Axonal Outgrowth within the Abnormal Scaffold of Brain Tracts in a Zebrafish Mutant

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SUMMARY

The role of specific axonal tracts for the guidance of growth cones was investigated by examining axonal outgrowth within the abnormal brain tracts of zebrafish *cyclops* mutants. Normally, the earliest differentiating neurons in the zebrafish brain establish a simple scaffold of axonal tracts. Later-developing axons follow cell-specific pathways within this axonal scaffold. In *cyclops* embryos, this scaffold is perturbed due to the deletion of some ventromedial neurons that establish parts of the axonal scaffold and the development of an abnormal crease in the brain. In these mutant embryos, the growth cones projected by the neurons of the nucleus of the posterior commissure (nuc PC) are deprived of the two tracts of axons that they sequentially follow to first extend ventrally, then posteriorly. These growth cones respond to the abnormal scaffold in several interesting ways. First, nuc PC growth cones initially always extend ventrally as in wild-type embryos. This suggests that for the first portion of their pathway the axons they normally follow are not required for proper navigation. Second, approximately half of the nuc PC growth cones follow aberrant longitudinal pathways after the first portion of their pathway. This suggests that for the longitudinal portion of the pathway, specific growth cone/axon interactions are important for guiding growth cones. Third, although approximately half of the nuc PC growth cones follow aberrant longitudinal pathways, the rest follow normal pathways despite the absence of the axons that they normally follow. This suggests that cues independent of these axons may be capable of guiding nuc PC growth cones as well. These results suggest that different guidance cues or combinations of cues guide specific growth cones along different portions of their pathway. © 1994 John Wiley & Sons, Inc.

Keywords: growth cone, zebrafish, brain, mutant

INTRODUCTION

Growth cones reach their targets by following cell-specific pathways in a variety of embryos (Goodman and Shatz, 1993). In many cases growth cones accomplish this by interacting with cues in their local environment to select appropriate pathways. Recent experimental manipulations demonstrated that growth cones utilize specific cues to select pathways in the early brain of zebrafish embryos as well (Chitnis and Kuwada, 1991; Chitnis et al., 1992). The brain of early zebrafish embryos contains a simple scaffold of tracts that are established by identifiable clusters of neurons (Fig. 1; Chitnis and Kuwada, 1990; Wilson et al., 1990). Within the diencephalon and mesencephalon, this axonal scaffold contains two longitudinal tracts, the dorsolateral tract of the postoptic commissure (TPOC) and the ventromedial medial longitudinal fasciculus (MLF). These tracts are intersected by a commissure, the posterior commissure (PC), that is located at the border of the forebrain and midbrain. The TPOC is established by neurons of the nucleus of the TPOC (nuc TPOC) located in the anterior diencephalon. These neurons extend axons posteriorly through the ventral diencephalon and tegmentum. The MLF is established by the posteriorly directed axons of the neurons of the nucleus of the MLF (nuc MLF). The nuc MLF is located ventromedially in the anterior tegmentum. The PC is established by the dorsally directed

Received July 19, 1993; accepted November 17, 1993
Journal of Neurobiology, Vol. 25, No. 4, pp. 345–360 (1994)
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CCC 0022-3034/94/040345-16

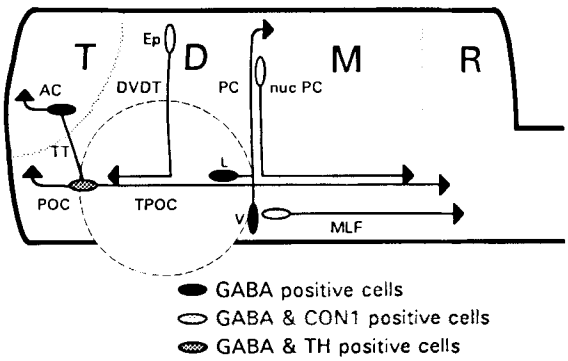

Figure 1 A schematic side view of the wild-type zebrafish brain (27 h PF) showing the distribution of neurons immunoreactive for GABA, TH, and CON1; and the pathways taken by growth cones through the early scaffold of axonal tracts. Nuc PC growth cones extend ventrally along the PC and then caudally along the TPOC; Ep growth cones first extend ventrally to establish the DVDT and then anteriorly along the TPOC. Unless otherwise stated, anterior is left and dorsal is up in all of the figures. The dashed circle represent the eye, and the dotted lines represent a fold or crease in the brain. *Abbreviations:* T = telencephalon; D = diencephalon; M = mesencephalon; R = rhombencephalon; AC = anterior commissure; POC = postoptic commissure; PC = posterior commissure; TT = telencephalic tract; DVDT = dorsoventral diencephalic tract; TPOC = tract of the postoptic commissure; MLF = medial longitudinal fasciculus; nuc PC = nucleus of the posterior commissure; Ep = epiphysial neurons; L = lateral cluster of posterior commissure neurons; V = ventral cluster of posterior commissure neurons.

axons of two clusters of neurons located in the ventral portion of the anterior tegmentum, the lateral and ventral PC clusters. Within this scaffold the growth cones of the approximately three to four neurons in the nucleus of the PC (nuc PC) (Chitnis and Kuwada, 1990) and of the two to three epiphysial (Wilson and Easter, 1991) neurons follow cell-specific pathways. The epiphysial neurons are located just lateral to the base of the epiphysis. They project growth cones that extend ventrally along the diencephalon toward the TPOC to pioneer the dorsoventral diencephalic tract (DVDT). Once at the TPOC, epiphysial growth cones turn anteriorly to extend along the TPOC. The nuc PC is located in the dorsolateral midbrain just posterior to the PC. They project growth cones that fasciculate with the PC axons and follow them ventrally into the anterior tegmentum. Once at the anterior tegmentum, they turn posteriorly onto the TPOC to fasciculate with these axons and extend along them into the hindbrain.

Although other trajectories are available at the intersection of tracts in the anterior tegmentum, nuc PC growth cones always turn posteriorly onto and extend along the TPOC. To see if nuc PC growth cones choose this pathway by selectively fasciculating with TPOC axons, the TPOC axons were surgically prevented from entering the midbrain (Chitnis and Kuwada, 1991; Chitnis et al., 1992). After this manipulation, 39% of the nuc PC growth cones followed aberrant pathways (usually the MLF). This signifies that cues associated with the TPOC axons normally play an important role in insuring nearly error-free pathfinding by these growth cones. However, a significant proportion of nuc PC growth cones extended on the endfeet of neuroepithelial cells along an apparently normal, dorsolateral longitudinal pathway despite the absence of the TPOC axons. This suggests that cues found along a dorsolateral longitudinal strip of endfeet may also be capable of guiding nuc PC growth cones. Furthermore, even growth cones that followed aberrant pathways usually turned and extended posteriorly through the tegmentum. This suggests that directionality cues are not limited to the TPOC axons and dorsolateral endfeet.

We have investigated these issues further by examining outgrowth by nuc PC growth cones in mutant *cyclops* embryos (Hatta et al., 1991). *Cyclops* embryos lack the floor plate in the midbrain, hindbrain, and spinal cord, and have eyes that are fused on the ventral side due to deletion of the ventromedial portions of the brain (Hatta et al., 1991, 1992a). In the diencephalon the deletion includes ventral neurons and longitudinal axonal tracts. Here we show that these abnormalities prevent the normal formation of the PC and TPOC, the tracts nuc PC growth cones normally follow. Despite the absence of the PC, nuc PC growth cones all extend ventrally to the anterior tegmentum. However, approximately half then followed aberrant pathways while the rest followed an apparently normal pathway in the absence of the TPOC. Our results confirm our earlier findings. Furthermore, the results suggest that different cues or combination of cues guide specific growth cones along different portions of their pathway.

METHODS

Animals

Zebrafish embryos were collected from a laboratory breeding colony. Embryos were raised at 28.5°C accord-

ing to the protocol described in Myers et al. (1986) and were staged from the estimated time of fertilization. Homozygous *cyclops* embryos were generated by crossing heterozygous parents and were identified by their cyclopean head phenotype (Hatta et al., 1991).

