Aspects of Mauthner Cell Differentiation in the Axolotl, Ambystoma mexicanum

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SYNOPSIS. In premetamorphic amphibians, the Mauthner cells (M-cells), a single pair of large neurons, are present in the medulla. M-cells differentiate early, are easily recognized morphologically, and in the axolotl embryo, may be approached experimentally: This system is a unique one for the study of neuronal development.

The withdrawal of a neuron from the cell division cycle is an early event in its differentiation. Gastrulae, neurulae and tailbud embryos were each given a single injection of 3H-thymidine. Radioautographs of larvae showed label over M-cell nuclei when injections were made before the end of gastrulation, but not when injections were made at later stages. Thus, the cells that give rise to M-cells cease DNA synthesis during late gastrulation.

Unilateral rotations of prospective hindbrain through 180° were performed to see if M-cell axes are specified during neurulation. Rigid axial polarization of the M-cell does not appear to occur in the neurula: The rotated cell regulates and develops normally with respect to its axes.

A major source of input to the M-cell is from the ipsilateral vestibular system. To study the interaction of the M-cell with ingrowing axons, unilateral implants of otic vesicles were made anterior to the otic vesicle in host midtailbud embryos. Preliminary data suggests a mechanism for the formation of specific neuronal connections not dependent upon position-time relationships: The ectopic vestibular axons enter the medulla and course caudad to terminate in the region of the ipsilateral M-cell. Whether these axons actually form synapses on the M-cell remains to be established.

INTRODUCTION

In premetamorphic amphibians, as well as in fish (Diamond, 1971), a single pair of neurons, the Mauthner cells (M-cells), are present in the medulla at the level of entry of the vestibular or VIIth cranial nerve (Fig. 1A). The M-cells are the largest neurons in the brain of the axolotl (Ambystoma mexicanum), and as in other species, their axons decussate and run caudad in the contralateral medial longitudinal fasciculus. The M-axons innervate the motoneurons of the axial musculature and control tail flips to either side, movements that constitute an important escape reflex.

The axolotl M-cell is clearly a polarized cell: It is long in its mediolateral axis and narrow in both its anteroposterior and dorsoventral axes. It has a very large lateral dendrite and a large medial one, and its axon always leaves the cell at its mediodorsal margin (Figs. 1B and 2). The M-cells receive afferent input from several groups of neurons whose axons terminate on its cell body and dendrites. Axons from the ipsilateral vestibular system (Figs. 1 and 2) and from the ipsilateral lateral line system provide major sources of sensory input (Herrick, 1914), and in the axolotl, they synapse specifically on the ventral (Figs. 1B and 2) and dorsal surfaces of the lateral dendrite, respectively. Afferent connections appear during the period of M-cell differentiation: The earliest that can be recognized are those of the ipsilateral vestibular axons (Leghissa, 1941). Later, by early feeding stages, these synapses are identifiable in the light microscope (Figs. 1B and 2, at arrowheads) and in the electron microscope (Kimmel and Shabtach, 1974) as large club endings.
WHY USE THE AXOLOTL M-CELL FOR STUDIES OF NEURONAL DEVELOPMENT?

Large neurons are generally produced before small ones (M. Jacobson, 1970), and the M-cell, being the largest central neuron in the axolotl, differentiates very early relative to the nervous system as a whole. Because the M-cell is striking in size and distinctive in form, and its location in the medulla is precisely defined, it is easily distinguished morphologically. With respect to afferent inputs, there is clearly a regional organization of synaptic connections on the surface of the M-cell, and thus there is topographic order at the level of a single cell. Groups of afferent synapses can be identified as to origin on the basis of characteristic fine structure. Most importantly, the M-cell can be approached experimentally during many stages of development, and the results can be assayed with morphological, behavioral and physiological methods. Thus, the M-cell has proved to be a unique preparation for the study of various aspects of neuronal development, and it is some of these aspects that I discuss below. I emphasize those investigations that use the axolotl M-cell as an experimental system, but bring in others when I think it useful to do so.
TIME OF ORIGIN OF THE AXOLOTL M-CELL

The withdrawal of a neuron from the cell division cycle is an early event in its differentiation. The time at which this occurs appears to be programmed in that a given population of neurons seems to have a fixed time for the cessation of DNA synthesis (M. Jacobson, 1968a, 1970). In Xenopus laevis, the cells that give rise to M-cells cease DNA synthesis during late gastrula stages (Vargas-Lizardi and Lyser, 1974), a finding that has been confirmed (Spitzer and Spitzer, 1975) and extended to a teleost (Kimmel et al., 1978). Billings and Swartz (1969) have shown that the Xenopus M-cell is a diploid cell, thus a mitosis occurs after the final round of DNA synthesis, probably within an hour or two (Vargas-Lizardi and Lyser, 1974). M-cell determination apparently happens shortly thereafter, early in neurulation (Stefanelli, 1947, 1951).

