Neuronal adjustments in developing nuclear centers of the chick embryo following transplantation of an additional optic primordium

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SUMMARY
Following transplantation of an additional optic primordium into the orbital mesenchyme of chick embryos of approximately 2 days of incubation age, the changes in cell number in the ciliary ganglion, accessory oculomotor and trochlear nuclei were studied at various stages of development. Cell counts were made at 1-day intervals from days 9 through 15 for ciliary ganglion, and from days 13 through 15 for the accessory oculomotor and trochlear nuclei. Cell counts for the ciliary ganglion on days 9 and 11 were similar on the operated and control sides which suggests that grafting of an additional optic primordium, and thus enlarging the periphery, is not involved in the control of proliferation. Comparison of the number of cells for the ciliary ganglia and the accessory oculomotor nuclei at days 13 and 15 showed an increase on the affected side ranging from 8 to 27%, and 9 to 33% respectively. We interpret this increase on the experimental side as a reduction in the number of degenerating cells that occur in normal development, as a result of an enlargement of the peripheral field of innervation. Three cases showed an increase in the number of cells in the trochlear nucleus ranging from 9 to 29%. This increase was attributed to an increase in the size of the superior oblique muscle of the operated side as determined by volumetric measurements. On the basis of the evidence we conclude that an enlarged periphery acts by regulating the level of naturally occurring cell death by reducing the amount of cell loss, leading to a corresponding increase in final cell number.

INTRODUCTION
Cell loss in the developing nervous system is a naturally occurring phenomenon during embryogenesis. In recent years it has become clear that neuronal death plays a significant role not only in determining neuronal numbers, but also as a major factor in the organization of the nervous system as a whole. The concept that neuronal death plays a major role in shaping developing neuronal centers, stems from the findings, largely on the development of the amphibian and avian nervous system (Hamburger & Levi-Montalcini, 1949;
Hughes, 1961, 1965; Prestige, 1965, 1967). In a detailed analysis of degenerative processes which take place in the developing sensory spinal ganglia, Hamburger & Levi-Montalcini (1949) showed a massive degeneration of neurons in cervical and thoracic ganglia, but not in the limb innervating sensory ganglia of brachial and lumbo-sacral levels. However, after extirpation of the fore and hind limb-buds in 2-day-old chick embryos, a massive degeneration of cells occurred in the sensory ganglia of these levels. The similarity in the topographic distribution of degenerative changes and the fact that it occurred at approximately the same developmental stages in the normal as well as experimental conditions, led them to suggest that perhaps a similar peripheral mechanism was operative in both instances. They proposed the hypothesis that, in general, axons which fail to establish contacts of some sort at the periphery, undergo retrograde degeneration which eventually results in the breakdown of their perikarya (Hamburger, 1958, 1975). For the first time, a clear and logical explanation of neuronal death in the embryonic nervous system emerged, namely, that cells were dying in both normal and experimental situations for the same reason. In the normal case, too many cells were being produced and only a few which make suitable peripheral contact are selected to survive. In the second case, most of the cells die because the peripheral field had been significantly reduced experimentally. Similar observations on cell death have been reported in the development of several other brain stem nuclei and motor columns (Hughes, 1961, 1968; Cowan & Wenger, 1967, 1968a, b; Prestige, 1970; Rogers & Cowan, 1973), and have been the subject of an excellent review by Cowan (1973). The hypothesis of Hamburger & Levi-Montalcini (1949) can only be put to a critical test by peripheral overloading that might accommodate additional fibers and thus reduce the level of cell loss.

Until recently, the experimental investigation of Shieh (1951) had provided perhaps the most convincing evidence that peripheral effects on early neuronal development act by regulating neuronal death. By transplantation of the cervical segment of the spinal cord of 9–25 somite chick embryos to the thoracic level of host embryos of the same age which had been previously operated on for the extirpation of the thoracic segment of the spinal cord, Shieh showed that the transplanted segment of the cervical spinal cord underwent a differentiation similar to the differentiation characteristic of the thoracic segment. In his experiment, survival and migration of nerve cells, doomed to die under normal conditions, were obtained when a suitable peripheral field was provided for the cells to innervate. The elucidation of mechanisms by which the periphery interacts with a neuronal population within a developing neuronal center has since become a central problem in neuroembryology.

