and replaced with homologous tissues containing a cell marker (Johnston, 1966; Noden, 1975, 1978a). The segregation is not without exception. Noden (1978a) observed an occasional neural crest cell undergoing neurogenesis among the distal placode population and displaying typical placodal cell morphology, and Johnston (1966) found some placodal cells contributing to the peripheral layer of trigeminal sensory neurons in the proximal part of the ganglion.

Studies on developing vertebrate nervous systems have shown that axon formation is restricted to postmitotic neurons (Angevine, 1970; Altman, 1972; Rakic, 1973; Vargas-Lizardi & Lyser, 1974), although neuron-specific enzymes may be present in proliferating neuroblasts (Polak, Rost & Pearse, 1971; Rothman, Gershon & Holtzer, 1978). Since many trigeminal sensory cells, including those of neural crest origin, have extensive peripheral projections by the end of the first week of incubation (Noden, 1980a, b), these cells must cease dividing very early in embryonic development. In this study tritiated thymidine has been applied to chicken embryos at various stages of incubation and the birthdates of neurons located in each part of the ganglion have been defined.

Between 11 and 15 days of incubation the topographical separation of small

and large cells disappears and both bipolar and pseudounipolar neurons of various sizes are observed throughout the ganglion (Gaik & Farbman, 1973*b*). Shortly before hatching, the dark and light neuronal cell types characteristic of the mature ganglion can be recognized. These cells are distributed throughout the ganglion (Ciani, Contestabile & Villani, 1973; Gaik & Farbman, 1973*a*, *b*; Finkelbrand & Silberman, 1977). The generally smaller, dark neurons are characterized by dense cytoplasm with Nissl substance distributed evenly throughout. Light neurons are larger and contain individual clumps of Nissl substance distributed in a pale cytoplasmic matrix with many neurofilaments and neurotubules between the islands of Nissl material (Gaik & Farbman, 1973*b*). Similar cell types have been observed in avian and mammalian ganglia (Hess, 1955; Yates, 1961; Tennyson, 1965) and in the trigeminal ganglia of the rat and mouse (Peach, 1972, 1973). The distinction between dark and light neurons in rhesus monkey trigeminal ganglia is disputed (Carmel & Stein, 1969; Pineda, Maxwell & Kruger, 1967), and apparently absent in the cat (Pineda *et al.* 1967; reviewed by Cammermeyer, 1962).

According to Gaik & Farbman (1973*a*, *b*) the ratio of dark to light neurons in the adult trigeminal ganglion is 62:38, which is similar to the ratio of small to large cells (70:30) in the embryo. At first glance these data suggest that the small cells might become the dark neurons and the large cells become the light neurons. If this is the case, the cells must undergo extensive morphogenetic rearrangements during the last week of embryonic development, from a largely segregated to a well-mixed distribution. This type of migration by postmitotic neurons is well documented in the central nervous system (Levi-Montalcini, 1964; Angevine, 1970; LaVail & Cowan, 1971*a*; Hollyday & Hamburger, 1977).

Alternatively it is possible that each cell in the immature trigeminal ganglion has more than a single developmental potential. That is, the neural crest and placode-derived cells remain in their original locations and, during maturation, each population forms both types of neurons seen in the adult. This alternative is suggested by comparison with avian spinal ganglia which are derived solely from the neural crest, yet contain segregated populations of small and large cells at early stages (Hamburger & Levi-Montalcini, 1949) and larger, light and smaller, dark neurons at later stages. By comparing the locations of labeled and unlabeled neurons in trigeminal ganglia fixed at 18 days of incubation with those in ganglia fixed at 11 days of incubation, it has been possible to show that this second alternative is the case.

<div style="text-align:center">MATERIALS AND METHODS</div>

Chick embryos of the White Plymouth Rock strain were incubated in a forced-draft incubator at 38 °C and 70 % relative humidity. At the desired stage 25 μCi of tritiated thymidine (New England Nuclear) in a volume of 0·25 ml sterile

chick saline and F12 nutrient medium was applied in three successive doses over a period of 6 h. This was done by dropping the isotope directly on the embryo through an opening in the shell. At the time of isotope application, embryos were staged according to the Hamburger & Hamilton (1951) stage series. Some of the older embryos (6–10 days) with their investment of extra-embryonic membranes and deeper position within the egg were staged according to the length of the incubation period. The eggs were sealed with a cover-glass and paraffin wax and returned to the incubator.

The method of cumulative labeling is used in order that the tritiated thymidine will be available for a sufficient amount of time to label all presumptive trigeminal neurons (Fujita, 1964; Langman & Haden, 1970; LaVail & Cowan, 1971b; Kahn, 1974). To assess whether the above procedures resulted in the labeling of all neuroblasts, several 2-day embryos received an additional 5 μCi of tritiated thymidine at 24 and 48 h after the regular injections. The labeling patterns of these embryos were the same as in embryos given the standard dosage.