To establish the time of origin of the prospective M-cell in the axolotl, blastulae, gastrulae, neurulae and tailbud embryos were each given a single intra-archenteron injection of 3H-thymidine and allowed to grow to early feeding stages (Model, unpublished data). Radioautographs showed label over M-cell nuclei when injections were made prior to the termination of gastrulation (Fig. 3A), but not when injections were made at later stages (Fig. 3D). Injections during late gastrulation, at stage 12 (see Harrison, 1969, for staging series of A. maculatum, a related species), produced a variable labeling pattern: Some M-cell nuclei were labeled (Fig. 3B), whereas others were not (Fig. 3C). In all cases, radioautographic silver grains were present over the nuclei of adjacent cells (Figs. 3A-D) showing the availability of isotope to cells in the region of the prospective M-cell throughout the experimental series. Since the isotope will label only those nuclei which are synthesizing DNA at the time that 3H-thymidine is present in the system, the absence of silver grains over M-cell nuclei allows the determination of the stage at which the precursor cell's final round of DNA synthesis occurred. Thus, in the axolotl, as in other species, the cells that give rise to M-cells cease DNA synthesis near the time that gastrulation ends.

REGULATION FOLLOWING UNILATERAL ROTATION OF THE PROSPECTIVE HINDBRAIN IN AXOLOTL NEURULAE

How nerve fibers make specific connections with other cells during the course of development is a central unanswered question in developmental neurobiology. There are several possibilities as to the nature of the mechanisms by which spatially ordered sets of nerve cells form appropriate interconnections. Some investigators believe that each nerve cell and fiber must have its own biochemical label (after Sperry, 1963), whereas others believe that the apparent accuracy with which nerves form connections can be ascribed to a well-ordered spatial and temporal sequence of development (e.g., Lopresti et al., 1973).

In the nervous system, a pattern of connections often develops between two groups of neurons so that each neuron in one group connects with a topographically equivalent neuron in the other group. The projection of the ganglion cells of the retina to the optic tectum in amphibians is an example of this (M. Jacobson, 1968a). An important observation to emerge from the study of this system is that early critical

FIG. 3. Phase micrographs of radioautographs of Epon-embedded, 4-μm thick cross sections through the M-cells of 21 mm larvae that had been injected with 3H-thymidine at different stages during development. Id, lateral dendrite; md, medial dendrite; a, axon; C, axon cap. A. Right M-cell from an animal injected with 3H-thymidine at Harrison stage 11, midgastrula. The M-cell nucleus is labeled. B. Left M-cell from an animal injected at stage 12, late gastrula. The M-cell nucleus is labeled. C. Left M-cell from an animal injected at stage 12, late gastrula. The number of silver grains over the M-cell nucleus approximates that of background. D. Right M-cell from an animal injected at stage 13, very early neurula. The M-cell nucleus is not labeled. In all cases, silver grains are present over the nuclei of cells adjacent to the M-cell.
events determine the axial polarity of the retina with respect to its connections with the tectum (M. Jacobson, 1968a). Sperry's (1963) hypothesis of neuronal specificity suggests a mechanism by which this selectivity might occur: Two or more gradients successively superimposed upon the retinal and tectal fields could account for an orderly cytochemical mapping. These gradients would mark each cell with respect to its position in a chemical code with matching values between the retinal and tectal maps. Evidence for Sperry's hypothesis comes from the demonstration of a preferential adhesion of dissociated neural retina cells for the optic tectum that mimics the retinal projection along the dorsoventral (d-v) axis (Barbera et al., 1973; Barbera, 1975) and from recent biochemical data that implicate two complementary molecules as effectors of this d-v adhesive specificity (Marchase, 1977). The accumulated evidence supports an interpretation of neuronal specificity dependent upon cell-surface interactions with specificity-conferring molecules being located on the surfaces of both retinal and tectal cells.

In the Introduction, I noted that with respect to afferent connections, there is a regional organization of synaptic input on the surface of the M-cell: for example, terminals from the ipsilateral vestibular axons are localized on the ventral surface of the lateral dendrite. Whether this topographic specification is due to regional differences on the surface of the M-cell remains to be established, but there are several possible experimental tests that can be addressed to this question. Regionally appropriate connections following displacement of the prospective vestibular apparatus, or following rotation (or displacement) of the prospective M-cell would suggest a mechanism based upon regional specification of the M-cell surface rather than one based upon position-time. Normally, the long axis of the M-cell is oriented approximately perpendicular to the long axis of the nervous system, and, thus, the origin of the M-axon and medial dendrite defines its medial pole and that of the lateral dendrite, its lateral pole. Given the clear polarity of the M-cell, the experimental approach that I chose was rotation of the prospective hindbrain to obtain a lateral projection of the M-axon and medial dendrite, and a medial projection of the lateral dendrite. But first, the time of axial polarization of the M-cell and its immediate tissue environment had to be established.