While most neuroembryologists are agreed that a certain proportion of axons degenerate, having failed in the competition for suitable attachment sites, it is now known whether the limitation is imposed by the number of postsynaptic sites available during development. There are only speculations as to the under-
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lying mechanism, and the nature of the interactions or exchanges that must occur, if a neuron is to ‘know’ and to react to the peripheral mass it finds itself in. The question of the nature of the influence of the periphery has not in fact been resolved, and it was this question and the conceptual issues related to it that prompted the present experimental study.

It is clear that to analyze and relate the nature of the influence of the periphery one would like a readily characterizable neuronal center, preferably involving a well defined peripheral field of innervation. The influence should be subject to experimental manipulation or control, and the responding neuronal center should be capable of analysis both quantitatively and qualitatively during development, including the critical period of normally occurring cell loss affecting the center. Many of these advantages, we find, are provided by the developing visual system. On the other hand, the assessment of peripheral effects involving the addition of a supernumerary limb (Hollyday & Hamburger, 1976) is hampered by difficulties in the interpretation of motor neuron distribution along the lateral motor column, and the peripheral distribution of nerves supplying the hindlimbs on the experimental side.

Therefore, we chose the developing visual centers for our analysis since considerable information has been accumulated on the magnitude of cell loss in some of the visual centers in normal development as well as following removal of the eye (Cowan & Wenger, 1967, 1968a, b). This paper is our first report on the mechanism underlying peripheral control of cell number in brain centers. It details the effects of transplantation of an additional optic primordium on cell loss in the ciliary ganglion, accessory oculomotor nucleus and trochlear nucleus in the chick embryo, and compares the quantitative data from experimental and control sides. In general, the results indicate that peripheral effects on early development are mediated by the regulation of neuronal death, and that the enlargement of a peripheral field of innervation promotes the survival of a significant number of neurons which, otherwise, would have been doomed to die under normal conditions.

MATERIALS AND METHODS

The embryos used in this investigation were from the Babcock strain of fowls maintained in the animal care facility at this medical center. Fertile eggs obtained from this flock were incubated in a commercial forced-draft incubator at 37.5 ± 1 °C and 70–80% relative humidity.

Surgical procedure

The donor embryos for the operation were incubated for 40–45 h; stage 12 (Hamburger & Hamilton, 1951), i.e. 16-somite stage. The host embryos were incubated for 38–40 h; stage 11, i.e. 13-somite stage. This latter ratio between host and donor embryos was determined after trying several stages in order to ensure a synchronous and harmonious development of host and graft tissues.
The technique originally described by Narayanan (1970) for opening the egg and preparing the embryo was adopted for microsurgery. The operations were carried out aseptically. The vitelline membrane and the portion of the optic primordium of the donor embryo usually of the right side was lightly stained with nile blue sulfate impregnated in agar, while the right side of the host embryo was stained with neutral red. The differential staining of graft and host tissues helped greatly in the exact positioning and orientation of the graft while being maneuvered into the site which has been previously prepared on the host embryo. The implantation site was prepared in the following way: the site was always on the right side close to and just behind the native eye (i.e. the host eye on the operated side). The ectoderm together with adhering mesoderm of this region was carefully incised with the vibrating needle (Wenger, 1968). Using the point of the needle, the incision was widened sufficiently by a slight to-and-fro movement so as to form a pocket large enough to receive the graft. Utmost care was taken during this procedure not to damage the underlying endoderm or cause excessive hemorrhage.

The optic primordium from the donor embryo was then excised with the vibrating needle. Utmost care was taken to keep the graft as small as possible with minimum of adjacent mesenchyme or epithelium. The graft was transferred by means of a Spemann micropipette from donor to host embryo. The donor lens epithelium was always retained in its proper relation to the graft. The graft was pressed into the implantation site as securely as possible. To attain a good fit of the transplant against that of the host, it was found necessary to withdraw as much fluid as possible from the region around the host head, causing the graft to adhere to the adjacent host structures firmly and in the proper orientation. The window on the shell was then sealed with a cover glass and hot paraffin, and the embryo was returned to the incubator. The embryos were allowed to survive until they were recovered for fixation.