In the first series of experiments embryos ranging in age from 2 to 10 days were treated. Most of these embryos were sacrificed at 11 days of incubation (stages 36–38), which is the latest stage at which the two embryonic cell classes are topographically separated. Several of the 4-day experimental animals were fixed at daily intervals from 11 to 17 days of incubation. Neurons produced shortly after injection are heavily labeled, while those produced in subsequent generations retain less tritium. Neurons post-mitotic at the time of injection are unlabeled. The position of unlabeled neurons in the various 11-day ganglia indicates which neurons were formed on each day of development.

In a second series of experiments embryos injected in an identical manner were not sacrificed until day 18 of incubation (stages 43–44), by which time the two neuronal cell types characteristic of the mature ganglion are present. Comparison of the locations of the labeled and unlabeled cells in 11- and 18-day ganglia should reveal any cell rearrangements which occur during this period.

After the desired length of incubation trigeminal ganglia were excised and placed in Carnoy's fixative or 2% glutaraldehyde in 0·1 M phosphate buffer (pH 7·4) containing 5% sucrose. Following dehydration in a butanol:ethanol series the tissues were embedded in Paraplast. Sections were cut at 8 μm and affixed to acid-washed, albumen-coated glass slides. The sections were de-paraffinized and dried. Slides were prepared for autoradiography by dipping in Kodak NTB2 emulsion according to the methods of Kopriwa & Leblond (1962). After an exposure time of 12–14 days at 8 °C the slides were developed in Kodak D19 for 2½ min at 18 °C. Control slides for false positives and false negatives (Rogers, 1969) were included with each batch of autoradiograms. All slides were washed for 30 min in running tap water. Sections were stained in 0·25% thionine in sodium acetate buffer at pH 4·7 (Sidman, 1970).

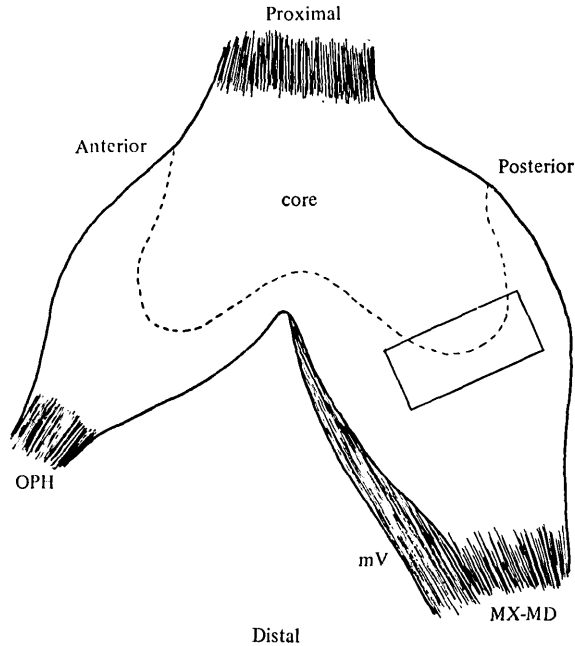Several ganglia were fixed in glutaraldehyde, dehydrated in a graded series of

Fig. 1. Lateral view of the left avian trigeminal ganglion illustrating the terminology used to describe particular regions of the ganglion. The solid rectangle represents the area depicted in Fig. 2A. In the text the term peripheral refers to the more superficial cells of the ganglion, central denotes cells deep within. MX-MD, Maxillo-mandibular lobe; OPH, ophthalmic lobe; mV mandibular (fifth) motor nerve.

ethanols, passed through three changes of propylene oxide, and embedded in a mixture of Epon and Araldite. Sections 1–2 μm thick were cut, affixed to glass slide and stained with 1 % toluidine blue in 1 % borax.

Histological analyses were done with the aid of a drawing tube. Cells with a distinct nuclear outline were classified as unlabeled (no grains above background), heavily labeled (> 40 grains), or lightly labeled (intermediate). The following results are based on the study of trigeminal ganglia from 39 11-day embryos, 36 18-day embryos, and 16 embryos sacrificed between 11 and 17 days of incubation.