In 1947, Stefanelli removed one side of the prospective hindbrains in *Rana esculenta* and reimplanted the pieces with the anteroposterior (a-p) and mediolateral (m-l) axes reversed relative to the axes of the nervous system as a whole. He found that when the rotations were carried out in midneurulae (3 cases), the orientation of the M-cell was normal, but when carried out in midtailbud embryos (4 cases), the orientation of the M-cell was inverted: The M-axon projected from the lateral border of the cell body. Although the number of experimental animals was small, it appears that regulation of the axial polarity of the prospective M-cell in the frog can occur during neurulation, but not at later stages. The same appears to be true for the salamander, *Ambystoma maculatum*, at least with regard to the a-p axis (Detwiler, 1940).
The results obtained by C.-O. Jacobson following rotation of pieces of the prospective hindbrain region of axolotl neural plate through 180°, either bilaterally (1964) or unilaterally (1976), are difficult to reconcile with those just discussed, and with my own (described below), particularly with respect to the degree of determination of the axes in the prospective medulla of neurulae. Jacobson found that structures within the grafted tissue developed according to origin (e.g., the large motor nuclei of the medullae were transposed relative to the a-p axis of the rest of the nervous system), and so he concluded that the axes of the medulla are determined in detail during neurulation.

Since in seven of the 17 cases in which C.-O. Jacobson (1976) unilaterally rotated pieces of the neural plate containing the prospective M-cell resulted in reversal of the cell’s m-1 axis (the axon emerged from the lateral pole instead of the medial one), it seemed reasonable to me to perform similar experiments to obtain abnormally oriented M-cells. Thus, I rotated one side of the prospective hindbrain of axolotl midneurulae (stages 15-16) through 180° (Model, 1968): Along the a-p axis, the grafts included the entire prospective medulla, and along the m-1 axis, they included all of the neuroepithelium (and sometimes the underlying mesoderm) from the median groove to the neural fold. I then examined cross sections through the brains of experimental larvae that had been allowed to feed and grow to 21 mm in length (Figs. 4-6).

In most of the animals (13 of 18), neither the a-p nor m-1 borders of the grafts could be defined, and no aberrations in the morphology of the medullae were evident: The rotated half of the medulla could not be distinguished from the control half (as in Fig. 4A). In one animal, the ependymal surface of the rotated side was slightly disturbed in the region of the M-cell (Fig. 4B). In all of these cases (14 of 18), the form and orientation of the M-cell in the rotated graft was normal in every respect (Fig. 4).

In three animals, the morphology of the rotated half of the medulla deviated from normal in that a wall of medullary tissue projected from the medial border of the graft to divide the IVth ventricle into two parts (Fig. 5A), and the M-cell, although normal in form, was oriented longitudinally along the a-p axis of the nervous system, with its axon and a dendrite arising anteriorly (Fig. 5B) from the cell body (Fig. 5C), and a large dendrite, posteriorly (Fig. 5D). The caudally directed dendrite was located at the level of entry of the vestibular axons (Fig. 5A), and large club-like endings resembling those of vestibular origin were present on its ventral surface in all three cases (Fig. 5D). It is likely that this dendrite is the lateral dendrite since its origin is opposite to that of the axon and thus, I believe that these observations offer preliminary evidence in support of regional specification of the M-cell surface.

In the remaining animal, the morphology of the medulla was grossly disturbed (see Fig. 6 and its Figure Legend for details) and the ipsilateral vestibular axons did not enter the graft (Fig. 6A). The M-cell was not present at its usual location, but instead, was located far caudad (Fig. 6B). Its shape was abnormal (Fig. 6B), the axon arose from its anterior border to follow an erratic course rostrad (Fig. 6A), and large, identifiable dendrites were absent. The M-cell in the control side was normal (Fig. 6A).