Successfully operated embryos showing good fusion and excellent differentiation of the eyes with clearly identifiable lens and iris in both graft and native eyes were fixed by transcardiac perfusion with Bouin’s fluid. The heads of experimental embryos were then dissected out from the rest of the body and immersed in Bouin’s fluid overnight, dehydrated in ethanol and processed for paraffin embedding. Each head was serially sectioned at 12 μm in a transverse plane, and stained with hematoxylin–eosin–orange G (Humason, 1962).

Cell counts

The number of cells in the ciliary ganglion, the trochlear nucleus and the accessory oculomotor nucleus on the affected and control sides were determined by counting the cells throughout the extent of each of the above. Cell counts were made in every section of embryos of 11–15 days of incubation age, and in every fifth section of embryos of 9 days of incubation age or younger. Each section was projected on to a white paper at an original magnification of 300 ×
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using a camera lucida drawing tube attached to a Wild M-20 binocular research microscope. The outline of each neuron with a distinct mass of chromatin in its nucleus was drawn while being viewed through the microscope and was simultaneously recorded on a hand tally digital counter. Raw counts were corrected for double counting by the method of Abercrombie (1946), using the section thickness and the average extent of the chromatin mass. All cell counts were made by the same investigator (Y.N.) for the sake of consistency in the criteria and the method used for cell counting. The data were analyzed statistically using a Student t-test.

RESULTS

1. General condition of the experimental embryos

Although every effort was made to be consistent in the microsurgical procedure with regard to the size of the optic primordium removed from the donor and the site of implantation on the host, variations were observed in the relative development of the grafted eye in some cases. Therefore, in order to assess the effect on cell loss following the transplantation of an additional eye, several criteria have been examined in the selection of our experimental material for this report. These were: (1) good differentiation of the native and grafted eyes surrounded by one orbit; (2) fusion of the native and grafted eyes; and (3) formation of central connexions by optic nerves emerging from both native and grafted eyes as observed in serial sections through the head region. Based on the above criteria, it was determined that 6% of the operations were successful. Twenty-two successful cases were selected from which sections of the orbital tissues and the extraocular muscles were available for histological examination. A typical case (Experiment DE 81; 13 days incubation age) is shown in Fig. 1. From this it can be seen that the grafted eye is well developed, has well formed lens, and is almost the same size as the native eye. Both the native and grafted eyes on the operated (right) side are enclosed in one enlarged orbit showing excellent healing of the surrounding tissues. Figure 2 is a low power photomicrograph of a frontal section through the head of a 13-day experimental embryo, DE 187, showing good fusion of the eyes on the operated side, and Figs 3 and 4 are low power photomicrographs of transverse sections through the head of a 11-day experimental embryo, DE 355, showing portions of the optic nerves of the native and grafted eyes.

2. The effects of transplantation, qualitative and quantitative aspects

The magnitude of normally occurring cell loss in the trochlear nucleus (Cowan & Wenger, 1967), in the accessory oculomotor nucleus (Cowan & Wenger, 1968), and in the ciliary ganglion (Levi-Montalcini & Amprino, 1947; Landmesser & Pilar, 1974a, b, 1976) have been so thoroughly investigated in the chick embryo that there is little likelihood of new information arising from
normal cell counts in the present study. However, as explained earlier, since some degree of variability in the relative development of the native and graft eyes is inevitable in such experiments, it became necessary to use quantitative estimates of the unaffected (control) sides of our experimental material, as a background against which variations in cell number of the affected side might be interpreted objectively. The results are presented in Table 1 for the ipsilateral ciliary ganglion, the ipsilateral accessory oculomotor nucleus and the contralateral trochlear nucleus, for the experimental cases, and corresponding values from the unaffected sides as controls. The figures refer to data obtained from embryos of 9, 11, 13 and 15 days for the ciliary ganglion; 13 and 15 days of incubation for the accessory and trochlear nuclei. The transplantation of an additional optic primordium inevitably results in some asymmetry, and consequently defining the boundaries of these nuclei is difficult at early stages. Therefore, no counts have been made of the cells in the trochlear nuclei of both sides in embryos of 9 and 11 days of age. However, for the sake of evaluating our data on cell loss, the number of cells in the accessory oculomotor nuclei of both sides were counted in one 11-day experimental animal (DE 298), and is included in Table 1. By day 13 of incubation, however, both the trochlear and accessory oculomotor nuclei are distinctly defined, and counts at these stages are comparatively easy.

