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IN SITU ANALYSIS OF NEURONAL DYNAMICS AND POSITIONAL CUES IN THE PATTERNING OF NERVE CONNECTIONS

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Summary

Recently developed imaging techniques permit individual cells to be uniquely labeled and followed over time as development proceeds in intact vertebrate embryos. Small groups of cells in the developing eye rudiment of the frog *Xenopus* have been labeled with the vital dyes DiI, lysinated fluorescein dextran (LFD) or lysinated rhodamine dextran (LRD). Individual optic axons and their growth cones were clearly visible in the intact living animal using confocal microscopy or epifluorescence microscopy with a low light level video camera and computer-based video image enhancement. To follow the dynamics of single optic nerve fiber terminal arborizations, small groups of cells, or even single retinal ganglion cells, were labeled with DiI, and the resulting labeled optic nerve fibers were imaged using a confocal microscope. The images show a profound alteration in morphology from day to day, demonstrating that optic nerve terminal arborizations are dynamic structures constantly extending and retracting branches.

To follow the topography of the developing projection and analyze the cues that guide its formation, small groups of eyebud cells from LFD- and LRD-labeled donor embryos were grafted to an unlabeled host in either a location equivalent to that from which they had been removed (homotopic grafts) or a non-equivalent location (heterotopic grafts). Axons from homotopic grafts projected to the tectum as expected from the adult topography of the retinotectal projection. Dorsoventral topography was present from the time that the optic nerve fibers were observable in the tectum, in agreement with previous work. Nasotemporal topography was subtle or absent for the first few days, and then slowly refined. The importance of positional cues was tested by performing heterotopic eyebud grafts, in which the labeled eyebud cells are grafted to inappropriate places in the host eyebud. The heterotopic grafts appeared to integrate with the ectopic site in the eyebud in a functional manner. They should, therefore, project to the tectum together with their new neighbors if neighbor interactions or activity-based cues were of primary importance in the initial patterning of the map. However, the experiments showed that the axons from heterotopic grafts always behaved in a fashion appropriate to their position of origin in the donor, regardless of their final

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position in the host. These observations indicate that small groups of eyebud cells (as small as a single cell) possess positional information that plays a dominant role in guiding the optic nerve fibers to their target sites in the tectum.

Introduction

A key aspect of synaptogenesis is the selection of the proper targets upon which to synapse. Not only must the presynaptic element recognize the proper class of postsynaptic target cell, it must also find a target cell in the correct spatial position to form a functional set of connections. The ordering of neuronal projections can be very striking. In many cases, neurons navigate over relatively long distances to connect in a predictable orientation and order. Central as these processes are to correct neuronal function, our knowledge of their genesis remains fragmentary. In most systems, the events that mediate the ordering of neuronal projections are difficult to observe directly; therefore, these processes are typically explored in the vertebrate central nervous system by neuroanatomical examination of populations of axons in fixed tissue. Such an approach has been valuable, leading to several inferences about the events that are important for neuronal patterning. Some experiments suggest a role for target recognition mechanisms in which the axons select appropriate target sites based on markers on the postsynaptic cell (e.g. Sperry, 1963); other experiments have suggested that an orderly set of connections is established by selectively stabilizing those that are functionally appropriate. It is now of critical importance to test these inferences by directly assaying the roles of cellular dynamics and positional cues in the formation of neuronal maps.

In situ light microscopy of growing nerve fibers offers the possibility of following directly the events critical for the formation of topographic nerve connections. By combining low light level video microscopy and confocal microscopy with the more classical techniques of experimental embryology, we have been examining the patterning of nerve connections in the development of the visual system in lower vertebrates. The results demonstrate that the optic nerve fiber terminal arborizations are continually rearranged, sprouting and retracting several of their branches each day. Grafting experiments indicate that these dynamics are guided largely by positional cues in the cells of the developing eye.

