Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4476–4480, June 1990
Developmental Biology

The microenvironment created by grafting rostral half-somites is mitogenic for neural crest cells

(avian embryo/dorsal root ganglia/mitogenesis/sclerotome/segmentation)

RONALD S. GOLDSTEIN*, MARIE-AIMÉE TEILLET†, AND CHAYA KALCHEIM*‡

*Department of Anatomy and Embryology, Hebrew University of Jerusalem, Hadassah Medical School, Jerusalem, P.O.B. 1172, 91010, Israel; and †Institut d'Embryologie Cellulaire et Moléculaire, Centre National de la Recherche Scientifique et Collège de France, 49 bis Avenue de la Belle Gabrielle, 94736 Nogent-sur-Marne, Cedex, France

Communicated by Nicole Le Douarin, March 20, 1990

ABSTRACT **Chicken embryo neural crest cells that migrate into a paraxial mesoderm constructed of multiple rostral half-somites from quail embryos form unsegmented "polyganglia," instead of distinct dorsal root ganglia (DRG). We report here that the environment that is created by grafting rostral somitic (RS) moieties not only is permissive for neural crest cell migration and consequent DRG formation but also is mitogenic for the DRG precursor cells. On embryonic day 3.5 (E3.5), 1 day after surgery, there is a 42% average increase in volume of the polyganglia compared with the corresponding DRG on the unoperated side. The volume increase is accounted for by an increased number of DRG cells—an average of 46% more cells are found in the polyganglia. The increases in volume and cell number are still present a day later at E4.5 (38% and 52%, respectively) and are observed in both limb-forming and non-limb-forming regions of the embryonic axis. The mechanism for this increase in cell number and volume in the polyganglia is enhanced proliferative activity. On E3.5 the proportion of cells incorporating thymidine of the total DRG cell number is 45% higher in the polyganglia than the control side, when embryos are given a short pulse before sacrifice. This indicates that the rostral sclerotomal environment stimulates the crest cells to proliferate. The difference in volume between the polyganglia and the normal DRG continues to grow until at least E8, when the polyganglia are twice as large as the control DRG. The continued increase in volume can also be accounted for by the mitogenic effect of the RS grafts, since on E4.5 the percentage of thymidine-labeled cells compared with the total cell number in DRG is 28% higher in the polyganglia than in control ganglia. This study demonstrates that the somitic microenvironment regulates the proliferation of neural crest cells in the nascent DRG.**

Dorsal root ganglia (DRG) arise from a transient embryonic structure, the neural crest (1, 2). Although the initial migration of the neural crest cells from the neural tube is continuous rostro-caudally (3–5), segmented DRG eventually form because of the constraint placed on the further migration of crest cells by the adjacent mesodermal structures, the somites. Neural crest cells and motor axons are able to enter only the rostral portion of the sclerotome arising from each somite (5, 6–11). In contrast, the caudal half-somites inhibit the entry of neural crest cells, exiting the neural tube opposite them. Being unable to migrate laterally, the crest cells migrate either rostrally or caudally where they contribute to two adjacent DRG (5).

It has been shown that experimentally modifying the cranio-caudal organization of the somitic mesoderm impairs the segmental migration of crest cells and segmentation of motoneuron axons into individual nerves (12). In the case of the DRG, construction of a continuous rostral somitic (RS) mesoderm led to the development of large, relatively unsegmented DRG termed polyganglia (13). In contrast, mesoderm consisting of only caudal somitic (CS) halves caused small, dorsally located DRG to develop.

In the present study, the effects on DRG development of the RS mesoderm are further investigated. The volume of the polyganglia with respect to the contralateral, control DRG was found to be increased at several developmental stages. This difference in DRG volume is due to the enhanced proliferation of neural crest-derived cells that develop in contact with both the grafted RS mesoderm and the neural tube. Therefore, the RS mesoderm, alone or in concert with the neural tube, is not only permissive for migration but also is mitogenic for neural crest cells that become DRG.

MATERIALS AND METHODS

Embryos of chicken (*Gallus gallus*) and Japanese quail (*Coturnix coturnix Japonica*) were used for this study. Eggs obtained from commercial sources were kept in a humidified incubator at 38 ± 1°C.