Ciliary ganglion

In our experimental material the ciliary ganglion of the operated side, as revealed in sections, is somewhat irregular in outline compared to the smooth contour and oval shape of the ganglion of the control (left) side (Fig. 5). A low power photomicrograph of a section through the ciliary ganglion in one of these experimental cases (DE 187; 13 days of age) is shown in Fig. 6. In the majority of cases the ganglion has developed lobe-like extensions as seen in Fig. 6. No significant differences are observed in counts of the number of

**Figures 1-4**

Fig. 1. A typical example DE 81 (incubation age: 13 days) to illustrate the results of grafting an additional optic primordium into the orbital mesenchyme of the right side of a chick embryo. Observe the excellent fusion of the grafted eye (GE) with the native eye (NE) and located in one enlarged orbit.

Fig. 2. The grafted eye (GE) and the native eye (NE) in experiment DE 187 (incubation age: 13 days) showing the fusion between the two eyes as observed in a frontal section.

Fig. 3. Light micrograph of a transverse section through the native eye (NE) in experiment DE 355 (incubation age: 11 days) showing emergence of the optic nerve (ON). Scale 1 mm.

Fig. 4. Light micrograph of a transverse section through the grafted eye in experiment DE 355 (incubation age: 11 days) at the level of the optic chiasm to show the crossing of the optic nerve (ON) of the grafted eye (GE). Scale 1 mm.
neurons (Table 1) made on four cases on the two sides of embryos between 9 and 11 days of incubation. The ganglion on the operated side is similar in size and general appearance to that of the unoperated (left) side (DE 338: 9 days; DE 298, DE 367, DE 355: 11 days). Based on these observations and cell counts, it seems reasonable to conclude that transplantation of an additional optic primordium has no effect on proliferation of cells or their differentiation in the ciliary ganglion during the earlier stages of development when the ganglion supposedly attains numerical completion.

The next 48 h is characterized by a dramatic cell loss on the unoperated side. Three experimental cases of 13-day embryos are available: DE 81, DE 187, and DE 190. Counts at this age range between 5513 and 5613 cells in the ganglion on the control (left) side. Compared with the cell counts made on DE 338: 9 days (see Table 1) there is a 32% cell loss on the unoperated side at this age. However, the number of cells in the ciliary ganglion on the operated (right) side in all three cases at this age (13 days) range between 6431 and 6911 cells, and when compared with the 9-day count on the operated side, shows a 20% cell loss. Further, the cell counts at 13 days on the operated side correspond very closely with the figures for the right and left sides of the 11-day embryos (Table 1). When compared with cell counts of 11-day embryos, there is a slight increase on the average of 54 cells (approximately 0.8%) on the transplanted side of our 13-day embryos as against a more pronounced loss on the average of 1320 cells (approximately 19%) on the control side.

If we now compare the number of cells on the affected side with those of the control values on day 13 in the three experimental cases (Table 1) there is a marked increase in the number of cells ranging between 818 and 1464 cells (approximately 15 and 27%). Figure 9 summarizes our data on ‘hyperplasia’

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**Figures 5-8**

Fig. 5. Low power photomicrograph of a section through the ciliary ganglion of the control side in experiment DE 187 (incubation age: 13 days). Observe the rather smooth, oval contour of the ganglion. Scale 100 µm.

Fig. 6. Low power photomicrograph of a section through the ciliary ganglion of the operated side in experiment DE 187 (incubation age: 13 days). Observe the irregular contour of the ganglion and the lobe like extensions (arrows). Scale 100 µm.

Fig. 7. Low power photomicrograph to show the appearance of the trochlear nucleus in experiment DE 187 (incubation age: 13 days). At this stage the nucleus on the experimental side (E) is considerably larger. In the experiment the nucleus showed an increase of 11% in the number of cells as compared with those of the control side (C). Scale 1 mm.