RESULTS

For standardization most of the autoradiographs presented in this paper are from parasaggital sections of the trigeminal ganglion. Fig. 1 schematically shows the left ganglion from a lateral perspective, and indicates the terminology we have adopted to describe certain regions within the ganglion. The terms medial and lateral are ambigious because the medial surface of the ganglion at early stages of development corresponds to the inferior or ventral surface in the mature chick. Since the only asymmetries of mitosis involving the medio-lateral axis involve cells adjacent to the mandibular motor nerve, which
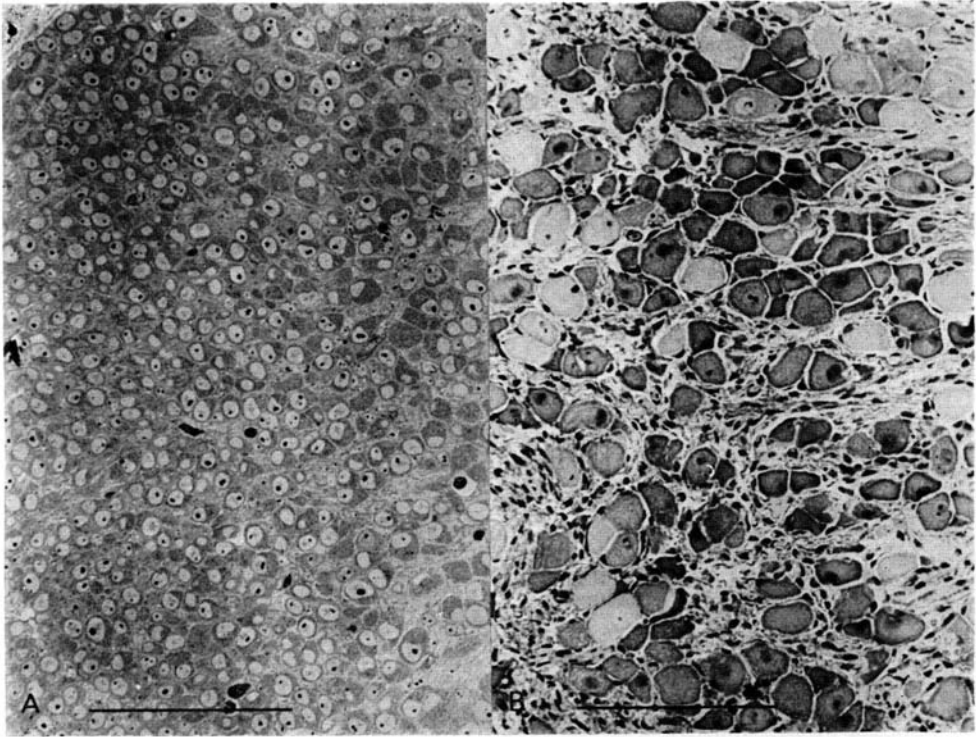
Fig. 2. Sections of Epon:Araldite-embedded trigeminal ganglia taken from the area indicated in Fig. 1. (A) Section through the MX-MD lobe of a 7-day embryonic ganglion showing the small, proximal cells on the left and the larger, distal cells on the right. Differences in cell density are also apparent. (B) Section through the MX-MD lobe of an 18-day embryonic ganglion. Upper and lower aspects of this micrograph correspond to proximal and distal aspects of the lobe. The enlargement of the neurons, growth of sensory nerve fibers, and the emergence of light and dark cells are visible. Bar equals 100 μm.

generally passes along the antero-medial margin of the maxillo-mandibular lobe, this difficulty of terminology can be avoided.

The histological appearance of the avian trigeminal ganglion early in the second week of incubation is illustrated in Fig. 2(a), which is from the interface between the placode-derived, large-cell (10–15 μm diameter) population and the neural crest-derived, small-cell (6–10 μm diameter) population. It is evident in this micrograph that there are some cells of intermediate size in the ganglion.

The histology of the 18-day ganglion (Fig. 2b) is considerably different. Light and dark neurons, which range in size from 15 to 30 μm in diameter, are interspersed throughout the entire ganglion. All cells cannot be clearly identified as belonging to one or the other class in this micrograph. In addition the neurons are separated into cords or clusters by bundles of nerve fibers which radiate through the ganglion from both the proximal (dorsal root) and distal (peripheral nerve) margins.
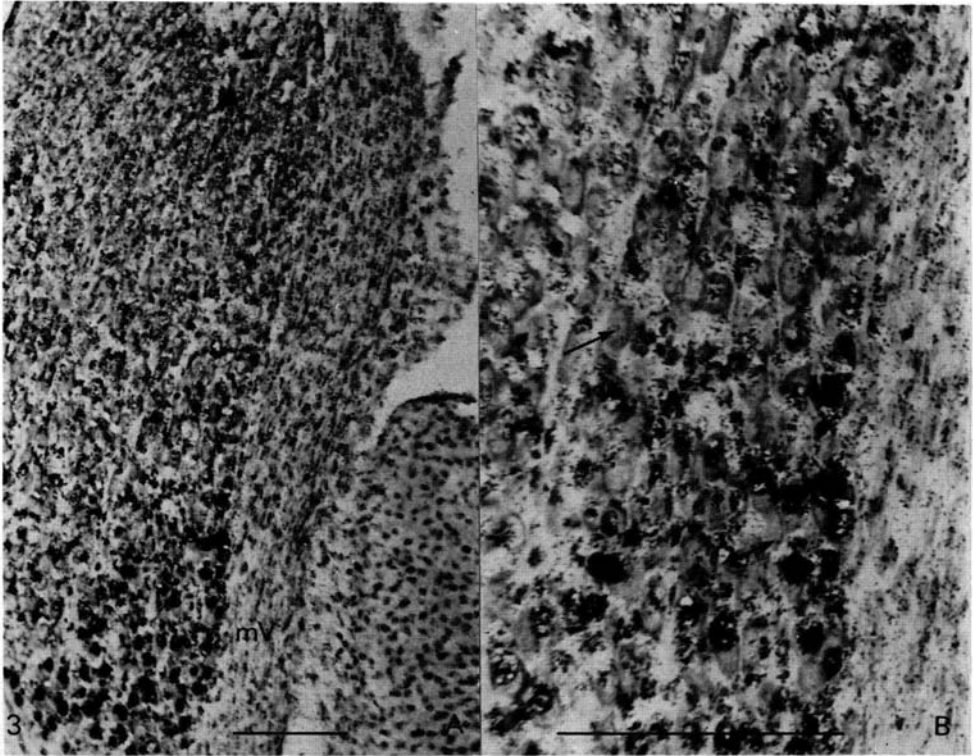
Fig. 3. Low (A) and high (B) magnification sections through MX-MD lobe of a right ganglion from an 11-day chick embryo injected at stage 14 (2 days). As in all subsequent illustrations, the proximal part of the ganglion is uppermost. Heavily labeled cells are visible in the distal region of the lobe and along the motor nerve (mV). Apparently unlabeled cells in B (arrows) are artifacts of the plane of section; examination of serial sections confirms that all cells underwent DNA synthesis following isotope application at stage 14. Bars indicate 100 μm.