Embryonic Microsurgery. Surgery in this study was essentially the same as has been described (13). Briefly, the rostral halves of the three or four most recently formed somites were excised from 20- to 24-somite-stage quail embryos. A drop of 50% pancreatin (GIBCO) in Tyrode buffer (pH 7.4) was applied locally to facilitate dissection. The somitic halves from several donor quails were pooled in Tyrode buffer until implantation.

The three or four most recently formed somites on the right side of chicken embryo recipients were removed, and the space produced was filled with two to five quail RS. In some animals, unsegmented paraxial mesoderm equivalent in length to two somites was removed, along with the last detached somite(s). Recipients were from two groups. The first group was composed, in keeping with the previous study (13), of embryos of 11–15 somites at the time of implantation, and the DRG that formed facing the quail mesoderm were at cervical levels. The second group of recipients were at the 19- to 22-somite stage at the time of implantation. In these animals the DRG that formed opposite the graft were the brachial DRG 14 to 16, that contribute to the sensory innervation of the forelimb.

[^{3}H]Thymidine Labeling and Tissue Processing. Application of [^{3}H]thymidine was performed *in ovo* by the method of McMillan-Carr (14). The eggs were opened, and 12.5 μCi (1 μCi = 37 kBq) of thymidine (specific activity, 45–47 Ci/mmol; Amersham) was applied in two 50-μl drops of Tyrode

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CNS, central nervous system; CS, caudal somite/somitic; DRG, dorsal root ganglia; E, embryonic day; RS, rostral somitic.

‡To whom reprint requests should be addressed.

buffer on top of the chorionic membrane. The eggs were then sealed and incubated at 38°C for 70 min to allow uptake of the marker.

After incubation, the embryos were pinned out in Tyrode buffer, fixed in 4% formaldehyde in phosphate-buffered saline overnight at 4°C, and then dehydrated and embedded in paraplast. Serial 5- or 7-μm transverse sections were made at the level of the surgery, and the sections were stained by using the Feulgen–Rossenbeck reaction to distinguish the quail marker (15). Sections were counterstained with Fast green to better delineate the ganglia from surrounding tissues.

After determination of the limits of the graft and quantification of DRG size and cell number (see below), the incorporation of [^{3}H]thymidine into DRG cells was examined. Coverslips were removed by soaking the slides in xylene, and the sections were rehydrated to distilled water and allowed to air dry. The slides were then dipped in Kodak NTB-2 emulsion diluted 1:1 with distilled H_2O and exposed for 3–7 days. After development of the grains, the slides were restained in Fast green or toluidine blue to facilitate delimitation of the ganglia.

Quantification and Data Analysis. DRG were drawn from the serial sections with a camera lucida. The cross-sectional areas of the DRG were determined with the program SIGMASCAN (Jandel Scientific) using a high-resolution digitizing pad (Summagraphics M1201). Cell number in the DRG was quantified by counting all nuclei in every section in embryonic day 3.5 (E3.5) embryos and in every second section in E4.5 embryos. The DRG that were surrounded by a quail sclerotome were measured on the operated side and compared with the DRG on the left, unoperated side. For the purpose of quantification, ganglia on both sides that continued beyond the rostro-caudal extent of the graft were measured to completion (Fig. 1). All quantitative data are expressed as the percent difference between the operated (right) and control (left) sides ± SEM. Significance of results was determined using Student's paired *t* test.

We were unable to count nucleoli, as is conventionally done for estimation of cell number, because the formaldehyde fixation used did not produce distinct staining of nucleoli in these early embryos. In addition, many cells were still dividing and would not have had nucleoli. Since the average differences observed in cell number between the sides were substantially larger than the differences that occur naturally (see *Results*), and since in the E4.5 embryos we counted cells in only every second section, the likelihood of counting a cell twice was low. Therefore, statistical compensation for double-counting was not performed.

Three-dimensional reconstructions of the neural structures were made in a few chimeras. Camera lucida drawings were entered into the HVEM-3D program developed by the University of Colorado at Boulder Laboratory for high-voltage Electron Microscopy.