Fig. 8. Low power photomicrograph to show the appearance of the accessory oculomotor nucleus (outlined) in experiment DE 254 (incubation age: 15 days). The nucleus on the affected side (E) as a whole is larger in size than the nucleus of the control side (C). In the experiment the nucleus showed an increase of approximately 18% over the figures for the nucleus of the control side. Scale 1 mm.

All sections shown here have been stained with hematoxylin–eosin and orange G.
following transplantation of an additional optic primordium. The stippled area shows clearly the uniform increase in the number of cells which survive on the affected side. Also, the time course of cell loss in both control and affected sides appear to be the same and the general trend in the affected ganglia is essentially similar to that of the ganglia on the control (left) side at all ages (Fig. 9).

In the animals sacrificed on day 15 of incubation (DE 132 and DE 254) the increase in the number of cells on the affected side is maintained, ranging between 287 and 722 (approximately 8 and 20 %) over the values for the ganglia of the unaffected (left) side (Table 1).
Table 1. Summary of data on hyperplasia in the ciliary ganglion, accessory oculomotor nucleus, and trochlear nucleus following transplantation of an additional optic primordium

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age in days at fix</th>
<th>Ciliary ganglion</th>
<th>Accessory oculomotor nucleus</th>
<th>Trochlear nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unoperated</td>
<td>Operated</td>
<td>Difference (%)</td>
</tr>
<tr>
<td>DE 338</td>
<td>9</td>
<td>8166</td>
<td>8336</td>
<td>+2</td>
</tr>
<tr>
<td>DE 298</td>
<td>11</td>
<td>6918</td>
<td>7064</td>
<td>+2</td>
</tr>
<tr>
<td>DE 367</td>
<td></td>
<td>6958</td>
<td>6548</td>
<td>-6</td>
</tr>
<tr>
<td>DE 355</td>
<td></td>
<td>6655</td>
<td>6345</td>
<td>-5</td>
</tr>
<tr>
<td></td>
<td>$\bar{x} \pm$ s.e. 6844 ± 95</td>
<td>6652 ± 214</td>
<td>$P &gt; 0.40$ N.S.</td>
<td></td>
</tr>
<tr>
<td>DE 81</td>
<td>13</td>
<td>5513</td>
<td>6777</td>
<td>+23</td>
</tr>
<tr>
<td>DE 187</td>
<td>13</td>
<td>5447</td>
<td>6911</td>
<td>+27</td>
</tr>
<tr>
<td>DE 190</td>
<td>13</td>
<td>5613</td>
<td>6431</td>
<td>+15</td>
</tr>
<tr>
<td></td>
<td>$\bar{x} \pm$ s.e. 5524 ± 48</td>
<td>6706 ± 143</td>
<td>$P &lt; 0.01\ast\ast$</td>
<td>2275 ± 20</td>
</tr>
<tr>
<td>DE 132</td>
<td>15</td>
<td>3700</td>
<td>4422</td>
<td>+20</td>
</tr>
<tr>
<td>DE 254</td>
<td>15</td>
<td>3813</td>
<td>4100</td>
<td>+8</td>
</tr>
</tbody>
</table>

* Significant at 0.05 level (Student t-test).