Birthdate analyses of 11-day ganglia

Application of isotope at stage 14 (2 days of incubation), by which time the neural crest cells have finished their migration to the site of trigeminal ganglion formation and the placodal cell population has just been formed, results in labeling of all cells in the ganglion. As illustrated in Fig. 3 all cells have not retained identical amounts of the isotope. Heavily labeled cells, those which ceased dividing shortly after incorporating the labeled thymidine, are concentrated in the distal regions of both the ophthalmic and maxillo-mandibular lobes. The remaining cells in the ganglion are lightly labeled, indicating that they continued to divide many times after stage 14. Heavily labeled cells occasionally are found in the core region and, more often, adjacent to the motor nerve as it passes through the ganglion, as shown in Fig. 3(*b*).

Beginning at stage 16 (2½ days of incubation) there are some cells in the distal regions of the ganglion which are postmitotic. These are identifiable in Fig. 4 as
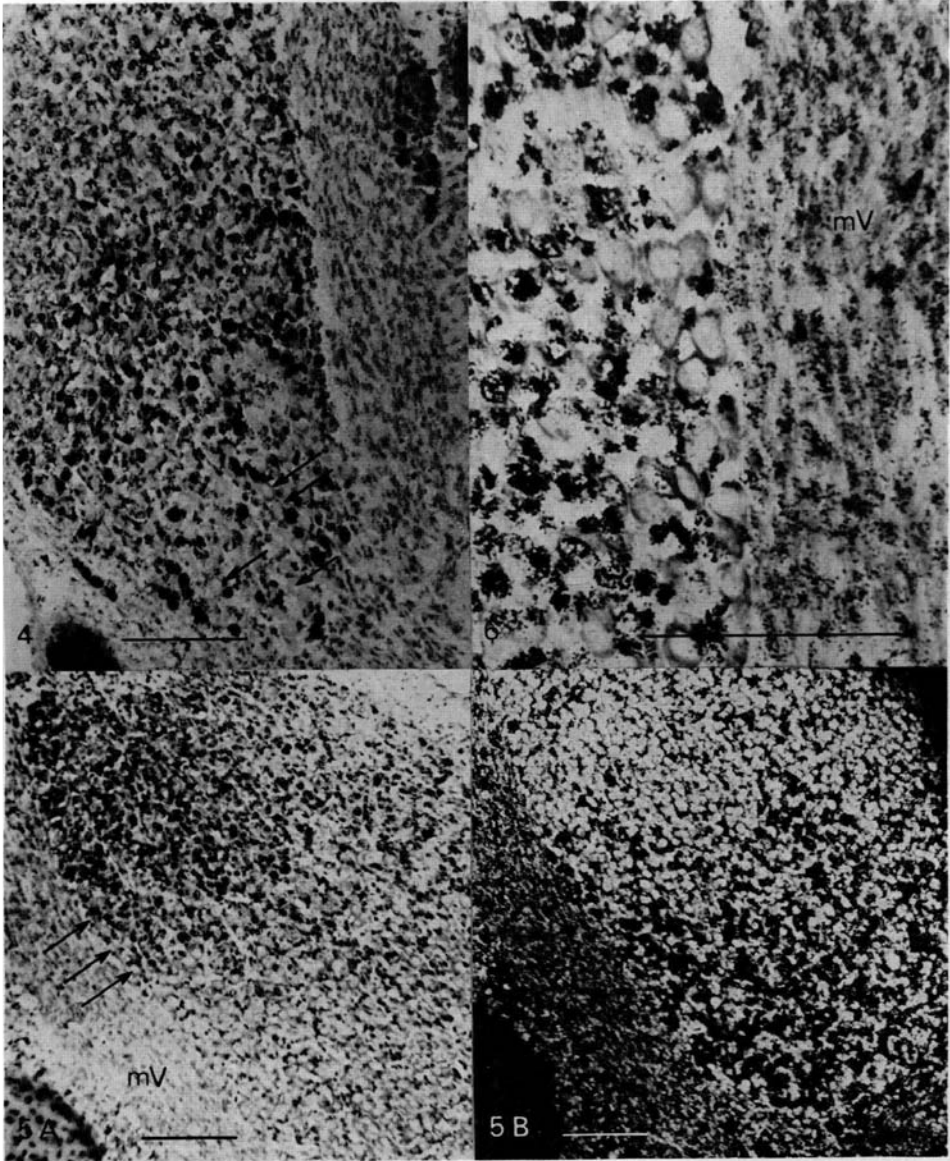
Fig. 4. Section through MX-MD lobe of right trigeminal ganglion from 11-day chick embryo injected at stage 16 (2½ days). Postmitotic neurons (arrows) can be seen in the distal region of the lobe. Bar equals 100 μm.