Since 14 of 18 rotated grafts developed normally, there can be little doubt that at...
neurulation, the major axes of the medulla are not yet irrevocably determined. It is especially significant that in the 14 animals in which regulation was complete, the boundaries between graft and host tissues could not be distinguished. The four instances in which normal development did not occur are instructive: Displacement of the M-cell away from its normal orientation was accompanied by gross distortion of the morphology of the rotated medulary tissue and by conservation of the boundaries between the graft and host tissues as defined by the transition from perturbed to normal morphology. In no case was there an M-cell that showed rotation through 180°. C.-O. Jacobson (1976), however, did observe such inversions with relatively high frequency after the same experimental procedure. He also observed that the morphology of the grafted tissue in those animals that showed rotation of the M-cell was abnormal, and indeed, the abnormalities that he observed are similar to those obtained in my experiments.

My data suggest that while there may be axial gradients present in the medulla at neurulation, the tissue is labile and under the appropriate host influences, it can successfully reorient when grafted after rotation through 180°. Disturbance of the morphology of the medulla after rotation can be interpreted as a healing response that has interfered with the regulatory mechanism. I do not as yet know whether the abnormalities result only from surgical trauma, or whether both trauma and rotation are needed to lead to them: Of four animals that were subjected to sham operations (grafting without rotation), none showed medullary aberrations, but the number of cases may not be statistically significant. At this juncture, I interpret my results and those of C.-O. Jacobson (1976) to mean that axial polarization of the medulla is not final at neurulation, and that in the absence of an interfering healing response, regulating factors from the surrounding tissues can override the original polarity of the graft to bring it into accord with the axes of the system as a whole.

**IMPLANTATION OF AN EXTRA OTIC VESICLE INTO AXOLOTL TAILBUD EMBRYOS**

The developmental processes that give rise to the characteristic shape of neurons appear to involve factors that arise progressively through the interaction of the differentiating cell with its environment as well as those that are intrinsic to it and its genome (M. Jacobson, 1970). For example, as connections form between neurons, there is the possibility of one neuron stimulating another across synapses. The correspondence in time between the growth and ramification of the M-cell lateral dendrite and the formation of the first synaptic connections which are vestibular in origin (Leghissa, 1941) together with the constancy with which the M-cell is anatomically (and functionally) associated with the vestibular system suggests that the differentiation of the M-cell may depend to some extent upon the presence of vestibular axons. Piatt (1969, and earlier) found that removal of an otic vesicle (the prospective vestibular apparatus) from large numbers of midtailbud (stage 27) *Ambystoma* embryos resulted in the absence of the M-cell on the deprived side in about 20% of cases. When the control side is normal, the M-cell on the rotated side appears to have regulated to form two fairly complete medullae that are fused at the midline. Two ventricles are present. Axons from an unidentified ganglion (top center) enter the fused halves dorsally. The M-cell (within rectangle) in the rotated graft is globular in form and its axon arises from its anterior border to follow an erratic course rostrad (see A). Large identifiable dendrites are absent. OC, otic capsule. (From Model, 1978)
a third of the experimental animals. In the remaining animals, the deprived M-cell developed normally. While aware of the apparent contradictions in his data, Piatt concluded that the presence and final maturation of the M-cell is in part (at least) dependent upon its interaction with the vestibular axons. Other evidence denies a necessary relationship: The M-cell can develop in the absence of the vestibular axons and be missing when the vestibular axons are present (Detwiler, 1927, 1933; Oppenheimer, 1941; Piatt, 1943; Stefanelli, 1951).

Ingrowing vestibular axons may, however, stimulate local branching of the M-cell lateral dendrite. Recently, Kimmel et al. (1977) removed an otic vesicle from late tailbud (stage 34) axolotl embryos and found that although M-cells were always present on the deprived side, there was a significantly reduced development of the lateral dendrite in the region normally supplied by vestibular axons. Since the ablations were performed prior to the time that vestibular connections form, Kimmel et al. (1977) suggested that interaction between the developing M-cell and its vestibular input is essential to the former's morphogenesis.

To further examine the extent of regulation imposed by ingrowing axons on the branching pattern of the M-cell lateral dendrite, my student, Alger Chapman (unpublished data) implanted an otic vesicle in approximately correct orientation, just anterior to the otic vesicle in host midtailbud (stage 27-28) axolotl embryos.
Subsequent examination of the heads of 21 mm experimental larvae showed that the grafted (extra) vestibular apparatus developed fairly normally and that the ectopic vestibular axons entered the medulla, often in conjunction with a near host cranial nerve root, to course caudad in the longitudinally running tracts and terminate in the region of the ipsilateral M-cell (Fig. 7). Since we don’t as yet know whether these axons actually form synaptic connections on the M-cell lateral dendrite, we can not attribute a role to excess ves-
ticle input on the morphogenesis of the M-cell lateral dendrite. However, the pas-
sage of the ectopic vestibular axons through foreign medulla to reach the ip-
silateral M-cell suggests that the formation of connections between them may not de-
pend upon undisturbed position-time relationships.

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