** Significant at 0.01 level (Student t-test).

N.S. = Not significant.
Trochlear nucleus

No counts have been made on the number of neurons in day-9 or day-11 embryos for reasons explained earlier (see p. 59). Cell counts from three experimental cases of 13 days incubation age are available from both sides of chick embryos DE 81, DE 187, and DE 190. The trochlear nucleus in the animals DE 187 and DE 190 gave a figure of 959 and 1010 cells respectively on the affected side, an increase of 92 cells (approximately 11%) and 227 cells (approximately 29%) over that of the cell counts for the control sides in each of the embryos. Figure 7 is a low power photomicrograph of a section through the trochlear nucleus of a 13-day experimental embryo, DE 187, showing increase in size of the nucleus on the affected side. Volumetric measurements of the superior oblique muscle were determined in the above two cases. DE 190, in which the trochlear nucleus shows a 29% increase in the number of cells on the affected side (Table 1), the superior oblique muscle showed a 30% increase over that of the control side (0.530 and 0.408 mm³ on affected and control sides respectively). In DE 187, in which the number of cells on the affected side (Table 1) had increased 11%, the corresponding muscle showed an increase of 8% (0.466 and 0.432 mm³ respectively). In DE 81, in which the number of cells was not significantly different between the two sides, the volumes of the muscle on the two sides were found to be identical (0.44 and 0.46 mm³ respectively). In the 15-day embryo, DE 254, in which the trochlear nucleus showed a 9% increase on the affected side, the corresponding muscle showed an increase of 11% (0.552 and 0.496 mm³ respectively). This indicated that in the three cases above (DE 187, DE 190, and DE 254), the increase in the number of nerve cells is probably due to a proportionate increase in the number of muscle cells and the 'hyperplasia' therefore is a reflection of the increase in the size of the peripheral field of innervation. In all the other cases, the transplantation of an additional optic primordium has little or no effect on cell loss on the affected side and the values are close to those of cell counts for the unaffected side. These figures are in agreement with the cell counts reported earlier for the trochlear nucleus by Cowan & Wenger (1967) for days 13 and 15.

Accessory oculomotor nucleus

It has been fairly well established that the axons of the cells of the accessory oculomotor nucleus terminate on the cells of the ciliary ganglion (Cowan & Wenger, 1968a; Narayanan & Narayanan, 1976). Therefore it seemed of interest to us to study the effect of transplantation of an additional optic primordium on the number of neurons in this nucleus.

One experimental embryo of 11 days, DE 298, for which we have cell counts of the accessory oculomotor nucleus, has 2925 cells for the unoperated (left) side, and 2943 cells on the operated (right) side. There is only a difference of 18 cells between the two sides (approximately 0.6%). Three experimental
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animals of 13 days incubation age are available: DE 81, DE 187, and DE 190. The number of cells in the accessory oculomotor nucleus on the operated (right) side in all three cases (Table 1) range between 2488 and 3087, while the figures for the unoperated (left) side range between 2274 and 2310 cells. There is a marked increase in the accessory oculomotor nucleus of the operated (right) side between 214 and 777 cells (approximately 10 and 33 %) over the values of the control (left) side. When compared with cell counts of the 11-day embryo, there is a loss on the average of 650 cells (approximately 22 %) on the unoperated side of our 13-day embryos, as against a loss of only 169 cells (approximately 5 %) on the operated side.

In embryos fixed on day 15 of incubation (DE 132 and DE 254), the accessory oculomotor nucleus shows an increase in the number of cells on the affected (right) side ranging between 9 and 18 % respectively (Table 1). Figure 8 is a low power photomicrograph of a section through the accessory oculomotor nucleus of a 15-day experimental embryo, DE 254, showing increase in the size of the nucleus on the affected side. The data on the degree of hyperplasia following transplantation of an additional optic primordium are summarized in Fig. 9. While the time course of cell loss is essentially similar in its general trend in both operated and control sides for the accessory oculomotor nucleus, the uniform increase in the number of cells which survive during the same time period on the affected side is especially noteworthy.

DISCUSSION

The purpose of this experiment has been to clarify the role of the periphery as a regulatory mechanism in the control of cell number within a population during development. This was done by the surgical creation of an enlarged periphery by grafting an additional optic primordium into the presumptive orbital mesenchyme of one side in chick embryos. The response to the experimentally altered situation at the periphery was studied in the ciliary ganglion, accessory oculomotor nucleus and the trochlear nucleus. Within the limitations of the method used and the restricted material to which it has been applied, the results of our quantitative analysis based on cell counts seem to lead to the conclusion that, in general, there is a reduction in neuronal cell loss following the transplantation of an additional optic primordium.

