A pioneering growth cone in the embryonic zebrafish brain

(axon outgrowth/epiphysis/central nervous system/axonal tract/neuroepithelium)

STEPHEN W. WILSON* AND STEPHEN S. EASTER, JR.

Department of Biology, University of Michigan, Ann Arbor, MI 48109-1048

Communicated by Dale Purves, December 21, 1990 (received for review October 24, 1990)

ABSTRACT During development of the nervous system, growth cones navigate very precisely to their appropriate, often distant, targets. In insects, the task of establishing the earliest pathways is accomplished by a small number of neurons, termed pioneers. These neurons have axons that lay down an early scaffold, which provides a substrate for many later-developing axons. Here we show that a similar type of cell exists in the embryonic vertebrate brain. Using light- and electron-microscopic techniques we have examined the formation of one of the earliest tracts in the zebrafish brain. We find that it is pioneered at a precise time by the growth cone of a single neuron present in a predictable location. These observations show a fundamental similarity in the establishment of axonal pathways in the central nervous systems of both invertebrates and vertebrates.

The neuronal growth cone was first described by Ramon y Cajal 100 years ago (1) and has been the subject of intensive study ever since. A distinction has been made between the first growth cones to advance along a path, termed pioneers, and those that appear later, the followers (2–6). In insect embryos, the pioneering growth cones are few in number and frequently belong to identifiable neurons (4–6). These neurons are responsible for establishing axonal pathways when distances are at a minimum. Pioneer neurons and growth cones have also been identified in the vertebrate nervous system (7–16). Indeed in the zebrafish peripheral nervous system, the growth cones of identified neurons are responsible for pioneering peripheral pathways (7, 8).

There have been relatively few studies of pioneer neurons or their growth cones in the vertebrate central nervous system (11–16), and in the brain it is still largely unclear how the extremely complex pattern of axonal tracts is established. Previous work has shown that the embryonic zebrafish brain is an excellent system in which to analyze tract formation (17, 18). At early developmental stages, there is a very simple scaffold of axonal tracts in the midbrain and forebrain. This scaffold may provide an important substrate for the growth cones of thousands of axons extended at later developmental stages (17). In this study, we have examined the formation of one of the earliest pathways in the brain. In whole-mounted brains, we used the lipophilic dye 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate (diI) to label the first growth cones present in the dorsoventral diencephalic tract (DVDT) (17). The DVDT is an axonal tract that runs from the epiphysis to the tract of the postoptic commissure (TPOC) in the ventral diencephalon. We found that a single growth cone pioneered the entire tract. This growth cone extended through a cellular environment consisting of columnar neuroepithelial cells. There was no indication of any morphological specialization in the neuroepithelium ahead of the pioneering growth cone that might act as a channel to guide the growth cone.

The results demonstrate a striking similarity between development of the DVDT and development of early insect axonal pathways.

MATERIALS AND METHODS

DiI Labeling. Zebrafish (*Brachydanio rerio*) embryos were staged by age and also by counting somites. They were fixed in Pipes (Sigma)-buffered 4% formalin for several hours, after which the eyes and skin overlying the brain were removed. DiI (19) (Molecular Probes) was dissolved in *N,N*-dimethylformamide (Sigma) and allowed to dry onto glass. A fragment of this diI (diameter, 5–40 μ m) was maneuvered onto the epiphysis with tungsten needles. Very small fragments labeled one, or a very few, cells, and larger fragments labeled the entire epiphysis. Preparations were generally left overnight at 4°C to allow the diI to diffuse. The fluorescence in the labeled axons was photoconverted to a brown reaction product by excitation of the dye in 0.05% diaminobenzidine (16), after which preparations were washed and cleared in 70% (vol/vol) glycerol.

Electron Microscopy. For electron microscopy, embryos were prepared as described above except that 0.1% glutaraldehyde was added to the primary fixative. After photoconversion, they were drawn and photographed, postfixed in 3% glutaraldehyde, and prepared for electron microscopy (17). Each specimen was serially sectioned at ≈ 0.15 μ m with occasional 0.5- μ m sections interspersed.