The visual system of the frog and of other lower vertebrates has played a central role in investigations of neuronal patterning. The retinotectal projection, the major projection of the visual system in lower vertebrates, displays a topographic order (see reviews by Fraser, 1985; Schmidt, 1985; Udin and Fawcett, 1988). Ganglion cells from the dorsal portion of the retina project optic nerve fibers to the lateral (ventral) portion of the optic tectum; those from the ventral tectum project to the medial (dorsal) tectum, from the nasal (anterior) retina to the caudal tectum, from the temporal (posterior) retina to the rostral tectum. The popularity of the system originates from the accessibility of both the eye and the optic tectum throughout development, as well as the ability of the optic nerve fibers to

regenerate. These advantages permit experiments using classical embryological techniques to be performed in parallel with experiments on post-embryonic stages. This latter approach, which is perhaps unique to lower vertebrates, may provide insights into both the regeneration and the initial development of neural maps.

Results obtained from the regenerating retinotectal projection of both frogs and fish have been taken as support for the roles of both position-based cues and activity-based cues in the formation of topographic connections (Fraser and Perkel, 1990). Regenerating optic nerve fibers reform a correctly ordered retinotectal projection within weeks; this robust process cannot be disrupted by either moving the eye into an abnormal orientation or disrupting the normal pathways of the optic nerve (Sperry, 1965). The role of the optic tectum in the targeting of the optic axons has been tested by transposing or rotating a portion of the optic tectum. The fibers can find and synapse upon the grafted tectal fragments, suggesting the presence of positional markers on the tectal surface (Yoon, 1973). Such results cannot be taken as conclusive proof of the *de novo* presence of cues on the tectal cells, however, because the tectum had been innervated previously by optic nerve fibers. It remains possible that the apparent positional cues resulted, at least in part, from the previous pattern of optic innervation.

Synaptic function also appears to play a role in the patterning of optic nerve fibers. Abolition of neuronal activity in the optic nerve by intraocular injection of tetrodotoxin (Schmidt and Edwards, 1983; Meyer, 1983) or alteration of the activity by rearing the animals in stroboscopic lighting (Schmidt and Eisele, 1985; Cook and Rankin, 1986) results in the regeneration of a more crudely organized retinotectal projection. The finding that the activity of neighboring retinal ganglion cells is correlated (Arnett, 1978) has been used to argue that correlated activity could serve as a means for the optic axons to recognize axons from neighboring retinal positions. In this hypothesis, the topographic order of the projection results, in part, from the fibers following the simple rule: 'fibers that fire together, synapse together'. In support of both the hypothesis and the possible role of the *N*-methyl D-aspartate (NMDA) receptor, the specific NMDA receptor blocking agent amino-phosphonovaleric acid (APV) has been found to disrupt the topography of the retinotectal projection (Cline and Constantine-Paton, 1989).

Axon tracing techniques have permitted an analysis of the initial formation of the retinotectal projection and the interactions that might be involved. Following the trajectories of a subset of the retinal ganglion cells and the developing optic axons provides an unambiguous assay of developing retinotopy. In this way it has been demonstrated that the dorsoventral topography of the projection forms as the fibers first reach the developing tectum (Holt and Harris, 1983; Sakaguchi and Murphey, 1985). Neither the natural order of fiber ingrowth (Holt, 1984) nor the presence of normal neural activity (Harris, 1980) is required for the formation of a projection with proper dorsoventral topography. Therefore, by eliminating some possible alternative explanations, it has been argued that positional cues in the cells of the eye must play the dominant role in the formation of the projection.

An *in situ* analysis of retinotopy

To permit more detailed analyses of the developing topography of the retinotectal projection, we have developed vital-dye fiber-tracing techniques for use in live *Xenopus* tadpoles (O'Rourke and Fraser, 1986). In one of these techniques, *Xenopus* embryos were labeled in their entirety by microinjecting the one-cell embryo with fluorescent dextrans. The injected animals develop normally but contain the fluorescent vital dye in all their cells. Small groups of eyebud cells, excised from these labeled embryos, were transplanted to the same location (homotopically) in an unlabeled host (Fig. 1). Both lysinated fluorescein dextran (LFD) and lysinated rhodamine dextran (LRD) are too large and charged to escape from the cells that contain them, and some of the dye is transported from the retinal ganglion cells along the optic nerve fibers to their terminal arborizations. Because the tissues surrounding the optic tectum are nearly transparent, the labeled optic nerve fibers can be imaged in the intact embryo and larvae by placing it on the stage of an epifluorescence microscope. Phototoxic effects of imaging the fluorescently labeled cells can be minimized by using a low light level