1. Changes in cell number after grafting an additional optic primordium

The most common technique employed in studies dealing with the problem of cell loss during normal development is a reduction of the peripheral field of innervation by the radical extirpation of either the optic vesicle (Cowan & Wenger, 1967, 1968) or the limbs (Hamburger, 1958). What has been shown in these experiments is the rather severe cellular hypoplasia affecting the neuronal center but essentially following the same time course as in normal development.
In contrast, the grafting of an additional optic primordium would provide an enlarged periphery for the cells to innervate. For the ciliary ganglion (Table 1), our evidence based on cell counts of a 9-day experimental chick embryo shows that there is no significant difference between the two sides, and this trend continues through day 11 of incubation. It would appear then, that the addition of an optic primordium has not produced an increase in the number of cells during this period when it would normally be expected to occur, if the experiment had any effect on mitotic activity in the ciliary ganglion. Additionally, the rather slight increase of cells in the unoperated side as compared with those for the ganglion of the operated side would argue against this possibility. Our results seem to point to the conclusion that enlargement of the periphery has not influenced proliferative activity in the ciliary ganglion. Similarly, no difference in cell counts was observed in the accessory oculomotor nucleus of a 11-day embryo. Since the same experimental animals were used in our analyses of other visual centers it seems reasonable to infer that this conclusion is also valid for the affected accessory oculomotor nucleus and the trochlear nucleus.

A difference in the number of cells in the ciliary ganglion, the accessory oculomotor nucleus and in the trochlear nucleus is observed on the experimental side from day 13 onwards well after proliferative activity has ceased. There is sufficient evidence derived from studies on normally occurring cell loss in each of these neuronal centers that the changes affecting the cells in later stages of development are rather regressive in nature. In normal development, the later stages are characterized by severe cell loss as shown for the trochlear nucleus (Cowan & Wenger, 1967), for the accessory oculomotor nucleus (Cowan & Wenger, 1968), and for the ciliary ganglion (Landmesser & Pilar, 1976). The difference between the two sides in our cell counts for the ciliary ganglia, accessory oculomotor nuclei and the trochlear nuclei appears as an increase in the experimental side due to a reduction in the level of cell loss. In our experimental animals, the increase in the number of cells ranges between 15 and 27% for the ciliary ganglion, and between 10 and 33% for the accessory oculomotor nucleus in 13-day embryos. This trend continues to 15 days of incubation where the figures range between 8 and 20% for the ciliary ganglion and between 9 and 18% for the accessory oculomotor nucleus. It may be pointed out that although the increase is fairly consistent in the accessory oculomotor nucleus, it is interesting to note that no strict correlation exists between the percentage of increase in the accessory oculomotor nucleus and that of the ciliary ganglion in each case (Table 1). This variability which is also evident in the 15-day experimental cases (Table 1) between the percentage increase in the ciliary ganglion and the accessory oculomotor nucleus is not unexpected for several reasons. The number of cells in the accessory oculomotor nucleus of the control (left) side is less than half the number of cells in the ciliary ganglion at corresponding stages of development (Table 1). There is probably a good deal of branching of the preganglionic fibers as stated by Cowan & Wenger (1968), and
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the relationship between the cells of the accessory oculomotor nucleus and those of the ciliary ganglion is probably not on a one to one basis (Landmesser & Pilar, 1976).

As for the trochlear nucleus, three cases showed a significant increase in the number of cells on the affected (left) side. In these cases a corresponding increase in the number of muscle fibers comprising the superior oblique muscle on the operated (right) side was observed. It is very likely that a small amount of mesenchyme might have been included with the graft. In all other cases, very little or no orbital mesenchyme was included with the graft which would account for the absence of additional muscle fibers. This is reflected, in turn, in the figures of cell counts for the two sides which are very similar (Table 1).

Two central conclusions can be drawn from the foregoing analysis of our data. The first, based on cell counts, is that grafting of an additional optic primordium has no effect on proliferative activity in the neuronal centers chosen for experimental analysis. It may be relevant to point out that removal of the eye in extirpation experiments has also failed to show any effect on cellular proliferation (e.g. the trochlear nucleus, Cowan & Wenger, 1967) as also in cases where the optic tectum is deprived of its afferent input (Cowan, Martin & Wenger, 1968). The second conclusion is that the effects of the enlarged periphery on early neuronal development in these centers are by the regulation of neuronal death. Thus a reduction in the level of cell loss leads to corresponding increase in the size (= cell number) of a neuronal population.