Immunocytochemistry

A guinea pig polyclonal antibody against GABA (Eugene Tech), a mouse monoclonal antibody (MAb) against tyrosine hydroxylase (TH), a MAb against acetylated α-tubulin (Piperno and Fuller, 1985) that labels many if not all axons in the zebrafish embryo (Chitnis and Kuwada, 1990), and a mouse MAb (MAb CON1) that recognizes a specific subset of early zebrafish neurons (Bernhardt et al., 1990) were used to label embryos using the whole-mount procedure as previously described (Kuwada et al., 1990). To detect the GABA antibody, a horseradish peroxidase- (HRP)-conjugated anti-guinea pig IgG antibody was applied followed by initiation of the reaction using diaminobenzidene (dab) as the chromagen. The acetylated α-tubulin and CON1 antibodies were detected using dab as the chromagen following the application of the biotin-/avidin-based Vectastain kit (Vector Research Corp.). In some cases, anti-acetylated α-tubulin-labeled embryos were refixed, dehydrated, embedded in plastic, and sectioned (1-μm semi-thins).

Embryos were labeled with both the GABA and acetylated α-tubulin antibodies by visualizing the GABA antibody using dab as the chromagen following application of HRP-conjugated anti-guinea pig IgG antibody and the acetylated α-tubulin antibody using a TRITC-conjugated anti-mouse IgG antibody. The embryos were incubated in the two primary antibodies at the same time followed by the two secondary antibodies at the same time. The HRP/dab reaction did not markedly interfere with the TRITC signal.

The data in this report are based on the examination of up to 24 wild-type and 10 *cyclops* embryos with anti-GABA; 17 wild-type and 17 *cyclops* embryos with anti-TH; 21 wild-type and 32 *cyclops* embryos with MAb CON1; and 24 wild-type and 34 *cyclops* embryos with anti-tubulin. 24 wild-type embryos were double-labeled with the MAb against acetylated α-tubulin and anti-GABA.

DiI Labeling of nuc PC and Epiphysial Neurons and All Other Axons with Anti-Acetylated α-Tubulin

Cyclops embryos at either 26 or 28 h PF were dechorionated, pinned in a Sylgard-lined embryo holder, fixed for 4 h with 4% paraformaldehyde in 75 m*M* phosphate buffer (pH 7.4), washed with 100 m*M* phosphate buffer (pH 7.4), and mounted on a fixed-stage compound microscope. Nuc PC or epiphysial neurons were visualized with differential interference contrast (DIC) optics, and labeled by pressure ejection from a micropipette of a small amount of a 0.2% DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) dissolved in N,N-dimethylformamide (Honig and Hume, 1986) into the nuc PC or the base of the epiphysis. DiI was allowed to spread for 10–14 h, and the labeled cells were marked with the brown dab reaction product by photooxidation (Maranto, 1982; Kuwada et al., 1990). The other axons in the embryos were then labeled with the acetylated α-tubulin antibody as described above, except a blue-violet dab product was produced by using chloronaphtol as the chromagen. Alternatively, other axons were marked with a blue dab reaction product by a heavy-metal intensification technique (Adams, 1981) following the acetylated α-tubulin antibody. In these embryos the DiI-labeled growth cones and axons were brown and could be readily distinguished from the other axons which were blue-violet or blue.

RESULTS

A Subset of the Neurons in the Brain Are Labeled by Antibodies Against GABA, TH, and CON1

The early zebrafish brain contains a simple scaffold of axonal tracts that is established by clusters of neurons located at stereotyped locations (Chitnis and Kuwada, 1990). The scaffold can be readily discerned by labeling axons with a MAb against acetylated α-tubulin (Piperno and Fuller, 1985). Anti-GABA, anti-TH, and MAb CON1 label subsets of these neurons (Fig. 1). At 26 h PF, a comparison of the pattern of neurons labeled with the MAb against acetylated α-tubulin with that labeled with anti-GABA demonstrates that many but not all the neurons in the forebrain and midbrain are GABA immunoreactive (Fig. 2). The cell bodies and axons of neurons in the lateral and ventral PC clusters, nuc TPOC in the anterior diencephalon, and in the telencephalon are GABA immunoreactive. Additionally, some neurons in the nuc MLF in the anterior tegmentum may also be GABA immunoreactive (see below). However, the epiphysial neurons that are located at the base of the epiphysis and pioneer the dorsoventral diencephalic tract (DVDT) nor the nuc PC neurons in the dorsolateral midbrain are labeled by anti-GABA. Consequently, axons in the PC, TPOC, MLF, anterior commissure, and telencephalic tract are labeled; the DVDT is not. Neurons in the telencephalon had previously been shown to contribute axons to the anterior commissure and the telecephalic tract (Chitnis and Kuwada, 1990). Although it is clear