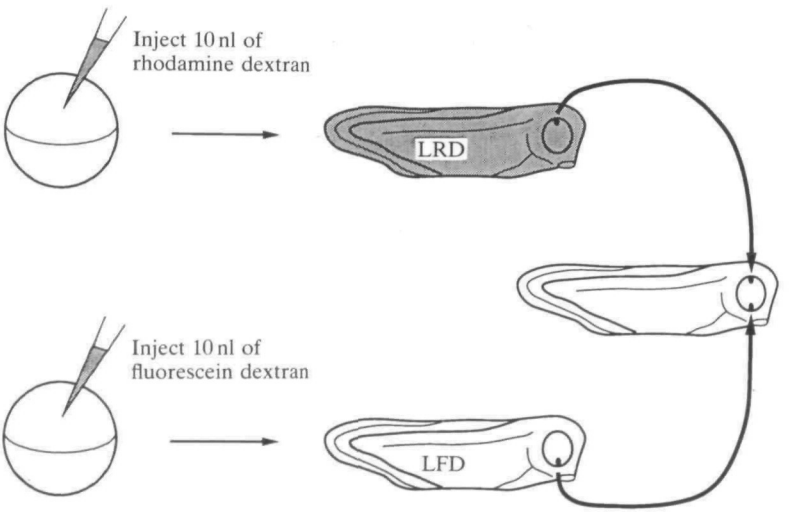

Fig. 1. Schematic drawing of a grafting operation. Donor embryos were labeled with lysinated rhodamine dextran (LRD) or lysinated fluorescein dextran (LFD) by microinjecting a fluorescent dextran solution (100 mg ml^{-1}) into a one-cell stage embryo. Because the total volume of the embryo does not increase significantly during the early stages of development, this yields an embryo with significant concentrations of the dye in all its cells. Homotopic grafts of labeled dorsal and ventral eyebud cells were performed by exchanging them for equivalent groups of cells in an unlabeled host. Heterotopic grafts (not shown in the diagram) involved the exchange of labeled cells with non-equivalent cells in the unlabeled host. All grafting operations were performed in a solution of anesthetic using sharpened watchmaker's forceps; the grafts were stabilized during healing by resting small fragments of coverglass on top of the graft site.