2. Results of peripheral overloading, other studies

To our knowledge, the only other experiment that has been reported recently dealing with the central problem of peripheral effects on neuronal cell loss is by Hollyday & Hamburger (1976). These investigators studied the effect of enlargement of the periphery by implantation of a supernumerary leg on the lateral motor column (l.m.c.) of chick embryos of 6, 12, and 18 days of incubation. Their study may be regarded as the most comparable to our own since, in both cases, the basic design is similar and is concerned with the effects of enlargement of the periphery upon an efferent system. They have provided evidence to show that the implantation of a supernumerary leg leads to an increase in the number of cells ranging between 11 and 27 %. An important observation made by these authors in the above experiment, which is relevant to our own investigation, is the fact that they excluded any direct effect or ‘remote control’ as they have termed it, on proliferation in the lateral motor column. This conclusion was based on cell counts which was found to be essentially similar between experimental and control embryos at stage 28 (6 days) after the l.m.c. is formed but preceding the onset of normally occurring cell loss in the development of the l.m.c. (Hollyday & Hamburger, 1976). One feature which may be relevant in this context, is the possible effect of sensory input on cell loss since transplantation of an additional limb does involve both motor and sensory fibers. It is well
known that basal plate elements are usually generated several days ahead of the alar plate or neural crest derivatives (Hamburger, 1948; Cowan et al. 1968), and the reflex circuit is closed at 6–6½ days by the establishment of synaptic connexions between collaterals from the dorsal sensory tract and internuncial neurons (Visintini & Levi-Montalcini, 1939). Experiments to determine whether sensory input has any influence on normally occurring cell loss in the l.m.c. of hindlimb levels by removal of neural crest precursors of spinal ganglia in chick embryos are currently in progress (Malloy & Narayanan, unpublished).

3. Conceptual issues related to peripheral control of cell number

The conceptual issues that have guided the investigation of normally occurring cell loss in various neuronal centers are versions of two basically different views as to why so many cells undergo early differentiation even to the point of eliciting responses and then die. These have been reviewed by Prestige (1974, 1976). The first of these, the redundancy hypothesis, derives from the work of Hamburger & Levi-Montalcini (1949). It holds that there may be an early hyperneurotization with an excess of fiber endings for a limited number of available postsynaptic sites or some trophic agent. Consequently a number of axons are rendered ‘redundant’ and undergo a form of retrograde degeneration, ending in cell death. This would imply that most or all of the axons from a center have reached the periphery before the regressive changes begin, and that cellular breakdown is paralleled by axon degeneration (see Hamburger, 1975). The second basic concept, the rejection hypothesis, holds that the majority of axons having made connexion with the periphery even to the point of being functional, in the sense of being capable of transmitting impulses, are rejected from their sites of ‘initial’ contact, leading to the death of the cells subsequently. The term initial is used here to mean transient connexions.

One obvious question that arises is whether or not the axons have made connexions with the periphery. A second question is if connexions have been made, how the axons terminate, and form synaptic connexions on particular peripheral cells at precisely specified areas, on a permanent basis. The evidence bearing on this problem, point to the presence of axons in the peripheral field and have been confirmed by axon counts comprising the ventral roots in Xenopus (Prestige & Wilson, 1972, 1974). Prestige (1976) has produced evidence to show that at least some of the spinal motoneurons that die during normal development of Xenopus have already been peripherally connected before death. The qualitative data obtained by Hamburger (1958) for the chick embryos in leg bud extirpation experiments indicate that the motor roots are at first fully formed and then become gradually reduced in size corresponding to the level of cell loss in the l.m.c. Oppenheim & Heaton (1975) found that the first appearance of a positive HRP reaction coincided with the time when nerve processes are first detected in the limb-bud at 4-5 days of incubation. Direct evidence in support of connexions being formed to the point of being func-
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Eliciting responses has been provided by Landmesser & Pilar (1974a, b) for the ciliary ganglion and by Landmesser & Morris (1975) for the hindlimb of chick embryos.

What this means is that there is some mechanism, presumably in the periphery, which operated from the time axons have made contact to approximately the period of maximal cell loss, that determines the establishment of ‘final’ connexions. The mechanism by which nerve terminals make permanent contact with appropriate sites remains to be investigated. It is tempting to turn at once to new ideas on the nature of cell interactions, including cell adhesions and surface interactions and to those in which an exchange of materials is involved. Further experimental work is needed in order to clarify the fundamental nature of this important relationship between a developing neuronal center and its peripheral field of innervation.

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