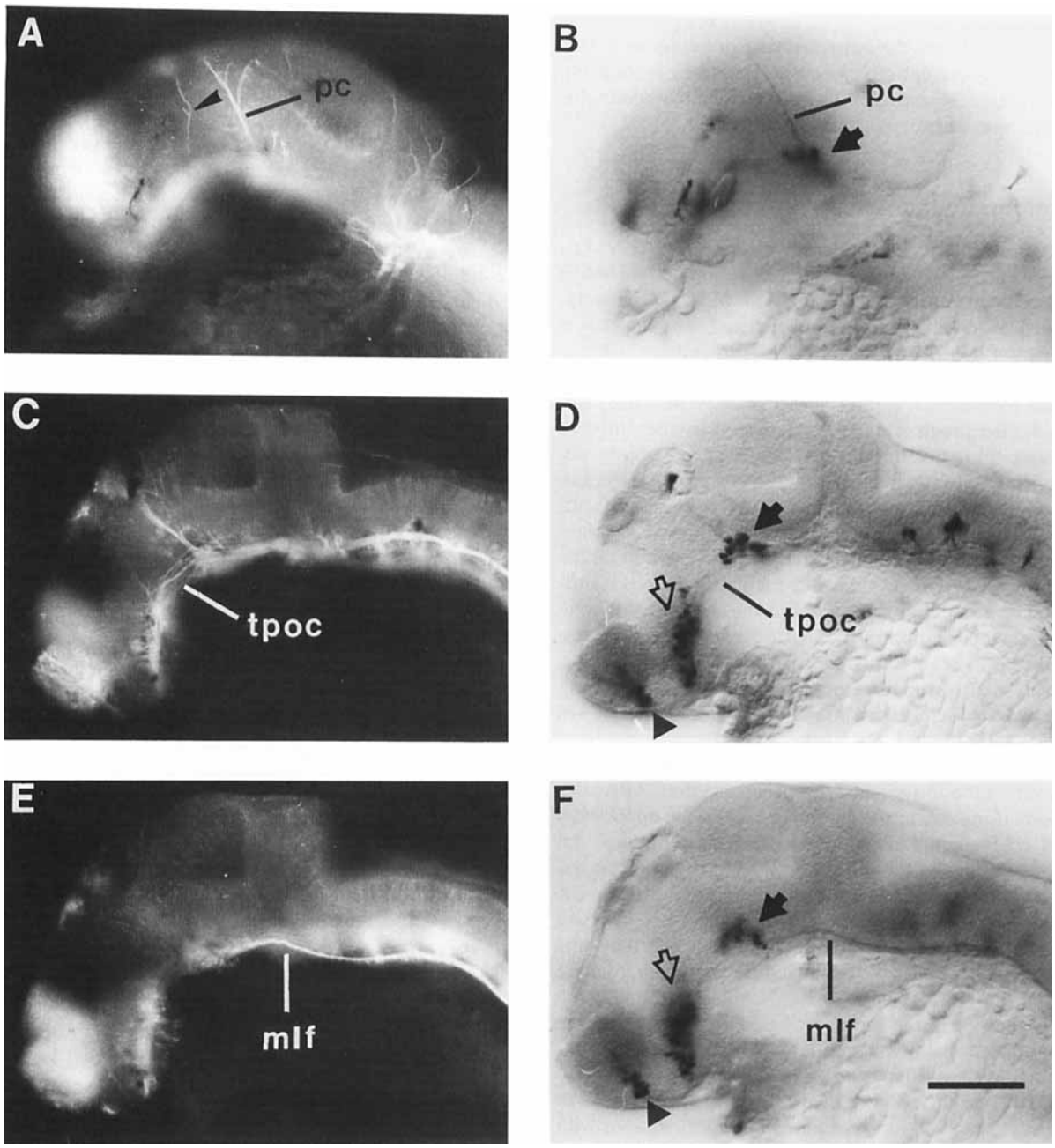

Figure 2 The antibody against GABA labels a subset of neurons in the early zebrafish brain. Embryos were double labeled with both antibodies against acetylated α-tubulin (A, C, and E) and GABA (B, D, and F) to confirm the identity of the GABA-immunoreactive cells. The acetylated α-tubulin-immunoreactive neurons were marked with TRITC and the GABA-immunoreactive neurons with a dab reaction product. The photomicrographs show a sideview of the brain: panels (A) and (B) are photomicrographs of the same embryo at 26 h PF and panels (C–F) are photomicrographs from another 26-h embryo. (A) and (B) Anti-GABA labels a group of neurons in the anterior tegmentum (filled arrow) that includes the lateral and ventral cluster of cells that give rise to the PC and possibly some nuc MLF neurons (see text); and axons in the PC. Neither the epiphysial axons (arrow head) nor nuc PC axons labeled by the acetylated α-tubulin antibody are labeled by the GABA antibody. (C) and (D) Anti-GABA labels a cluster of telencephalic neurons (triangle) and the nuc TPOC (open arrow) in the anterior diencephalon as well as the anterior tegmental neurons (closed arrow). (E) and (F) Anti-GABA labels axons in the MLF. Scale for all panels = 50 μm.

that anti-GABA labels axons in the MLF, at present it is unclear for two reasons whether anti-GABA-labeled MLF axons arise from either a subset of nuc MLF neurons or nuc TPOC neurons. First, nuc MLF neurons are contiguous with those in the ventral PC cluster, so identification of the GABA-immunoreactive neurons in this region cannot be made unambiguously solely on the position of their cell bodies. Second, a relatively small subset of axons that initially descend in the TPOC in the forebrain switch to the MLF in the tegmentum, so the GABA-immunoreactive axons in the MLF may arise from nuc TPOC neurons. The identity of the MLF axons that are GABA immunoreactive could be resolved by labeling embryos with both anti-GABA and MAb CON1 (see below).

MAb CON1 and anti-TH labeling is much more restricted than that by anti-GABA. MAb CON1 labels the cell bodies and axons of neurons in the nuc MLF, and anti-TH labels a subset of the neurons in the nuc TPOC (Fig. 3; Table 1). The TH-immunoreactive neurons were identified as nuc TPOC neurons by comparing the pattern of anti-TH labeling with that by the MAb against acetylated α-tubulin in double-labeled embryos [Fig. 3(A,B)]. At present we have not labeled embryos with both anti-TH and anti-GABA to see if there are nuc TPOC neurons that are both TH and GABA immunoreactive. However, since anti-TH labels a subset of the nuc TPOC neurons, it is clear that there exists nuc TPOC neurons that are GABA but not TH immunoreactive. Additionally, anti-TH labels a group of cells located over the yolk sack and lateral to the otocyst.

The TPOC Fails to Enter the Midbrain in *Cyclops* Embryos

Cyclops embryos are characterized by the deletion of cells at the ventral midline of the CNS (Hatta et al., 1991). In the forebrain this reduction leads to the cyclopean phenotype and is most severe in the anterior brain (Hatta et al., 1992a). To determine the identities of the neurons that are deleted in *cyclops* brains, *cyclops* embryos were labeled with antibodies against GABA and TH and compared with wild-type embryos labeled with the same antibodies. Unlike the two clusters of forebrain neurons (telencephalic and nuc TPOC neurons) labeled by anti-GABA in wild-type embryos, both anti-GABA and the antibody against acetylated α-tubulin labeled only a single cluster of neurons in the forebrains of *cyclops* embryos [compare Fig. 2(D); 4(A)]. At 26 h PF, there is a reduction in the number of GABA-immunoreactive neurons found in the forebrain of *cyclops* embryos: 11.1 telencephalic and 24.3 nuc TPOC neurons are GABA immunoreactive in wild-type embryos compared to 9.3 neurons in the single cluster in *cyclops* embryos (Table 1). Furthermore, it appears that the TH-immunoreactive nuc TPOC neurons are missing in *cyclops* embryos since anti-TH labeled no neurons in the brains of these embryos [Fig. 4(B)] compared with 6.0 neurons in wild-type brains (Table 1). The embryo shown in Figure 4(B) exhibits a high amount of background labeling since it was over-reacted for peroxidase activity to insure there was no TH-immunoreactive neurons. Anti-TH did label the cells overlying the yolk sack in *cyclops* embryos as it did in wild-type embryos (not shown).

To determine the pattern of axonal tracts in the brain of *cyclops* embryos, they were labeled with the antibody against acetylated α-tubulin since axons were only weakly labeled by anti-GABA and difficult to trace in *cyclops* embryos. The forebrain neurons labeled by anti-GABA in *cyclops* embryos are likely to be the telencephalic and/or nuc TPOC neurons that are labeled by anti-GABA in wild-type embryos (see Discussion). Regardless of their identity, these neurons extended posteriorly directed axons that failed to extend into the midbrain [Fig. 5(A)]. Instead, the axons formed an abnormal commissure and fasciculated with bilaterally homologous axons from the other side of the diencephalon. The abnormal commissure formed at an abnormal crease in the diencephalon. The crease extended dorsally from the ventral flexure towards a location anterior to the epiphysis [n = 40; Fig. 5(A)] and was evident by 16 h PF (n = 3; not shown), which is approximately the stage when brain neurons first project axons (Chitnis and Kuwada, 1990). This suggests that the aberrant crease prevents the axons of the GABA-immunoreactive neurons from extending into the midbrain.

The PC Is Missing and MLF Axons Show Abnormal Trajectories in the *Cyclops* Embryo

Cyclops embryos exhibited abnormalities in the midbrain in addition to those in the forebrain. As mentioned above, anti-GABA labeled neurons in the lateral and ventral clusters of PC neurons and possibly some nuc MLF neurons in the anterior