RESULTS

The zebrafish embryo has, by 24 hr postfertilization, a simple scaffold of axon tracts and commissures in the midbrain and forebrain (17, 18). (All ages are expressed in hours postfertilization.) Axons from the epiphysis (pineal body) make up one of these, the DVDT, which courses ventrally ≈ 100 μ m to the TPOC. At the intersection, the axons in the DVDT turn rostrally toward the postoptic commissure (Fig. 1 and ref. 17).

DiI was applied to the epiphysis between 18 and 21 hr to label axons and growth cones in the developing DVDT. At 18 hr ($n = 8$) none were seen, in spite of the fact that the applications labeled many, perhaps all, of the cell bodies in the region of the epiphysis (Fig. 2*a*). At 19–21 hr ($n = 21$), a single leading axon tipped with a growth cone was labeled (Fig. 2*b* and *c*). At 19–20 hr it was typically one-third to one-half of the way to the TPOC (Fig. 2*b*). The shape of the growth cone varied, but it was generally elongated in the direction of advance and flattened parallel to the surface of the brain. Filopodia extended in many directions but were out of reach of any other tract. [The closest, that of the posterior commissure (17, 18), lay ≈ 50 μ m caudally.] At 20–21 hr ($n =$

Abbreviations: DVDT, dorsoventral diencephalic tract; TPOC, tract of the postoptic commissure; diI, 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate.

*To whom reprint requests should be addressed.

FIG. 1. Whole-mounted zebrafish brain showing labeled axons originating from the epiphysis at 24 hr. Rostral is to the right and dorsal is up. The epiphysial axons course ventrally in the DVDT to its intersection with the TPOC (dots), where they turn rostrally toward the POC. These are the only axons within the DVDT, whereas the TPOC contains others that run both rostrally toward the POC and caudally toward the midbrain (unlabeled in this preparation, but see ref. 17). E, epiphysis; POC, postoptic commissure; T, telencephalon; Te, tectum. (Bar = 50 μ m.)

25), the growth cone had almost reached the TPOC (Fig. 2c). In 3 of the 25 embryos, a second labeled growth cone trailed the first by 50 μ m or more. In most preparations, the leading axon was traced back to its site of origin in the caudal half of the epiphysis. This origin was confirmed by retrograde labeling with dil ($n = 6$) (Fig. 2d). In summary, the DVDT is pioneered between 19 and 21 hr by a single growth cone that is followed by a second within hours; by 48 hr, the tract contains hundreds of axons (unpublished observations).

The structure of, and environment encountered by, the growth cone were examined electron microscopically at 19–20 hr ($n = 4$). Examination of the growth cone's environment confirmed that it was a pioneer. The labeled axon, growth cone, and filopodia were unaccompanied by any other axons (Fig. 3). Examination of the region ahead of the growth cone showed no other axons between it and the TPOC, thus excluding the possibility that the labeled axon was following other unlabeled ones from elsewhere.

The growth cone occupied a relatively superficial position 1–2 μ m deep to the basal lamina but occasionally in direct

contact with it (Fig. 3d). Three of the four growth cones were flattened parallel to the surface (Fig. 3d), confirming the light-microscopic impression. This orientation is surprising, since the path of least resistance through the interstices of the columnar end feet would seem to be realized by flattening in the opposite direction. Filopodia extended both parallel and perpendicular to the surface of the brain. Although most filopodia were in the space between cells, some penetrated into them (Fig. 3c). The observation that some filopodia extended deeply suggests that the restriction of axons to the most superficial stratum was not due to a failure to explore more deeply.

We looked for structural specializations that might presage the path taken by the growth cone, but we found none. The neuroepithelium ahead of, and in contact with, the leading filopodia was indistinguishable from that elsewhere in other axon-free regions (Fig. 3c and e). The neuroepithelial cells were relatively simple, with tubular end feet that overlapped very little with their neighbors (Fig. 3a, c, and e). The extracellular space around and ahead of the filopodia was no

FIG. 2. Micrographs from whole-mounted embryonic brains in which dil had been applied to the epiphysis (a–c) or to the DVDT (d). Rostral is to the right and dorsal is up. Dashed lines show the dorsal surface of the brain. (a) At 18 hr, cell bodies are labeled but none possesses axons or growth cones. (b) At 19.5 hr, a single pioneering growth cone is labeled approximately halfway down the DVDT. (c) At 21 hr, the pioneering growth cone is almost at the intersection with the TPOC (dotted line). (d) At 20 hr, a single neuron in the caudal epiphysis (arrowhead) is retrogradely labeled after dil application to the DVDT. E, epiphysis; Dil, application site. (Bar = 10 μ m.)