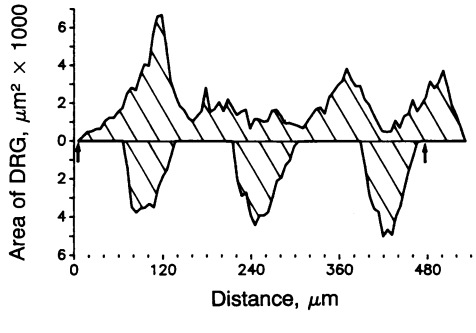

FIG. 1. The cross-sectional area of DRG as a function of distance along the rostro-caudal axis. The chicken embryo recipient, age 11 somites, received a graft of four quail RS halves in place of somites 9–11. The areas of the DRG on the control side are plotted downward and the areas of the polyganglion upward. The beginning and the end of the quail mesoderm are designated by arrows.

RESULTS

Polyganglia Have a Greater Volume and Contain More Cells Than Control DRG. In agreement with previous results (13) chimeras that contained a continuity of quail cells, and therefore a successful graft, gave rise to polyganglia in the operated region (Figs. 1 and 2*b*). In addition, as was first described by Stern and Keynes (12) using whole mount staining of younger embryos, the outgrowth of the motor axons on the grafted side was more or less continuous in the rostro-caudal axis (Fig. 2*d*).

The polyganglia that formed opposite the RS had a greater volume than the contralateral, control ganglia (Fig. 3). The average increase in volume of the polyganglia compared with the corresponding DRG on the control side immediately upon the condensation of the ganglia on E3.5 was 42 ± 8% and was significant ($P < 0.001$; $n = 7$).

On E4.5, when the ganglia were more distinct and outgrowth of sensory nerves had begun, the average increase in volume of the polyganglia compared with the control DRG was 38 ± 9% ($P < 0.005$; $n = 7$). Grafts in embryos sacrificed on E4.5 were made into two different portions of the neuraxis opposite ganglia 13–16, the ganglia that participate in the sensory innervation of the wing (16), and at cervical levels opposite ganglia 8–12. On E4.5, both groups had the same average increases in DRG size compared with controls—38 ± 5% for the brachial region ($n = 3$) and 38 ± 10% for the cervical region ($n = 4$). The difference in volume between polyganglion and the contralateral DRG in cervical-level grafts continued to grow until at least E8. The average elevations of polyganglia volume with respect to control DRG were 43 ± 3%, 57 ± 15%, and 98 ± 2% on days E5, E6, and E8, respectively (Fig. 3; $n = 3$ for E5 and E8; $n = 4$ for E6).

The elevation of polyganglion volume by grafted half-somites was specific to RS grafts. DRG that formed adjacent to grafted CS halves were substantially smaller than the control DRG as described previously (ref. 13; see also Fig. 3 showing the quantification of two representative CS grafts).

To determine the natural bilateral variation in ganglionic size, we measured the volume of three bilateral pairs of ganglia in four E4.5 unoperated animals. There was an average difference of 8.0% in the combined volume of the ganglia between the sides (9.1%, 10.4%, 4.3%, and 8.3%). However, unlike the situation in the operated animals, the side with the larger ganglionic volume was apparently random. Of these four animals, the first two had a larger volume on the left side, and the other two, on the right.

Counting DRG cells in the rostro-caudal extent of the grafts revealed that the increase in volume was largely due to an increase in the number of cells in the polyganglia compared with the contralateral control ganglia. The increase in cell number caused by the RS grafts was evident already on E3.5, when an average of 48 ± 6% more cells were observed. In E4.5 embryos, the average increase in cell number was 52 ± 10% (significant at $P < 0.005$), somewhat larger than that of the average volume increase. As was the case for volume, the increase in cell number in E4.5 embryos was evident in both brachial (59 ± 6%) and cervical (46 ± 14%) portions of the neuraxis.