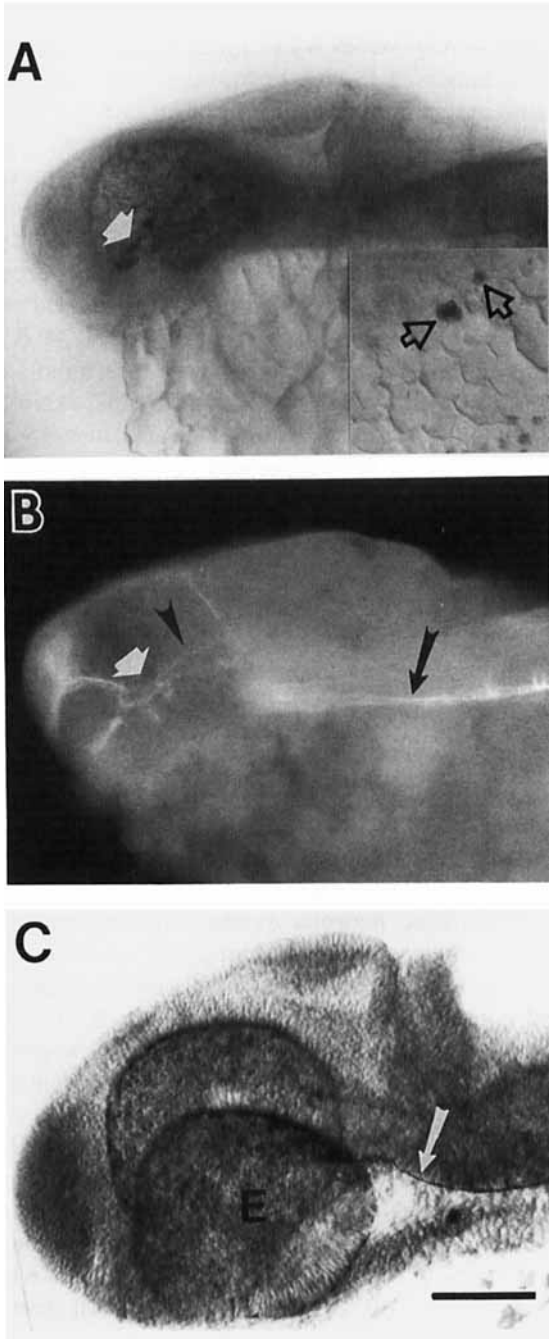

Figure 3 Antibodies against TH and CON1 label small subsets of neurons in the early brain. (A) and (B) A 26-h embryo double labeled with anti-TH (A) and anti-acetylated α-tubulin (B); shows that a small number of neurons in the anterior diencephalon (wide white arrow) that give rise to the POC and the TPOC (arrow head) and some cells overlying the yolk sack and lateral to the otocyst (open arrows) are TH immunoreactive. TH-immunoreactive cells were marked with a dab reaction product and acetylated α-tubulin-immunoreactive neurons with TRITC. In (A), the TH-immunoreactive TPOC neurons are obscured by the pigmentation in the overlying eye, and in (B), the black arrow denotes the MLF. (C) MAb CON1 labels the axons of the MLF (white arrow) in a 27-h embryo. The MAb CON1-labeled MLF on the other side can be seen out of the focal plane. This embryo is partly titled to one side and was flattened so that both eyes (E) are visible in this micrograph. Scale for all panels = 100 μm.

tegmentum of wild-type embryos. In *cyclops* embryos, anti-GABA labeled many fewer neurons in the anterior tegmentum than in wild-type embryos [Fig. 2(B,D,F); 4(A)]. At 26 h PF, 12.0 cells labeled in the anterior tegmentum of wild-type embryos, whereas 2.6 cells labeled in *cyclops* embryos (Table 2). Importantly, labeling axons in the brains of *cyclops* embryos with the acetylated α-tubulin antibody demonstrated that the PC was missing [Fig. 5(B)] in 38 of 40 embryos. In these embryos the ventrally directed axons of the nuc PC labeled with the acetylated α-tubulin antibody, but no axons were seen to run dorsal to the nuc PC. The high proportion of embryos observed in our experiments that were missing the PC may not be representative of *cyclops* embryos in general. The amount of fusion of the two eyes is somewhat variable in *cyclops* embryos (Hatta et al., 1991), and the embryos analyzed here generally had severe fusion of the eyes.

Along with the dramatic reduction in the number of GABA-immunoreactive cells, there were abnormalities in the position and number of nuc MLF neurons and the trajectories their axons followed in *cyclops* embryos as previously reported (Hatta, 1992b). Normally, the nuc MLF are bilaterally paired and located lateral to the ventral midline. Their axons extend posteriorly and ipsilaterally to establish the tightly fasciculated MLF (Kimmel et al., 1982). In *cyclops* embryos the nuc MLF were usually partially or completely fused (Fig. 6), and the number of nuc MLF neurons labeled with MAb CON1 was reduced in number. Wild-type embryos contained 14.6 neurons in the two bilaterally paired nuclei, while there were 10.4 neurons in *cyclops* embryos (Table 2). Furthermore, nuc MLF axons extended along a variety of pathways in *cyclops* embryos (Fig. 6). Of the 171 nuc MLF axons assayed, 56% extended posteriorly in a loosely fasciculated tract around the ventral midline [Fig. 6(B)]; 26% of the axons extended dorsally into the tectum and then extended posteriorly [Fig. 6(D)]; 13% of the axons extended anteriorly and dorsally into the forebrain [Fig. 6(C)] and in three embryos nuc MLF axons formed an aberrant dorsal commissure near the normal site of

Table 1 Comparison of the Number of GABA- and TH-Immunoreactive Neurons in the Forebrain of Wild-type and *Cyclops* Embryos at 26 h PF

	Wild-type, Telencephalic Neurons	Wild-type, nuc TPOC Neurons	*Cyclops*, Forebrain Neurons
GABA-Immunoreactive Neurons	11.1 (10–12) $n = 15$	24.3 (22–28) $n = 15$	9.3 (8–11) $n = 10$
TH-Immunoreactive Neurons	0 (0) $n = 9$	6.0 (5–7) $n = 9$	0 (0) $n = 17$

Note. For each category, the mean is followed by the range in parentheses.

the PC; and 5% of the axons exited the CNS completely.

Epiphysial Growth Cones Extend Ventrally but then Make Errors in *Cyclops* Embryos

Epiphysial axons normally extend ventrally from the base of the epiphysis to establish the DVDT and then turn to extend anteriorly along the TPOC (Fig. 1; Chitnis and Kuwada, 1990; Wilson and Easter, 1991). In *cyclops* embryos acetylated α-tubulin ($n = 23$) or DiI- ($n = 26$) labeled epiphysial axons extend ventrally as they normally do and do so posterior to the abnormal crease in the diencephalon [Fig. 5(C), 7]. After extending ventrally, DiI-labeled epiphysial axons either turn and extend posteriorly into the tegmentum (23%) or form an aberrant commissure at the abnormal crease (77%).

Nuc PC Growth Cones Extend Ventrally in the Absence of the PC but Make Errors in the Absence of the TPOC

Labeling *cyclops* embryos with the antibody against acetylated α-tubulin ($n = 34$) indicated that the dorsolaterally located nuc PC cells were present. This allowed us to examine axonal outgrowth by nuc PC neurons in the absence of the tracts they normally follow. In wild-type embryos, nuc PC growth cones first extend ventrally along the PC to the intersection of tracts in the anterior tegmentum and then turn and extend posteriorly along the TPOC (Fig. 1). Due to the absence of the PC, which usually obstructs a clear view of the nuc PC axons, the behavior of the nuc PC could be clearly examined in acetylated α-tubulin-labeled embryos. In the 38 *cyclops* embryos (22–26 h PF) in which the PC was missing, all of the nuc PC

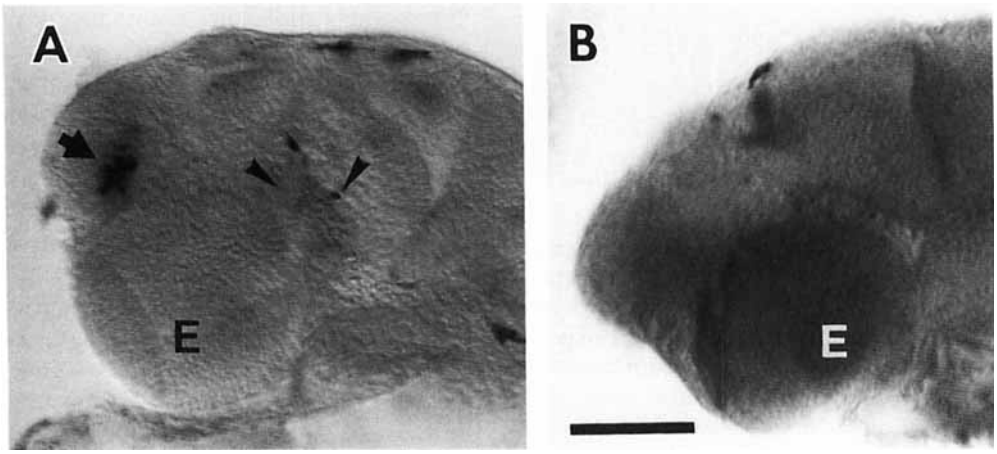

Figure 4 All the TH-immunoreactive and many of the GABA-immunoreactive neurons are missing in the brains of *cyclops* embryos (26 h). (A) Anti-GABA labels a single cluster of cells in the *cyclops* forebrain (arrow) rather than the two clusters labeled in the wild-type forebrain, and a much smaller cluster of cells in the anterior tegmentum (arrowheads) than normal. The single cluster of forebrain GABA-immunoreactive cells appear to be located in the telencephalon. (B) Anti-TH labels no cells in the *cyclops* brain. *Abbreviation:* E = eye. Scale bar = 100 μm.