FIG. 3. Electron micrographs of the pioneering axon and growth cone from preparations fixed at 19–20 hr. The basal lamina is toward the top of each panel. The dashed lines outline the boundaries of cells. (a) Transverse section through the labeled axon (arrowhead) near the epiphysis. Serial sections showed that the processes (asterisks) associated with it originated from nearby neuroepithelial cells. (b) Transverse section through the labeled axon (arrowhead) just proximal to the growth cone. A small side branch extends superficially and is apposed to a cluster of vesicles in the neuroepithelial cell that is enveloping the axon. Similar clusters were also seen in neuroepithelial cells untouched by the labeled structure, so they are probably not an artifact of the labeling. (c) Transverse section through a leading filopodium (arrowhead) that is inserted into a neuroepithelial cell. Penetrating filopodia, such as this, were not associated with coated vesicles, as has been described in insects (20), or with any other subcellular organelles. (d) Longitudinal section through a labeled growth cone that was advancing toward the right of the panel. The growth cone is in contact with the basal lamina (arrow). Filopodia (arrowheads) are between neuroepithelial cells, and some extend into the deeper nuclear layer of the neuroepithelium. The surface of the neuroepithelium is elevated above the growth cone, presumably a mechanical deformation. (e) Transverse section through the leading filopodium (arrowhead) of the growth cone. CV, cluster of vesicles. (Bar = 1 μm .)

larger than in flanking regions, which fails to support the idea that pioneer axons advance along preformed channels (21).

The neuroepithelial end feet around the axon were more complex than elsewhere (Fig. 3a). We examined the morphology of the end feet in sections both parallel to and transverse to the direction of advance of the growth cone. In both cases, we found that there were more end feet processes arising from cells in contact with the labeled axon than from neighboring cells either ahead of or lateral to the pioneer axon. This suggests that the columnar end feet were altered by the passage of the growth cone, perhaps triggered to envelop the axon. Whatever the particular influences were, it is clear that the structural changes in the end feet followed, rather than preceded, the arrival of the growth cone.

DISCUSSION

In the Introduction, we raised the question of whether tracts in the vertebrate central nervous system were formed in a similar fashion to those in invertebrates (see also ref. 22). We have shown that the DVDT is pioneered by a single growth cone that traces out a stereotyped trajectory and originates from a neuron reliably found in the same location in all zebrafish embryos. These are very like the events in early invertebrate neural development (23).

While it is apparent that the DVDT is pioneered by a single growth cone, we do not know whether this growth cone always belongs to the same cell body. As yet we have no way

of unambiguously distinguishing different cells in the epiphysis. All of the early growth cones to arise from epiphysial neurons have very similar trajectories, and the neuronal cell bodies are also located very close together.

The second growth cone to enter the DVDT generally follows the axon of the neuron that pioneered the tract and this raises the question of whether the follower needs the pioneer for pathway guidance. A similar question has been asked experimentally in other systems by ablating the pioneer and determining whether the follower can still pathfind; both outcomes have been observed (11, 24–27). We have not done such an experiment on the DVDT, but similarly labeled whole mounts of embryos a few hours older than these very occasionally show a second axon from the epiphysis that has a trajectory to the TPOC independent of the pioneer (unpublished data). This suggests that the pioneer of the DVDT is not the only cell in the epiphysis capable of navigating to the TPOC.

The axon and growth cone were restricted to the most superficial few micrometers of the neural tube, although the filopodia penetrated more deeply. Presumably, the superficial region provides a hospitable environment for axonal growth, as other studies have suggested (17, 28, 29). This region's salient feature may be a positive one, the concentration of adhesion molecules that support axonal outgrowth (29, 30), or a negative one, the absence of other molecules, as yet unidentified, that are repulsive (31).