RS Grafts Have a Mitogenic Effect on Cells of the DRG Anlage. Two basic mechanisms could account for the increase in cell number in the polyganglia. The first is that natural cell death was reduced in the polyganglia. This was not responsible for the increase in DRG cell number observed here because <0.5% of the cells in the ganglionic structures on both sides of the E4.5 animals were pycnotic. This low level of cell death is in agreement with other authors, who

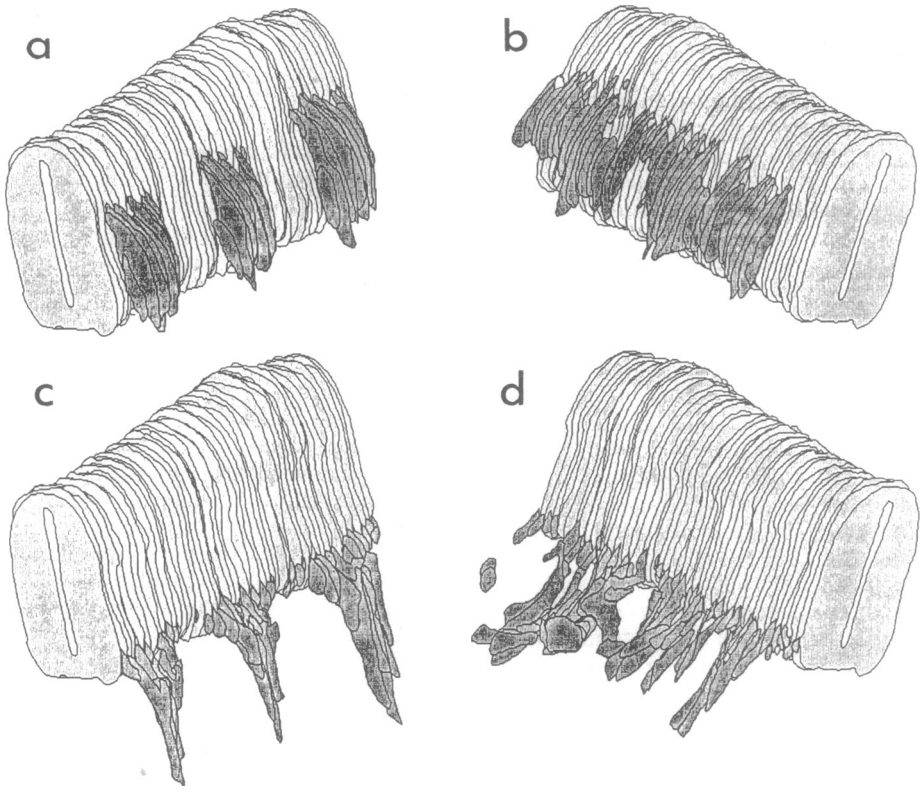

FIG. 2. Three-dimensional reconstruction of the neural tube and peripheral nervous structures in an E4.5 embryo receiving an implant of RS halves. (*a* and *b*) Sensory ganglia. Three normal DRG with their dorsal roots on the unoperated side of the animal are seen in *a*. *b* shows a continuous polyganglion that resulted from constructing a paraxial mesoderm of only RS moieties. (*c* and *d*) Nerves. In *c*, three motor nerves condense from axons growing out of the neural tube on the unoperated side. In contrast, motor axons emerging from the neural tube on the operated side do not form individual nerves (*d*).

found that E4.5 is before the period of massive cell death in the DRG (14, 17).

The other mechanism that can account for the observed increase in cell number opposite the grafted mesoderm is enhanced proliferation of crest-derived cells in the polyganglia relative to the unoperated side. To provide direct evidence that the RS environment elicited enhanced proliferation of the cells in the polyganglia, we examined the labeling of DRG cells by [^{3}H]thymidine autoradiography in the polyganglia and their contralateral DRG (Fig. 4). On E3.5, the proportion of DRG cells incorporating thymidine was 45% higher in the polyganglia (n = 3); an average of 47 ± 4% DRG cells in the polyganglia incorporated thymidine compared with the 32 ± 2% labeled DRG cells on the control side. Similarly, on E4.5, 36 ± 3.4% of the DRG cells incorporated thymidine in the polyganglia as opposed to 28 ± 1% of the DRG cells in control ganglia—an increase of 28% (n = 3).