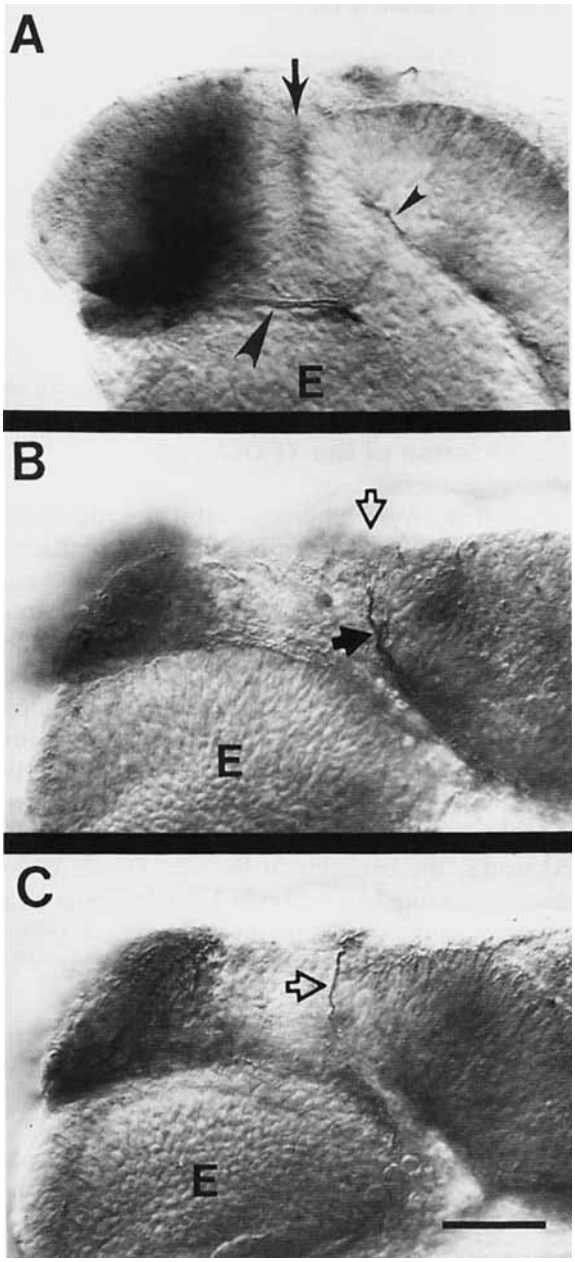

Figure 5 The scaffold of axonal tracts labeled with the antibody against acetylated α-tubulin is perturbed in the brains of *cyclops* embryos (26 h). (A) Axons (large arrowhead) from the anterior forebrain (see text) run posteriorly but form a commissure at the abnormal crease (arrow) found in the posterior forebrain of a *cyclops* embryo. Consequently, no TPOC axons extend into the anterior tegmentum in *cyclops* embryos. The posteriorly running portions of the axons of the epiphysial neurons and some MLF axons (small arrowhead) can be seen posterior to the crease. (B) The PC is missing but nuc PC axons are present and run ventrally (closed arrow) in another *cyclops* embryo. Open arrow denotes the lack of axons in the normal location of the PC. (C) The axons of the epiphyseal neurons are present in the same *cyclops* embryo as in (B) and run ventrally (open arrow) anterior to the nuc PC axons. *Abbreviation:* E = eye. Scale bar = 50 μm.

axons projected ventrally into the anterior tegmentum [Fig. 5(B)]. Since axons extended correctly, the PC axons are not required by nuc PC growth cones for this portion of their pathway.

What do nuc PC growth cones do once they arrive at the anterior tegmentum in *cyclops* embryos? In the acetylated α-tubulin-labeled embryos, it was often difficult to unambiguously determine the trajectory of nuc PC axons once at the anterior tegmentum since their axons were obscured by the epiphysial axons that extended abnormally (see above) and/or the MLF axons. Therefore, to determine the trajectories of nuc PC axons within the anterior tegmentum, nuc PC axons and growth cones were labeled by orthograde application of DiI and all the other axons were labeled with the antibody against acetylated α-tubulin (n = 30 embryos). In the doubled-labeled embryos, the DiI-labeled axons were marked with a brown reaction product and the acetylated α-tubulin-labeled axons marked with a blue or blue-violet reaction product (see Methods for details). The acetylated α-tubulin antibody not only allowed us to determine what tracts nuc PC growth cones extended in, but also confirmed the absence of the PC and the TPOC in every embryo used for this analysis.

The double-labeled embryos confirmed the observations made in the acetylated α-tubulin-labeled *cyclops* embryos. All of the 61 nuc PC axons first extended ventrally into the anterior tegmentum (Fig. 8). Once at the anterior tegmentum, however, nuc PC growth cones often made errors. Interestingly all axons eventually turned in the correct direction (posterior), but 52% of the growth cones incorrectly extended to the nuc MLF or followed the MLF while 48% followed an apparently normal dorsolateral tegmental pathway (dorsal to the MLF). Which longitudinal pathway a nuc PC growth cone followed was apparently independent of the pathway another growth cone followed in the same embryo. The longitudinal pathways followed by nuc PC growth cones within the same embryo were not correlated. Of 21 embryos where at least two nuc PC growth cones were assayed, growth cones followed only their normal pathway in 3 embryos, only the MLF in 6 embryos, and either their normal pathway or the MLF in 12 embryos. Furthermore, the nuc PC axons were

Table 2 Comparison of the Number of GABA-Immunoreactive and nuc MLF Neurons in the Midbrains of Wild-type and *Cyclops* Embryos at 26 h PF

	Wild-type	*Cyclops*
Anterior tegmental, GABA-immunoreactive neurons	12.0 (10–13) $n = 15$	2.6 (2–3) $n = 10$
Neurons in the bilateral pair of nuc MLFs	14.6 (12–18) $n = 10$	10.4 (9–13) $n = 10$

Note. For each category, the mean is followed by the range in parentheses.

more loosely fasciculated when extending longitudinally compared to nuc PC axons in wild-type embryos (not shown). These observations suggest that the TPOC axons are required to insure that nearly all nuc PC growth cones follow their correct longitudinal pathway.

DISCUSSION

The *Cyclops* Brain Contains an Aberrant Axonal Scaffold

A preliminary report demonstrated that *cyclops* embryos are missing the ventral-most region of the brain including ventral groups of neurons and longitudinal axonal tracts (Hatta et al., 1992a). Our findings confirm these findings and have identified the ventral neurons that are deleted and characterized the abnormal scaffold in *cyclops* embryos. In the *cyclops* midbrain the clusters of neurons that establish the PC are greatly diminished or are totally missing and, therefore, *cyclops* embryos have no PC. In the forebrain a single cluster of neurons as opposed to two separate clusters (telencephalic and nuc TPOC) in wild-type embryos are labeled by anti-GABA. Although we cannot unambiguously determine the identity of these GABA-immunoreactive neurons in *cyclops,* it seems likely that they represent the telencephalic cluster rather than the more ventral nuc TPOC since many ventral cells including the TH-immunoreactive nuc TPOC are missing in *cyclops* embryos. Regardless of the identity of these GABA-immunoreactive neurons, anterior forebrain axons fail to invade the anterior tegmentum probably because of the aberrant crease. Since the crease is evident at 16 h when TPOC growth cones are normally first projected, it is likely that potential TPOC growth cones never reach the anterior tegmentum. Importantly, these changes in the axonal scaffold deprive nuc PC growth cones of both tracts of axons they normally follow in the brain (Chitnis and Kuwada, 1990).

Nuc PC Growth Cones Utilize Different Combinations of Guidance Cues in Different Portions of Their Pathway

The pathways followed by nuc PC growth cones within the aberrant scaffold of the *cyclops* brain corroborate previous findings following surgical prevention of the TPOC from the anterior tegmentum in wild-type embryos (Chitnis and Kuwada, 1991). Furthermore, they suggest that these growth cones rely on axonal tracts for proper pathfinding to different degrees along different portions of their trajectory.