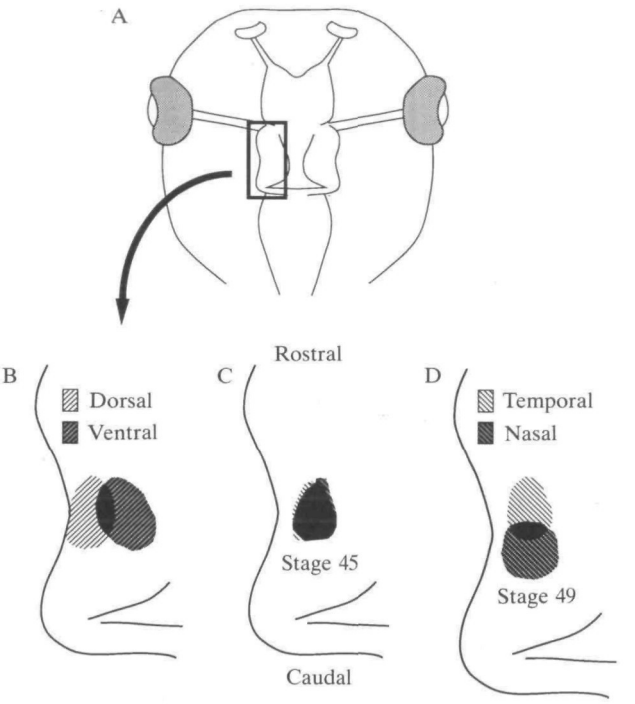

Fig. 2. Schematic drawing of the projections formed by the LRD- and LFD-labeled eyebud cells in the optic tecta of living larvae. (A) Dorsal view of a young *Xenopus* larva, showing the region of the optic tectum that was analyzed in detail. (B) The projection of LRD-labeled dorsal optic nerve fibers (light stipple) and LFD-labeled ventral optic nerve fibers (heavy stipple) in a representative animal. Note that the LRD-labeled dorsal fibers project to a more lateral position in the developing tectum, in agreement with the adult topography. (C) LRD-labeled nasal fibers and LFD-labeled temporal fibers project to the same region of the developing tectum at stage 45. (D) LRD-labeled and LFD-labeled fibers sort into a topographic order by stage 49, with nasal fibers (heavy stipple) projecting to the more caudal tectum.

video camera. In this way, the growth of the labeled cells has been followed for periods up to 4 weeks.

To follow the dorsoventral topography of the projection, ventral cells labeled with LFD were grafted to the ventral pole and dorsal cells labeled with LRD were grafted to the dorsal pole of the host eye rudiment (Fig. 1). By imaging the projection sites of the rhodamine- and the fluorescein-labeled fibers in the same animal, dorsoventral topography was observed at early stages, shortly after the fibers had entered the tectal neuropil (Fig. 2B; O'Rourke and Fraser, 1986). This early appearance of topography agrees with previous studies on fixed tissue (Holt and Harris, 1983; Sakaguchi and Murphey, 1985). The dextran results clearly demonstrated that the resolution of the initially formed projection was not highly defined; optic nerve terminals formed by ganglion cells from opposite poles of the eyebud were distinct but overlapped slightly in the tectal neuropil.

To follow nasotemporal (anteroposterior) topography, nasal and temporal cells were grafted to equivalent sites in the eyebud of an unlabeled host. In contrast to the results on dorsoventral topography, no anteroposterior topography was noticeable in the initial projection up to stage 45. Both the LRD- and the LFD-labeled fibers projected to the same portion of the developing tectum (Fig. 2C). Over the next few days (by stage 49), the LFD- and LRD-labeled fibers gradually established a clearly discernible anteroposterior topography (Fig. 2D). Again the results are consistent with previous findings on fixed tissue: in young larvae, optic nerve terminal arborizations were observed to fill most of the rostrocaudal extent of the tectum; in older animals the terminal arborizations did not innervate the most inappropriate portion of the tectum, reflecting the presence of a roughly topographic projection (Sakaguchi and Murphey, 1985). Repeated observations of the same animal, made possible by the non-invasive nature of the *in situ* fiber tracing technique, permitted the process of refinement to be followed. The nasal and temporal fibers overlapped to innervate the more rostral portion of the developing tectum. As development proceeded, the nasal fibers appeared to invade the more caudal portion of the tectum, leaving the temporal fibers behind and resulting in the establishment of topography. However, because the dextran technique follows two populations of optic nerve fibers, it was impossible to follow single optic nerve terminal arborizations in detail.

Confocal microscopy of optic nerve fibers

The patterning of dextran-labeled fibers suggests that rearrangements of the individual arborizations underlie the emergence of topography along the anteroposterior axis of the projections. To assess the dynamics of the terminal arborizations, individual retinal ganglion cells of a single optic nerve fiber were intensely labeled with the carbocyanine dye 1,1-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate (DiI). DiI is incorporated into the membrane of the cells it contacts, rendering both neurons and their growing axons fluorescent (Honig and Hume, 1986). Following a small extracellular injection of a solution of DiI in ethanol into the retina of a stage 40 larva, those animals with intensely labeled arborizations arising from a single cell were selected for detailed examination (O'Rourke and Fraser, 1990). Typical video microscopic techniques lack the resolution required to follow the evolution of a single optic nerve fiber as it becomes a more elaborate three-dimensional structure. Fluorescence from branches of the arborization above and below the plane of focus obscures details of the structure. To avoid this pitfall, we began to use a laser-scanning confocal microscope (White *et al.* 1987; Fine *et al.* 1988), which, because of its ability to eliminate out of focus information, permits the collection of unambiguous 'optical sections' deep into the larval tectum. A high-resolution image of the complex structure of the terminal arborizations was reconstructed by superimposing a set of optical sections through the entire depth of an arborization.