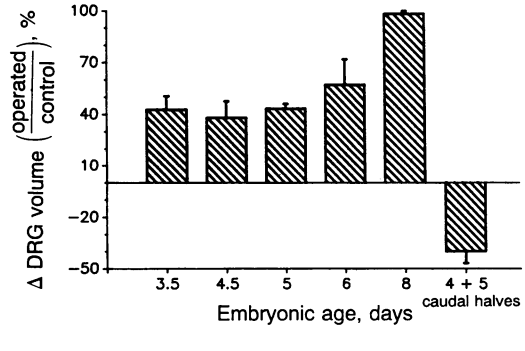

FIG. 3. Differences in volume between DRG on the operated and control sides in chimeric embryos of various ages. The first five bars show the average increase in volume of the polyganglia compared with the normal DRG in embryos with RS grafts. The data from E3.5 and E4.5 is pooled from brachial and cervical level ganglia, the data from E5, E6, and E8 is from cervical DRG only (see *Discussion*). The final bar (caudal halves) indicates the reduction of DRG volume on the operated side when the graft consists of CS halves. Error bars represent the SEM.

DISCUSSION

The above data demonstrate that the environment created by grafting RS moieties is mitogenic for neural crest cells that become DRG. This extends the observation of several groups that the RS is permissive for, and the CS inhibits the invasion of, neural crest cells and motor axons. This mitogenic effect

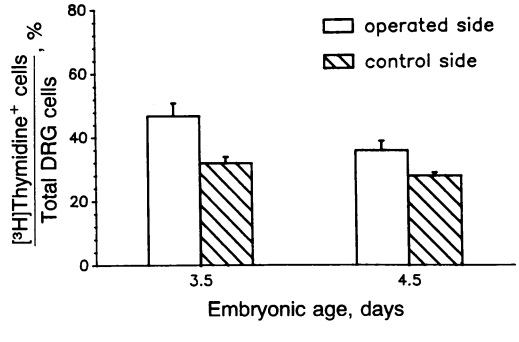

FIG. 4. Stimulation of [^{3}H]thymidine incorporation in embryos with multiple RS grafts sacrificed on E3.5 and E4.5. The open bars represent the proportion of thymidine incorporating cells to the total DRG cell population in the polyganglia, and the hatched bars represent the proportion in the control DRG. The percentage of dividing cells in the polyganglia as compared with the control DRG ([^{3}H]thymidine labeled in operated/control embryos) is 45% higher on E3.5 and 28% higher on E4.5.

is evident at least as early as E3.5 when there is a 45% higher proportion of thymidine-incorporating cells in the polyganglia than in the control DRG. The effect is likely to be present even earlier than E3.5, the time of the condensation of the DRG, because the nascent polyganglia from E3.5 embryos are already 42% larger and contain 46% more cells than the corresponding contralateral control ganglia.

Enhanced proliferation is still present on E4.5; the percentage of dividing DRG cells is 28% higher on the side of the graft. This results in a growing disparity in DRG size between the polyganglia and the control DRG until at least E8 in trunk regions (see below) as described (13).

Other experimental manipulations that lead to an increased number of DRG cells in chicken embryos are addition of supernumerary limbs (18) and treatment with nerve growth factor (17). Both of these experimental procedures produce DRG cell hyperplasia by rescuing neurons that otherwise would have degenerated because of natural cell death. However, the present results differ in that the increased cell number observed has been shown to be due to enhanced proliferation of DRG cells, rather than their rescue from natural cell death.

There are two possible sources for the mitogenic effect on DRG precursor cells observed here. First, enhanced proliferation of neural crest cells could be elicited directly by rostral sclerotome-derived factors such as matrix components, soluble factors, or a combination of both. Alternatively, this effect could be exerted by factors released from the neural tube. In this second possibility, the formation of a continuous area of contact between crest cells and the neural tube as a result of the graft of rostral mesoderm would provide greater accessibility of central nervous system (CNS)-derived factors to the developing DRG.

Early DRG have been shown to be dependent on CNS-derived factors for their survival (19–23). For instance, the interposition of an impermeable membrane between the neural tube and the DRG anlage causes in a specific manner the death of all neural crest cells remaining distal to the implant, whether adjacent to RS or to CS regions (20). Moreover, brain-derived neurotropic factor (24, 25) enhances the survival of neural crest cells *in vivo* (21) and those neural crest cells that develop into substance P immunoreactive neurons in culture (22). Another CNS-derived factor, basic fibroblast growth factor, rescues a population of neural crest-derived nonneuronal cells of DRG expressing the HNK-1 epitope both *in vivo* and in culture (23).