First, the PC axons seem not to play an important role in guiding nuc PC growth cones to the anterior tegmentum since they extended ventrally into the anterior tegmentum despite the absence of the PC in *cyclops* embryos. This suggests that cues independent of the PC axons are sufficient to guide these growth cones to the anterior tegmentum. This does not mean that the PC does not normally participate in guiding nuc PC growth cones. Since we did not assess the dynamic behavior of growth cones, it's possible that nuc PC growth cones may extend faster or with less exploration in the presence of the PC. This possibility can be assessed by time-lapse analysis of labeled growth cones in wild-type and *cyclops* embryos.

Second, once at the anterior tegmentum, half of the nuc PC growth cones aberrantly follow the MLF, while the rest follow an apparently normal, dorsolateral longitudinal pathway in the absence of the TPOC. The variable trajectories of nuc PC axons may be due to variability in the deletion of ventral tegmental cells in *cyclops* embryos. We disfavor this possibility for two reasons. First, surgical elimination of the TPOC in wild-type embryos produce results comparable to those seen in *cyclops* embryos (Chitnis and Kuwada, 1991). In these cases the variability of nuc PC trajectories cannot be due to variable damage to the tegmentum. The TPOC was prevented from invading the midbrain by cutting the embryo in the diencephalon; the tegmentum was not damaged. Second, the behavior of the nuc PC growth cones in the same embryo were not correlated. If variability of the *cyclops* phenotype were responsible for the variable pathways taken by nuc PC growth cones, then one might have expected to see all growth cones follow either a normal or abnormal pathway in the same embryo. Instead, the finding that nuc PC growth

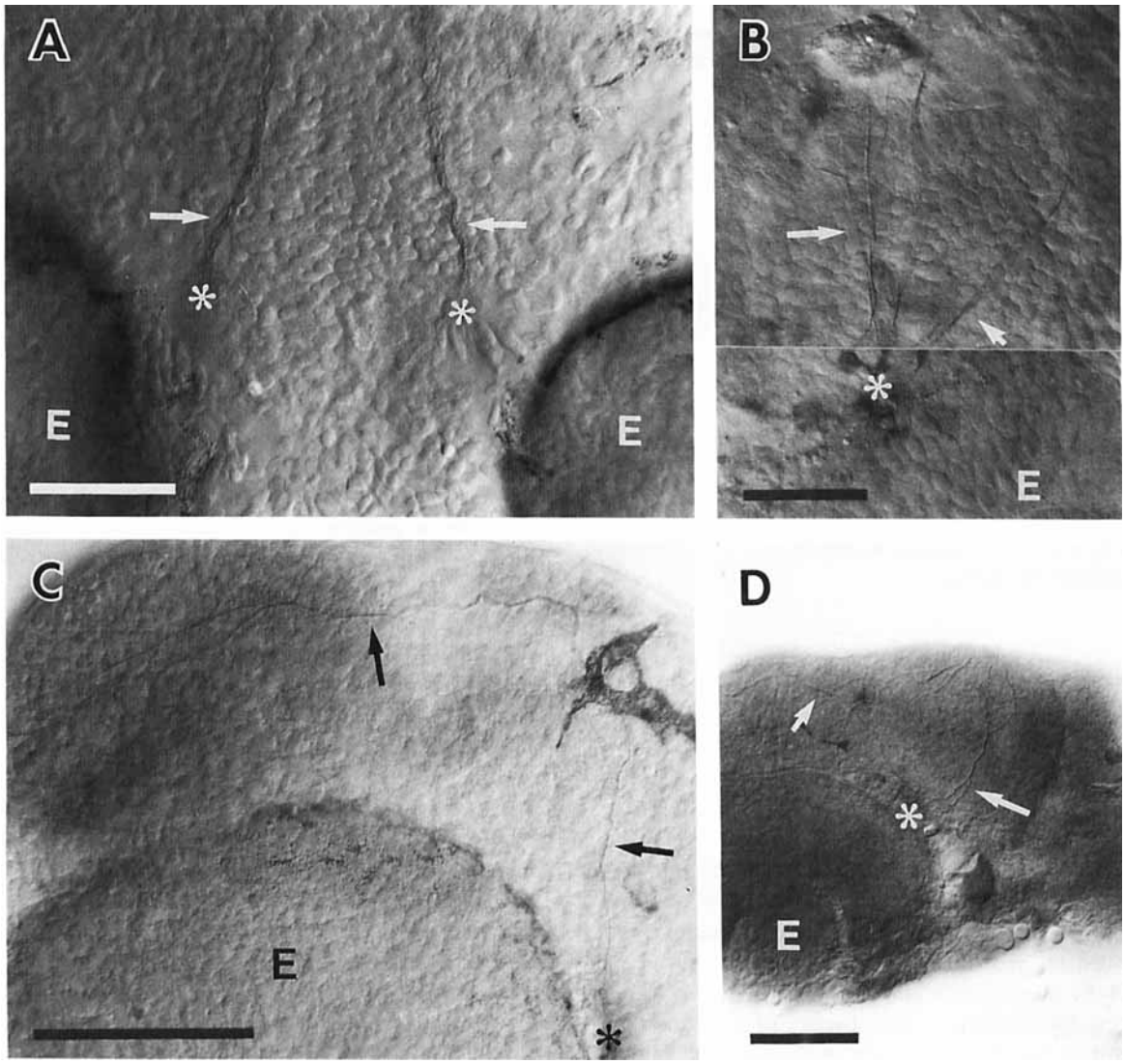

Figure 6 The normally bilaterally paired nuc MLF are fused, and their axons follow a variety of pathways in *cyclops* embryos. In all panels embryos were labeled with MAb CON1. (A) Ventral view of the midbrain showing the bilaterally paired nuc MLF (asterisks) and the tightly fasciculated MLF axons (arrows) in a 28-h wild-type embryo. In all panels, (E) denotes the eye; in panels (A) and (B), posterior is up. Scale bar = 50 μm. (B) Ventral view of the midbrain showing the fused nuc MLF (asterisk) and the poorly fasciculated MLF axons in a 28-h *cyclops* embryo. Note that several of the MLF axons extend posteriorly (long arrow), and one axon extends posteriorly and laterally (short arrow) in the tegmentum. Scale bar = 50 μm. (C) Side view of forebrain and anterior midbrain showing nuc MLF axons (arrows) running dorsally, then anteriorly along the forebrain in a 28-h *cyclops* embryo. In panels (C) and (D), anterior is to the left and dorsal is up, and asterisk marks the location of the fused nuc MLF. Scale bar = 50 μm. (D) Side view of the diencephalon and midbrain showing nuc MLF axons running dorsally (long arrow) into the tectum and dorsally and anteriorly (short arrow) into the diencephalon in a 28-h *cyclops* embryo. Scale bar = 50 μm.

cones greatly increase their error rate in the anterior tegmentum both in *cyclops* embryos and following surgical elimination of the TPOC in wild-type embryos suggests that the TPOC axons do participate in guiding nuc PC growth cones at the intersection.

The ability of nuc PC growth cones to follow the MLF further suggests that they are not normally

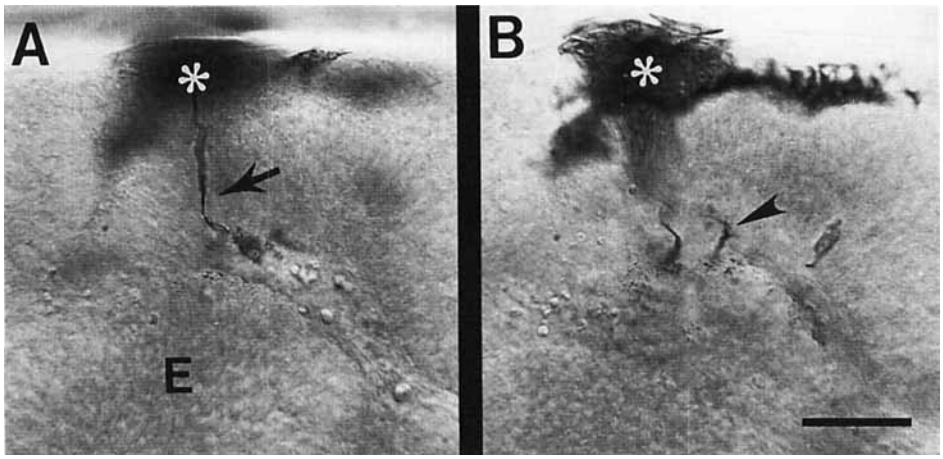

Figure 7 Epiphysial growth cones extend ventrally then turn posteriorly (A) or cross over to the contralateral side (B) at the site of the abnormal crease in *cyclops* embryos. Epiphysial axons and growth cones were labeled with DiI by injection of it to the epiphysis (asterisk) and subsequently marked with a dab reaction product by photooxidation. (A) Sideview of the diencephalon and anterior midbrain showing ventrally, then posteriorly running epiphysial axons (arrow) in a 27-h embryo. (B) Sideview of the diencephalon and anterior midbrain showing an epiphysial growth cone (arrowhead) that had crossed over from the other (contralateral) side of the brain in another 27-h embryo. A long filopodium can be seen extending anteriorly from the growth cone. The labeled axon anterior to the growth cone originated from the ipsilateral side and crossed over to the contralateral side. *Abbreviation:* E = eye. Scale bar = 50 μm.