Fig. 5. Bright- (A) and dark-field (B) photomicrographs of MX-MD lobe of left ganglion from 11-day embryo injected at stage 22 (4 days). Postmitotic neurons can be seen in the distal half of the lobe and adjacent to the motor nerve (arrows). Satellite cell labeling persists as can be clearly seen in the darkfield micrograph. (B) indicates the presence of a few unlabeled neurons in the more proximal part of the ganglion. Bar equals 100 μm.

Fig. 6. Unlabeled neurons are seen adjacent to the mandibular motor nerve (mV) in this ganglion, which was treated the same as that in Fig. 5. Bar equals 100 μm.
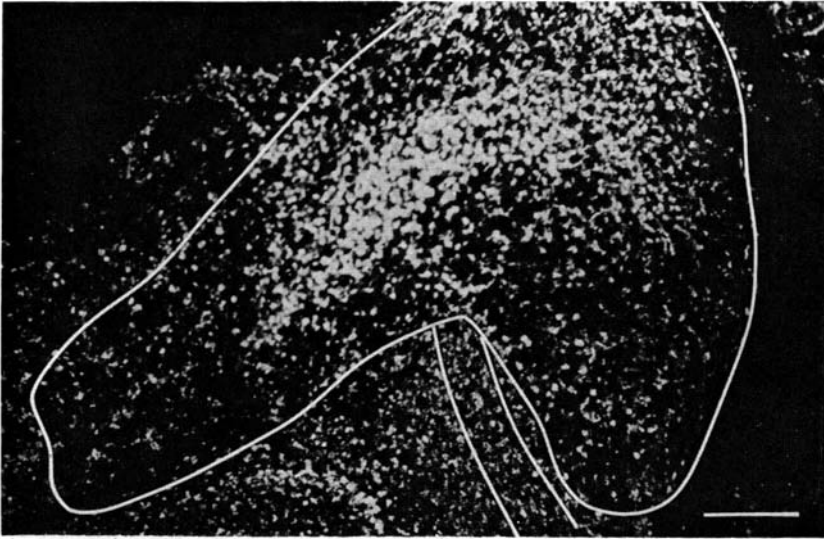
Fig. 7. Dark-field photomicrograph of left trigeminal ganglion from 11-day embryo injected at stage 25 (5 days). For orientation the outline of the ganglion is indicated. The distal to proximal wave of cessation of mitosis continues.

unlabeled neurons. During the third and fourth days of incubation, increasing numbers of neuroblasts withdraw from the proliferation pool. Most of these are in the distal regions of the lobes, though a few are scattered throughout the ganglion. Cells in the central regions of both lobes and in the zone of apposition of the lobes are now heavily labeled, while most proximal core cells are lightly labeled indicating that these cells continued to divide many times.

The position of labeled and unlabeled cells in an 11-day trigeminal ganglion treated with tritiated thymidine at stage 22 (4 days of incubation) is shown in Figs. 5 and 6. Cells in the proximal half of each lobe are heavily labeled except for a population of unlabeled, postmitotic neurons adjacent to the motor nerve and a few scattered unlabeled cells. As is easily seen by comparing bright- and dark-field micrographs (Fig. 5), most of the neurons in the distal half of the ganglion are postmitotic by stage 22.