Most likely, normal DRG development depends upon a successful interaction with both the neural tube and the somites. A putative factor originating from the RS could stimulate neural crest cells to proliferate provided the survival of these cells is first ensured by appropriate factors deriving from the CNS primordium.

Relatively large differences in the number of sensory neurons of bilaterally equivalent ganglia in normal animals have been described in several species (reviewed in ref. 26). The determination of the volume of pools of three continuous ganglia on each side of unoperated embryos in the present study revealed a difference of up to 10% in volume between the sides. This is only one-third to 1/10th the difference observed between polyganglia and their contralateral counterparts. In addition, the side on which the larger volume of DRG was found occurred randomly in these animals. Therefore, the differences in DRG volume observed here were not due to random developmental variations.

The mitogenic effect of the RS mesoderm occurs both in limb-forming and in non-limb-forming regions of the neuraxis. This is interesting in view of the observation that there is natural variation in proliferation rates in DRG and in the motor column in different portions of the neuraxis. In normal animals more DRG cells and presumptive motoneurons are born in limb-forming regions than in trunk regions as a result of different rates of proliferation (14, 17, 27).

Preliminary evidence suggests, however, that the polyganglia in brachial and cervical regions have different fates beyond E4.5. At cervical levels of the neuraxis, the polyganglia continue to grow until they become twice the volume of the contralateral DRG as described (13). In contrast, at brachial levels, the difference in size between the polyganglia and control DRG is reduced to <10% on E6 (unpublished results). This suggests that, although the neural crest cells from both limb-forming and non-limb-forming levels of the trunk are initially responsive to the mitogenic environment of the RS, intrinsic differences in the development of the DRG cells (28), spatial constraints, or competition for neurotrophic factors control the size of the ganglia at later developmental stages. Furthermore, the dynamics of polyganglion growth relative to that of normal DRG beyond E4.5, may result from a combination of changes in cell proliferation, cell death, and cell size, all of which should be quantified in order to characterize fully the polyganglion model at different axial levels.

Both neurons and satellite cells arise from the neural crest cells in the DRG. Since in this study the quantification of cell number was performed on E3.5 and E4.5 embryos before the differentiation of the satellite cells, it is not possible to know whether the enhanced proliferation produces larger numbers of glia cells, neurons, or both.

An interesting question that arises from our results is whether the mitogenic effect is specific for DRG cells. Another derivative of the trunk neural crest are the sympathetic ganglia, which also develop in association with the sclerotome. It is possible that the proliferation of sympathetic ganglion cells or non-crest-derived neurons such as motor neurons are also affected by the RS mesoderm. In addition, it is also possible that neurons in the dorsal horn of the spinal cord are stimulated to divide either directly by the mesoderm (29) or indirectly by the presence of additional DRG cells.

In addition to providing an experimental system for the study of initial segmentation and regulation of cell number in the nervous system, the polyganglia and unsegmented motor nerves provide a unique model to address several questions of central interest in neurobiology. For example, what are the roles of segmentation in determining which pathways axons take to their targets (30, 31)? Do the motor and sensory axons in areas of continuous rostral mesoderm lose positional information and have difficulty finding their normal targets in the periphery? Another question is that of the determination of the identity of nerve cells by their position along the neuraxis, which in turn may determine their peripheral targets. The possibility exists that the crest cells in the polyganglia, not being confronted with the barrier of caudal mesoderm, are free to move several segments within the confines of the graft. Will the sensory cells that end up several segments from their point of origin project to a target based on their new position or on their birthplace? Since sensory axons are at least in part guided by motor axons (32, 33), these questions would be best addressed in animals without motor neurons (i.e., after ablation of the ventral part of the neural tube).

Control of cell division in the DRG, the development of the surrounding vertebrae and muscles, and the competition for extrinsic survival factors are all processes that contribute to the determination of the size and shape of DRG in normal animals. Experimental perturbation of these processes by construction of chimeras with areas of continuous RS mesoderm, may prove a useful system to address central questions in development: how are the final size and form of an organ determined?