The terminal arborizations of labeled optic nerve fibers were observed daily for

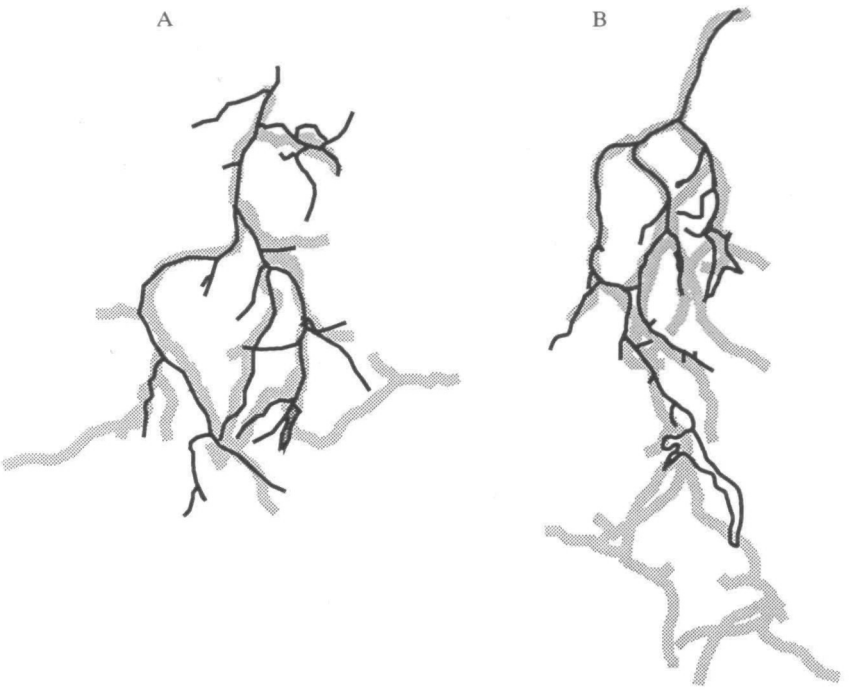

Fig. 3. Growth of optic fibers within the tectal neuropil. (A) Tracings of a laser scanning confocal microscope image of a temporal terminal arborization visualized at stage 45 (black line) and at stage 48 (stipple). (B) Tracings of a nasal terminal arborization visualized at stage 45 (black line) and at stage 48 (stipple). Note that the two different images of the temporal arborization are largely similar in rostrocaudal extent, whereas the nasal arborization has significantly elongated along the rostrocaudal axis. Medial is to the left, caudal to the top.

up to 5 days. All the arborizations underwent extensive remodeling, with the addition and loss of branches each day. In contrast to what might have been predicted, based on anatomical studies of fixed terminal arborizations, as many as half the branches could be lost or gained over a 24-h period. This dynamic rearrangement was present even in arborizations that did not change their overall size or position, suggesting that this may reflect a continuous process even in relatively mature projections.

The resolution of the confocal microscope images permitted a detailed evaluation of the arborizations during the time that nasotemporal topography was established. Although the nasal and temporal optic nerve arborizations were similar in many regards, including the number of branches and their overall dimensions (O'Rourke and Fraser, 1990), they displayed qualitatively different growth patterns (Fig. 3). Optic nerve fibers from both the nasal and the temporal retina branched to form terminal arborizations that covered most of the rostrocaudal extent of the tectal neuropil at stage 45. Temporal arborizations continued to rearrange from day to day, but remained in the same general position. In contrast,

nasal arborizations shifted over time to invade more caudal portions of the neuropil as the tectum grew (Fig. 3). Quantification of the number of branches in the tectum (O'Rourke and Fraser, 1990) confirmed this rearrangement, revealing that the nasal arborizations progressively increased their number of branches in the caudal tectum and decreased their number of branches in the rostral tectum.