inhibited from extending on the MLF. If this is correct, then specific attractive cues associated with the TPOC tract would be required to guide nuc PC growth cones at the intersection. On the other hand, since a significant proportion of the nuc PC growth cones followed an apparently normal pathway in the absence of the TPOC, additional cues not associated with the TPOC axons may also be able to guide nuc PC growth cones. Since these growth cones extend upon the endfeet of dorsolateral neuroepithelial cells, a longitudinal strip of endfeet may be a second source of guidance cues (Chitnis et al., 1992). Thus a combination of axonal and nonaxonal cues may work together to insure that nuc PC growth cones get off the PC and turn onto their normal pathway in the anterior tegmentum. Combinations of cues may act simultaneously to guide growth cones in the spinal cord of zebrafish embryos (Bernhardt et al., 1992a,b; Greenspoon et al., 1993) and in the nervous systems of other embryos as well (e.g., Schubiger and Palka, 1985; Tomaselli et al., 1988; Harrelson and Goodman, 1988; Elkins et al., 1990; Hedgecock et al., 1990).

Third, once on the correct longitudinal pathway, the TPOC axons act to keep the nuc PC growth cones from straying from their longitudinal pathway at a second intersection of tracts located in the anterior hindbrain (Chitnis et al., 1992). Normally, nuc PC growth cones extend through this second intersection of tracts with an occasional growth cone transiently turning dorsally onto axons running orthogonal to the TPOC. In the absence of the TPOC, those nuc PC growth cones that follow their normal longitudinal pathway through the tegmentum now greatly increase the probability that they will turn dorsally at this second intersection.

Does a Direct Effect of the Mutation or Changes in the Fate of Prospective nuc PC Neurons Cause Growth Cones to Follow Aberrant Pathways?

Could the variable behavior of the nuc PC growth cones in *cyclops* be due to a direct effect of the mutation on the nuc PC neurons rather than to the elimination of the PC and TPOC? A proper test of this possibility would require the generation of mosaic embryos in which nuc PC cells from *cyclops* embryos are orthotopically transplanted into wild-type embryos and vice versa. While these transplant experiments have so far not been done, several lines of reasoning argue against a direct effect

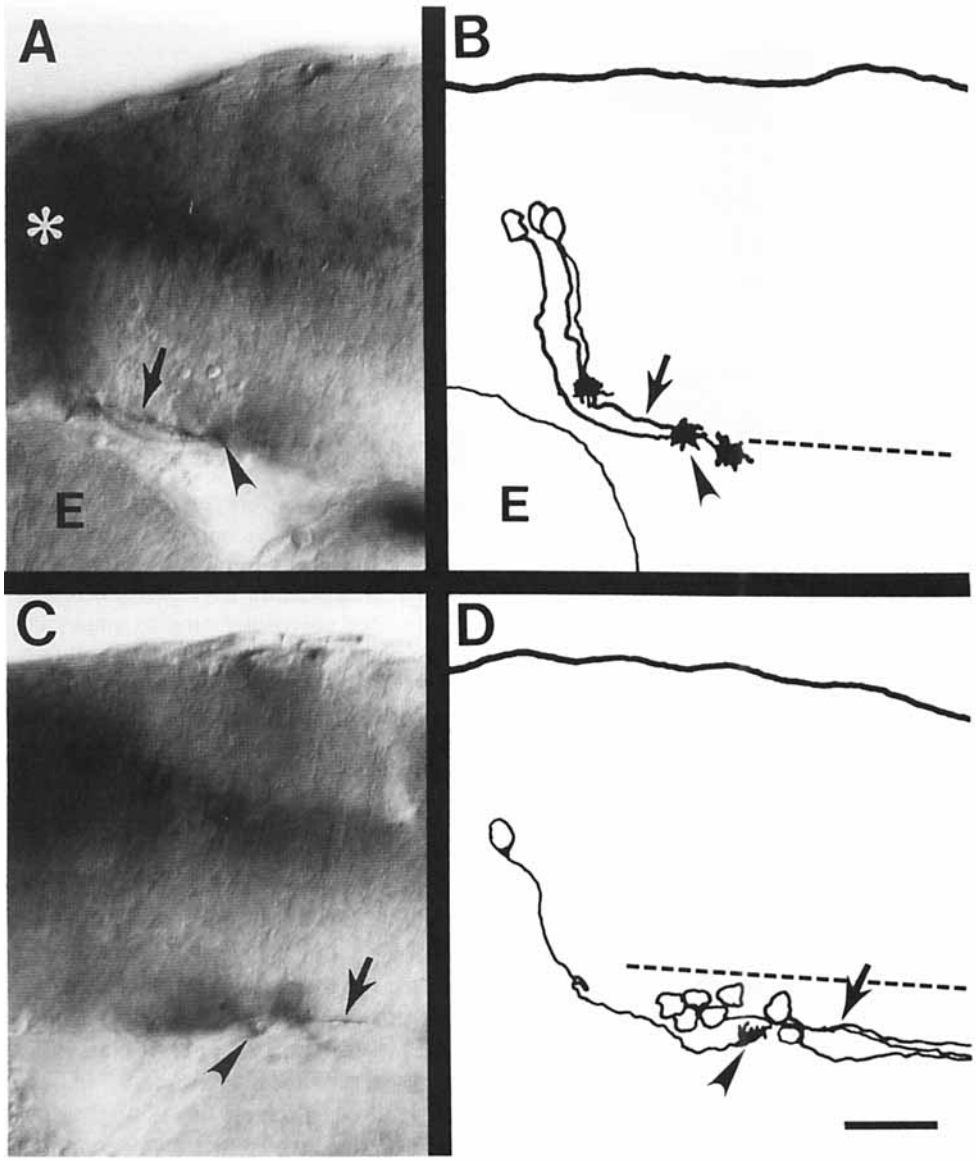

Figure 8 Nuc PC growth cones extend ventrally, then either follow an apparently normal longitudinal pathway in the dorsolateral tegmentum (A and B) or aberrantly extend to and follow some of the posteriorly running MLF axons (C and D) in the same *cyclops* embryo. Photomicrographs (A and C) and corresponding camera lucida drawings (B and D) show photooxidized, DiI-labeled nuc PC axons and growth cones in a 27-h *cyclops* embryo that had all other axons labeled with the antibody against acetylated α-tubulin. In this embryo four nuc PC growth cones were labeled with two following an apparently normal longitudinal pathway (A and B), one following the MLF (C and D), and one at the anterior tegmentum (A and B). In such embryos, DiI-labeled axons and growth cones that were brown could clearly be distinguished from the blue/black, acetylated α-tubulin-labeled axons (see Methods). Camera lucida drawings of the corresponding photomicrographs are shown because the axons and growth cones were at various planes of focus. In all panels sideviews of the anterior midbrain are shown. (A) Photomicrograph taken from a focal plane through the dorsolateral tegmentum showing an axon (arrow) from one nuc PC neuron and a growth cone (arrowhead) from another nuc PC neuron following an apparently normal pathway. *DiI injection site. *Abbreviation:* E = eye. (B) Camera lucida drawing of the axon and growth cone of the two nuc PC neurons shown in (A). Another nuc PC growth cone that had just reached the anterior tegmentum is also shown. The arrow and arrowhead denote the same axon and growth cone denoted in (A). Dashed line denotes approximately the normal position of the TPOC. (C) Photomicro-

of the mutation. First, nuc PC growth cones extend correctly along the ventral portion of their pathway 100% of the time in *cyclops* embryos. Second, surgical elimination of the TPOC in wild-type embryos is sufficient to phenocopy nuc PC trajectories in *cyclops* embryos. Furthermore, in the spinal cord the *cyclops* mutation appears to specifically delete the floor plate cells and generally not affect the fates of spinal neurons (Hatta et al., 1991; Bernhardt et al., 1992a). Spinal growth cones that normally make turns at the floor plate now err in the absence of the floor plate in *cyclops* embryos, and laser ablation of the floor plate cells in wild-type embryos phenocopies the aberrant behavior of these spinal growth cones (Bernhardt et al., 1992a). These observations also argue against the possibility that prospective nuc PC neurons have axons with atypical trajectories, because they have changed their fate in *cyclops* embryos.