During the fifth day of incubation this generally distal-to-proximal wave of cessation of proliferation continues. In addition, as shown in Fig. 7, the peripheral neuroblasts around the margins of both lobes stop dividing at this time. Thus, by stages 25–26 the only mitotically active neuroblasts are found within the proximal core regions of each lobe. The size of this proliferative population diminishes during the sixth (Fig. 8) and seventh days of incubation. In ganglia exposed to tritiated thymidine after stage 30 (7 days of incubation) there are no labeled neurons, although satellite cell labeling continues at least through the tenth day of incubation.
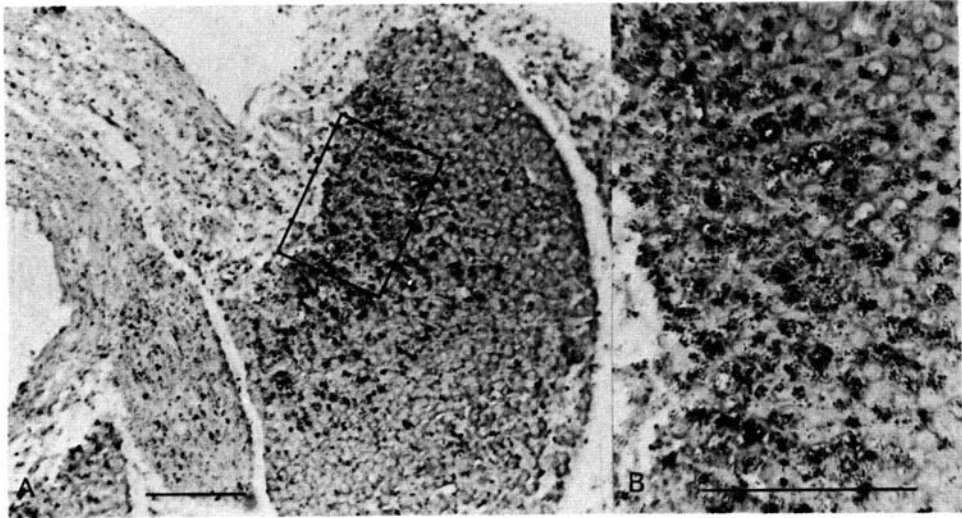
Fig. 8. Low (A) and high (B) magnification sections through left trigeminal ganglion from 11-day embryo injected at stage 28 (6 days). Only clusters of labeled neurons persist in the proximal core region. The location of (B) is indicated in (A). Bar equals 100 μm.

Birthdate analyses of 18-day ganglia

In order to examine the terminal positions of both the early formed distal neurons and the later-formed proximal neurons, ganglia from embryos injected with isotope on separate days during the first weeks of incubation were fixed and examined at 18 days of incubation. By this time, stage 44, the neuronal cytology is similar to that seen in adult trigeminal ganglia.

Generally the labeling patterns seen in the 18-day ganglia are the same as has been described for the 11-day ganglia. Figs. 9 and 10 illustrate the distribution of proliferating cells and post-mitotic neurons following treatment at stage 18 (3 days of incubation) or stage 25 (5 days). Comparing these with Figs. 4 and 7, respectively, clearly indicates that the location of neurons which cease dividing at each stage of embryonic development does not change during later stages. This was confirmed by examining a series of ganglia from embryos injected with [³H]thymidine on day 4 and fixed at daily intervals between days 11 and 17 of incubation.

Close examination of ganglia fixed at 11 days of incubation reveals that the proximally shifting interface between labeled and unlabeled cells is not precisely demarcated, but consists of an area in which dividing and postmitotic cells are intermingled. In the 18-day ganglia it is generally (but not exclusively) found that the unlabeled cells in this proximally moving zone are the larger neurons.
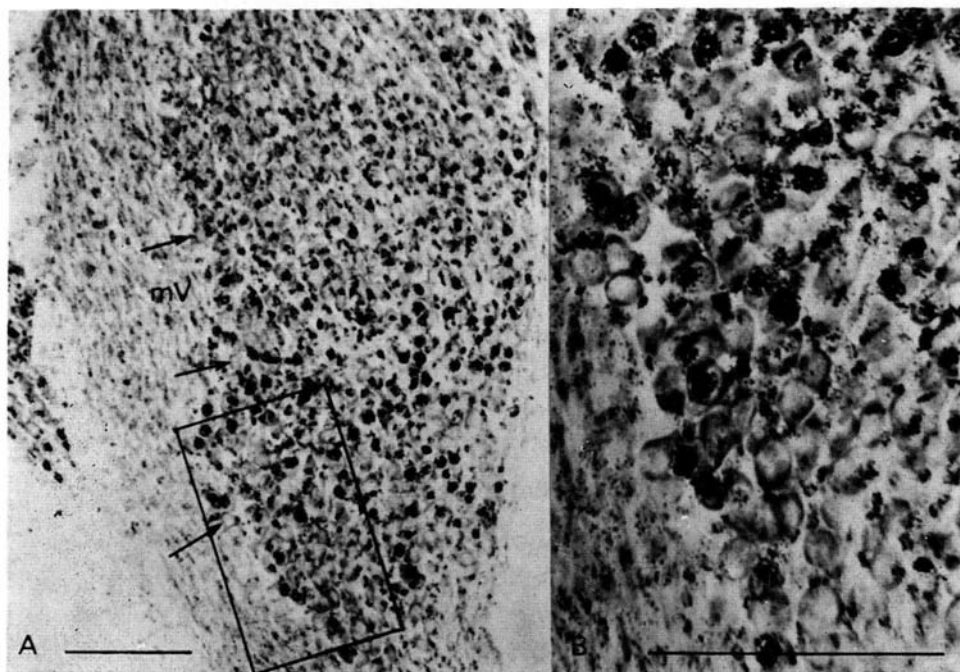
Fig. 9. Low (A) and high (B) magnification sections through MX-MD lobe of left trigeminal ganglion from 18-day embryo injected at stage 18 (3 days). The location of unlabeled cells is similar to that shown in Fig. 4. Unlabeled cells are observed along the motor nerve in this figure, unlike Fig. 4, consistent with the slightly older stage at injection. The location of (B) is indicated in (A). Light and dark neurons are not apparent in this or the next figure due to fixation with Carnoy's, which does not perserve this distinction. Bar equals 100 μm.