Is retinotopy guided by positional cues?

The data from experiments on the regeneration and development of the retinotectal projections provide evidence in support of both position-based cues and neighbor-based cues in the formation of an ordered retinotectal projection. For example, the results of tectal grafts, in which the optic nerve fibers follow transposed or rotated portions of the tectum, support the action of position-based cues; in contrast, results from experiments in which activity is altered or disrupted suggest the importance of activity as a means for neighbors to recognize one another. The fiber tracing techniques described above can be used to explore the relative roles of position-based and neighbor-based cues, because the two can be confronted by grafting small groups of eyebud cells from LRD- and LFD-labeled donors to non-equivalent sites in the eyebuds of unlabeled hosts. If cues based on the position of the eyebud cells are dominant in the formation of the initial retinotectal projection, the fibers should terminate in agreement with their position of origin. Conversely, if cues based on interactions between retinal neighbors are dominant, the optic nerve fibers should project to the tectum in accordance with their new neighbors. Grafts performed from the nasal, temporal, dorsal and ventral poles of the eyebud give consistent results: the heterotopically grafted cells project to the tectum as predicted by their position of origin in the donor, not their position in the host. That is, retinal axons from the dorsal portion of the eyebud projected to the ventrolateral portion of the tectum after being grafted to the ventral, nasal or temporal poles of the eyebud. The results demonstrate the dominant role played by positional cues in the initial formation of the retinotectal projection. In addition, they show that positional cues are present in the cells of the eyebud long before differentiation begins and that the cues are stable in the presence of abnormal neighbors.

Conclusions

The results described above demonstrate the power of combining new technologies with more classical techniques. Classical embryonic cell grafting techniques, combined with low light level video microscopy or laser-scanning confocal microscopy, permit a new level of analysis of the patterning of the retinotectal projection. The results from grafting experiments demonstrate convincingly that cell-autonomous positional cues in the developing eye play a role in the formation of the retinotectal projection. Because the grafted cells project to the tectum in accord with their position of origin in the donor, even when confronted with

improper neighbors, the cell-autonomous positional cues appear to be dominant in the initial formation of the retinotectal projection of *Xenopus*.

The experiments in which single, DiI-labeled optic nerve fibers were followed over a period of days demonstrate that the terminal arborizations are best viewed as dynamic structures. A significant fraction of the branches are added and removed in each 24-h period; more recent experiments suggest that individual branches may extend or retract in their entirety in times of the order of an hour. Subtle biasing of this continuous remodeling can play an important role in the appearance of retinotopy. Nasal arborizations preferentially prune branches in the rostral tectum while extending branches in the caudal tectum, resulting in a topographic projection. Because such subtle biases can profoundly alter the final pattern of the projection, many of the apparently disparate cues that have been proposed may play a significant role in forming the pattern of neuronal connections. Anything that alters the growth of the axons, the lifetime of terminal branches or the stability of synapses could, in some experimental settings, be intimately involved in the patterning of the projection.

In view of the dynamic nature of the optic nerve fiber terminal arborization, a complete understanding of its patterning must await more detailed analyses of potential interactions. For example, the results of regeneration experiments indicate the importance of competition between optic nerve terminals in re-establishing neuronal contacts, yet the role of competition in the initial development of the projection remains unclear. Preliminary experiments, analyzing the first 2 days of arbor growth, suggest that such competition plays little, if any, part in the earliest phases of retinotopy. In another example, the rearrangements of the terminal arborizations observed with the confocal microscope take place during periods when functional synaptic contacts are present. It remains an open and important question whether synaptic function has a role in any aspect of the dynamics of the terminal arborizations. Experiments now under way may provide the answers needed to refine this system into a setting in which synaptic dynamics can be followed in parallel with neuronal patterning.

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