The zebrafish brain offers many advantages for the future study of vertebrate axonal pathfinding. The pioneering growth cone we describe here is well separated in both time and space from any other growth cones or axons. This situation greatly facilitates study of the factors influencing its growth, particularly in the living animal.

We thank Ms. C. Malinoski for technical support, and Drs. R. Hume, J. Kuwada, K. Tosney, Mr. J. Burrill, and Ms. R. Marcus for useful comments. This work was supported by National Institutes of Health Research Grant EY00168 to S.S.E. and by Science and Engineering Research Council/North Atlantic Treaty Organization Fellowship BR/F8217 to S.W.W.

1. Ramon y Cajal, S. (1890) *Anat. Anz.* **5**, 609–613.
2. Harrison, R. G. (1910) *J. Exp. Zool.* **9**, 838–843.
3. Spiedel, C. (1933) *Am. J. Anat.* **52**, 1–75.
4. Bate, C. M. (1976) *Nature (London)* **260**, 54–56.
5. Ho, R. K. & Goodman, C. S. (1982) *Nature (London)* **297**, 404–406.
6. Bentley, D. & Keshishian, H. (1982) *Science* **218**, 1082–1088.
7. Eisen, J. S., Myers, P. Z. & Westerfield, M. (1986) *Nature (London)* **320**, 269–271.
8. Myers, P. Z., Eisen, J. S. & Westerfield, M. (1986) *J. Neurosci.* **6**, 2278–2289.
9. Tosney, K. W. & Landmesser, L. (1985) *J. Neurosci.* **5**, 2345–2358.
10. Stainier, D. Y. R. & Gilbert, W. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 923–927.
11. Kuwada, J. Y. (1986) *Science* **233**, 740–746.
12. Nordlander, R. H. (1984) *J. Comp. Neurol.* **228**, 117–128.
13. Holley, J. A. & Silver, J. (1987) *Dev. Biol.* **123**, 375–388.
14. Mendelson, B. (1986) *J. Comp. Neurol.* **251**, 172–184.
15. Yaginuma, H., Shiga, T., Homma, S., Ishihara, R. & Oppenheim, R. (1990) *Development* **108**, 705–716.
16. McConnell, S. K., Ghosh, A. & Shatz, C. J. (1989) *Science* **245**, 978–982.
17. Wilson, S. W., Ross, L., Parrett, T. & Easter, S. S., Jr. (1990) *Development* **108**, 121–145.
18. Chitnis, A. B. & Kuwada, J. Y. (1990) *J. Neurosci.* **10**, 1892–1905.
19. Honig, M. G. & Hume, R. I. (1986) *J. Cell Biol.* **103**, 171–187.
20. Bastiani, M. J. & Goodman, C. S. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1849–1853.
21. Singer, M., Nordlander, R. H. & Egar, M. (1979) *J. Comp. Neurol.* **185**, 1–22.
22. Easter, S. S., Jr., Purves, D., Rakic, P. & Spitzer, N. (1985) *Science* **230**, 507–511.
23. Harrelson, A. L. & Goodman, C. S. (1988) *Science* **242**, 700–708.
24. Klose, M. & Bentley, D. (1989) *Science* **245**, 982–984.
25. Keshishian, H. & Bentley, D. (1983) *Dev. Biol.* **96**, 116–124.
26. Eisen, J., Pike, S. H. & Debu, B. (1989) *Neuron* **2**, 1097–1104.
27. Pike, S. H. & Eisen, J. S. (1990) *J. Neurosci.* **10**, 44–49.
28. Easter, S. S., Jr., & Taylor, J. S. H. (1989) *Development* **107**, 553–573.
29. Letourneau, P. C., Madsen, A. M., Palm, S. L. & Furcht, L. T. (1988) *Dev. Biol.* **125**, 135–144.
30. Silver, J. & Rutishauser, U. (1984) *Dev. Biol.* **106**, 485–499.
31. Patterson, P. H. (1988) *Neuron* **1**, 263–267.