DISCUSSION

The cumulative labeling technique used in this investigation is a proven way of determining cell birthdates (Fujita, 1964; Pelc & Appleton, 1965), although interpretive difficulties can be encountered, as discussed by LaVail & Cowan (1971 *b*). In this study the criteria established by Sidman (1970) for the determination of cell birthdates have been met: (1) isotope injections have been made at a series of developmental stages and autoradiographic analyses have been performed at several times after injection, (2) all major cell types present in the ganglion have been accounted for, as evidenced by complete labeling following isotope application at 2 days of incubation, and (3) a coherent changing relationship between lightly and heavily labeled cells has been found in comparisons of results from animals labeled at various stages. That is, a given cell appears lightly labeled, heavily labeled, and then unlabeled as the isotope is applied well in advance of, immediately prior to, and then subsequent to its birthdate.

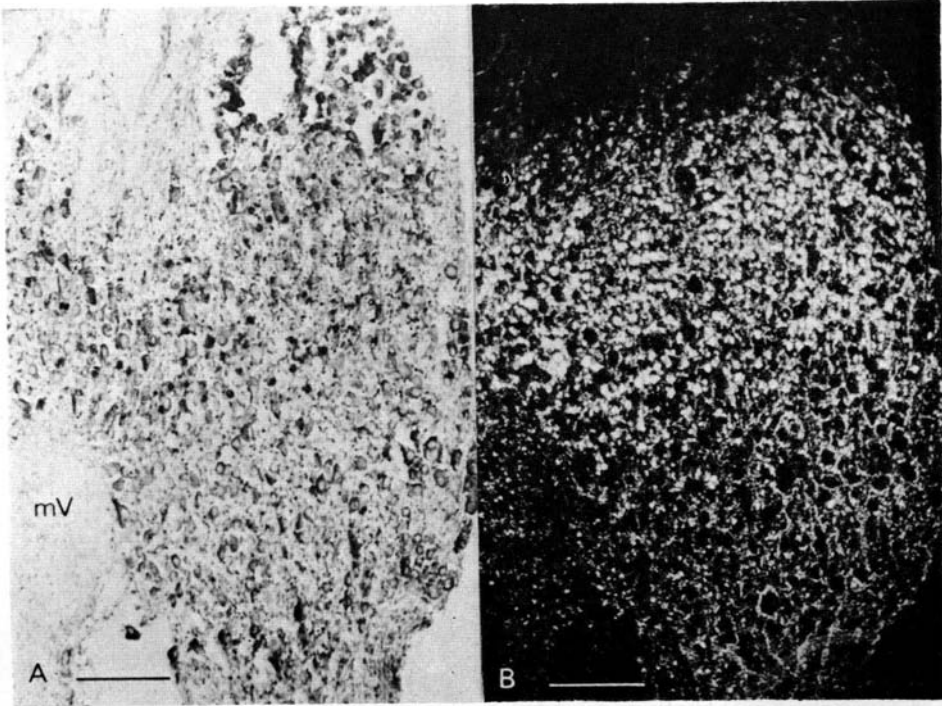The results of these experiments indicate that most neurons in the chick

Fig. 10. Bright-(A) and dark-field (B) photomicrographs of left trigeminal ganglion MX-MD lobe from an 18-day chick embryo injected at stage 26 (5 days). The presence of unlabeled cells distally and peripherally, and the scarcity of labeled cells around the motor nerve is similar to that observed in Fig. 7. Bar equals 100 μm.

trigeminal ganglion are formed between the second and seventh days of development in a distal-to-proximal and peripheral-to-central sequence, as summarized in Fig. 11. These waves of neuron production are not discrete and discontinuous but overlap both in position and time. Most of the cells which continue to divide after the first week of incubation appear to be neural crest-derived supporting elements, although the possibility that some of these cells may be small, immature neurons which do not develop until after hatching cannot be excluded.

These results reveal that there is a positive correlation between neuronal birthdates and embryonic origins of trigeminal sensory neurons. Neurons derived from placodal epithelium are the first to withdraw from the mitotic cycle, being formed between days 2 and 5 of incubation. Included in this population are the placodal cells described by Johnston (1966 and in preparation) that are located peripheral to neural crest-derived neurons in the proximal part of the ganglion.