The helpful discussions and critical reading of the manuscript by Prof. N. Le Douarin are gratefully acknowledged. We also would like

to thank Chana Carmeli for excellent technical assistance and Effy Rosenthal for his work on the three-dimensional reconstruction. This study was supported by research grants from the Muscular Dystrophy Association and the Israel Academy of Sciences and Humanities (to C.K.), the Israel Institute for Psychobiology (to R.S.G.), and by a grant from the National Council of Research and Development, Israel, and the Commission of the European Communities (to C.K. and M.-A.T.).

1. Weston, J. A. (1963) *Dev. Biol.* **6,** 279–310.
2. Le Douarin, N. M. (1982) *The Neural Crest* (Cambridge Univ. Press, Cambridge), pp. 160–165.
3. Weston, J. A. (1970) *Adv. Morphog.* **8,** 41–114.
4. Tosney, K. W. (1978) *Dev. Biol.* **62,** 317–333.
5. Teillet, M.-A., Kalcheim, C. & Le Douarin, N. M. (1987) *Dev. Biol.* **120,** 329–347.
6. Keynes, R. J. & Stern, C. D. (1984) *Nature (London)* **310,** 786–789.
7. Keynes, R. J. & Stern, C. D. (1985) *Trends Neurosci.* **8,** 220–223.
8. Rickmann, M., Fawcett, J. W. & Keynes, R. J. (1985) *J. Embryol. Exp. Morphol.* **90,** 437–455.
9. Bronner-Fraser, M. (1986) *Dev. Biol.* **115,** 44–55.
10. Newgreen, D. F., Scheel, M. & Kastner, V. (1986) *Cell Tissue Res.* **244,** 299–313.
11. Loring, J. F. & Erickson, C. A. (1987) *Dev. Biol.* **121,** 220–236.
12. Stern, C. D. & Keynes, R. J. (1987) *Development* **99,** 261–272.
13. Kalcheim, C. & Teillet, M.-A. (1989) *Development* **106,** 85–103.
14. McMillan-Carr, V. (1978) *J. Comp. Neurol.* **182,** 727–740.
15. Le Douarin, N. M. (1973) *Exp. Cell Res.* **77,** 459–468.
16. Stirling, R. V. & Summerbell, D. (1979) *Nature (London)* **278,** 640–642.
17. Hamburger, V., Brunso-Bechtold, J. K. & Yip, J. W. (1981) *J. Neurosci.* **1,** 60–72.
18. Hamburger, V. & Levi-Montalcini, R. (1949) *J. Exp. Zool.* **111,** 457–501.
19. Teillet, M. A. & Le Douarin, N. M. (1983) *Dev. Biol.* **98,** 192–211.
20. Kalcheim, C. & Le Douarin, N. M. (1986) *Dev. Biol.* **116,** 451–466.
21. Kalcheim, C., Barde, Y. A., Thoenen, H. & Le Douarin, N. M. (1987) *EMBO J.* **6,** 2871–2873.
22. Kalcheim, C. & Gendreau, M. (1988) *Dev. Brain Res.* **41,** 79–86.
23. Kalcheim, C. (1989) *Dev. Biol.* **134,** 1–10.
24. Davies, A., Thoenen, H. & Barde, Y. A. (1986) *J. Neurosci.* **6,** 1897–1904.
25. Hofer, M. M. & Barde, Y. A. (1988) *Nature (London)* **331,** 259–261.
26. Ygge, J., Aldskogius, H. & Grant, G. (1981) *J. Comp. Neurol.* **202,** 365–372.
27. Oppenheim, R. W., Cole, T. & Prevette, D. (1989) *Dev. Biol.* **133,** 468–474.
28. Marusish, M. F., Pourmehr, K. & Weston, J. A. (1986) *Dev. Biol.* **118,** 494–504.
29. Rothman, T. P., Gershon, M. D., Fontaine-Perus, J., Chanconie, M. & Le Douarin, N. M. (1987) *Dev. Biol.* **124,** 331–346.
30. Landmesser, L. (1984) *Trends Neurosci.* **7,** 336–339.
31. Scott, S. A. (1987) *Trends Neurosci.* **10,** 468–473.
32. Landmesser, L. & Honig, M. (1986) *Dev. Biol.* **118,** 511–531.
33. Swanson, G. J. & Lewis, J. (1986) *J. Embryol. Exp. Morphol.* **95,** 37–52.