We did observe a few neurons interspersed among the proximal, crest-derived population that are unlabeled following isotope application as early as the third day of incubation. Whether these are isolated placodal cells or pre-
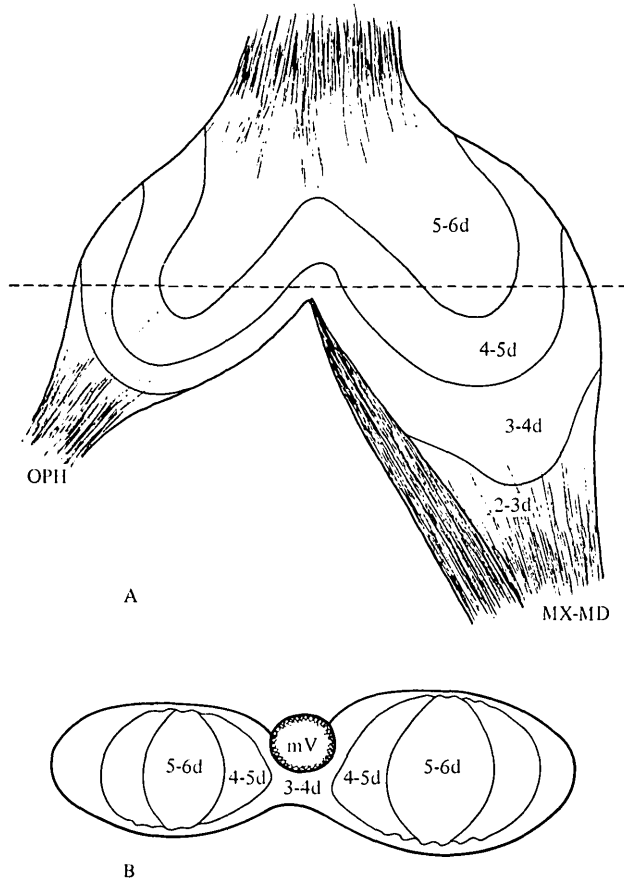
Fig. 11. Summary of the times of terminal mitosis of trigeminal ganglion neurons. (A) Schematic parasaggital section through left ganglion. (B) Frontal section at the plane shown by dashed line in (A). Times indicate days on which terminal mitosis of presumptive neurons occurs.

cociously postmitotic neural crest neurons is not known, and experiments in which the birthdates of identifiable crest and placode cells can be determined are now in progress.

The prospective trigeminal sensory neurons derived from the neural crest, which are located primarily in the proximal, central part of the ganglion, cease dividing between days 4 and 7 of incubation. In contrast to the placode cells which immediately acquire many of the morphological characteristics of sensory neurons, these proximal cells have been described as a mixture of small, immature neurons and undifferentiated cells through the end of the second week of incubation (Hamburger, 1961; Meyer, Wenk & Grosse, 1973; Ciani *et al.* 1973; Gaik & Farbman, 1973*b*). One possible explanation for this difference is that the crest-derived cells may be neurons which exhibit a long latency between the time of terminal mitosis and the onset of maturation, as occurs in some central

systems (Rakic, 1973). Alternatively, these cells may simply be differentiating neurons that are refractory to silver impregnation. This latter possibility is supported by recent horseradish peroxidase uptake analyses that have shown that many proximal as well as most distal neurons in the avian trigeminal ganglion possess extensive peripheral projections by 7 days of incubation (Noden, 1978b, 1980b).

In addition to establishing the times of terminal mitosis our study indicates that there are no significant morphogenetic rearrangements of sensory neurons in the avian trigeminal ganglion. The locations of labeled and unlabeled cells in ganglia fixed at 18 days of incubation are the same as those in ganglia fixed at 11 days. This proves that the dark and light neurons, which are interspersed throughout the mature ganglion, do not necessarily differ in their birthdate. Furthermore, no unique relationship exists between the two neuron types in the mature trigeminal and the two embryonic populations which initially form the trigeminal ganglion. Apparently, any cell in the 7-day embryonic ganglion, whether placode- or neural crest-derived, has the developmental potential to become a dark or light neuron.

Avian spinal sensory ganglia are cytologically similar to the trigeminal during both early embryonic and later stages (Hamburger & Levi-Montalcini, 1949). Using methods similar to ours Carr & Simpson (1978) found a similar distal-to-proximal sequence of neuronal birthdates in brachial dorsal root ganglia. Thus, early cessation of mitosis is not a property unique to sensory neuroblasts of placodal origin, which are absent in spinal ganglia, but rather is dependent upon a cell's position within the ganglion.

The situation in rodents appears to be quite different. In the trigeminal ganglia of albino rats (Forbes & Welt, 1979) and in the spinal ganglia of both mice (Sims & Vaughn, 1979) and rats (Lawson, Caddy & Biscoe, 1974; Theisen, 1979) the sequence of neuronal birthdate corresponds more closely to the size of the neuron in the *mature* ganglion, with the larger neurons of the adult being generated first and the smaller cells later. Since these populations are interspersed in the ganglia, it appears that cell type rather than cell position is more closely correlated with terminal mitosis in these mammals. The significance of this apparent difference is not known.

This study, in conjunction with HRP analyses of the neurons in the trigeminal ganglion (Noden, 1980a, b), provides baseline data for further experiments concerned with mechanisms of sensory gangliogenesis. By integrating these data with experiments involving transplantation of ganglionic precursors, it is possible to investigate the role of interactions among neural crest cells, placodal cells and the peri-metencephalic environment in both the determination of cranial sensory neurons and the establishment of normal trigeminal connections. In addition it is hoped that these experiments will eventually clarify the functional significance of both the dual embryonic origin and the dual cytology of trigeminal sensory neurons.

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