How Do nuc PC Growth Cones Know What Direction to Extend or Turn?

The existence of extrinsic cues that influence the direction that growth cones extend has been demonstrated by manipulating a variety of embryos in a number of different ways. For example, one of the classic demonstrations of directional cues involved the manipulation of Mauthner neurons in the hindbrain of amphibian embryos (Hibbard, 1965). Mauthner axons normally project posteriorly into the spinal cord. Portions of a donor hindbrain containing Mauthner cells can be grafted into the hindbrain of a host embryo with the graft having a reversed orientation along the anterior/posterior axis. In such cases, the grafted Mauthner axons initially extend according to the orientation of the graft, but reorient when they encounter host tissue. Similarly, when a graft containing a portion of the amphibian brain, which retinal axons normally traverse, is rotated, retinal axons will reorient (Harris, 1989). In the insect wing, imaginal disk directional information along the proximal/distal axis is associated with the wing epithelium (Nardi, 1983; Blair et al., 1987). Analysis of ectopic sensory neurons in the *Drosophila* wing indicate that the polarity cues to which sensory axons respond are strongest along the band of epithelium. This band serves as a preformed pathway and polarity cues fall off with distance from the band (Blair and Palka, 1989).

Directional cues can be widely distributed in the embryo. For example, directional cues can be supplied by long-distance cues that attract growth cones and are emitted by target or intermediate target tissue (Lumsden and Davies, 1986; Tessier-Lavigne et al., 1988; Bovolenta and Dodd, 1991; Heffner et al., 1990; Okamoto and Kuwada, 1991a,b; Yaginuma and Oppenheim, 1991). In these cases the directional cues are not restricted to the pathway followed by the receptive growth cones. Recently molecular genetic analysis of the nematode, *Caenorhabditis elegans,* suggests that polarity cues instruct growth cones to extend in a particular direction. Several mutations that affect the direction of extension of specific growth cones have been analyzed, and the corresponding genes also cloned and analyzed (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Ishii et al., 1992; Hamelin et al., 1993). These genes control extension of growth cones along the dorsal/ventral axis but not the anterior/posterior axis signifying the existence of separate cues for each axis. Furthermore, since the cells affected by mutations in these genes are widely distributed in the nematode, the directionality cues are likely to be widely distributed.

What is the nature of the directional cues in the zebrafish brain? It is unlikely that nuc PC growth cones were unable to extend dorsally due to a physical barrier in *cyclops* since axons can extend dorsally in the *cyclops* brain. Aberrant MLF axons extended dorsally within the superficial margin of the midbrain including sites near the normal location of the PC. Similarly, it is unlikely that a physical barrier prevents nuc PC axons from extending anteriorly once in the anterior tegmentum of *cyclops* embryos. Since epiphysial axons sometimes extended posteriorly into the anterior tegmentum from the diencephalon, axons are not prevented

graph taken from a focal plane near the midline of the tegmentum of the same embryo shown in (A) showing a nuc PC growth cone (arrowhead) extending along the fused nuc MLF and some of their posteriorly extending axons (arrow). (D) Camera lucida drawing of this nuc PC neuron and the nuc MLF neurons and their axons. The arrow and arrowhead denote the same axon and growth cone denoted in (C). Note that the axon made a loop near the site nuc PC growth cones would have normally turned posteriorly onto the TPOC (dashed line). Scale bar for all panels = 25 μm.

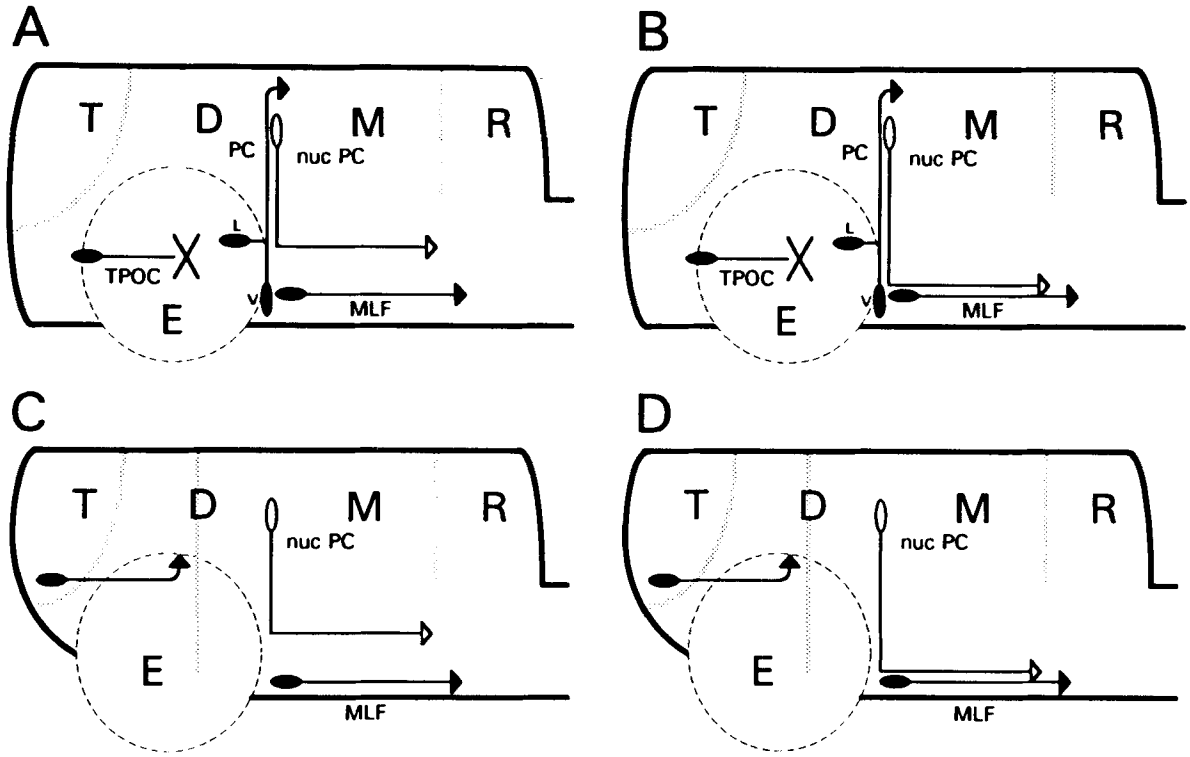

Figure 9 A schematic diagram illustrating that nuc PC axons either follow their normal (A and C) or an abnormal (usually the MLF; B and D) pathway following surgical prevention of TPOC axons from the anterior tegmentum in wild-type embryos (A and B) and in *cyclops* embryos (C and D). Arrowhead in C and D denotes the abnormal crease that appears to prevent axons from the anterior forebrain from extending into the tegmentum in *cyclops* embryos. For clarity, only the posteriorly projecting MLF axons are illustrated in (C) and (D). Abbreviations are as previously noted.

from extending through the region of the brain just anterior to the anterior tegmentum. It also seems unlikely that a chemotropic factor secreted by cells at or near the ventral midline that is deleted in *cyclops* embryos is required for extension by these growth cones toward the ventral brain. Our observations do not rule out the secretion of an attractive factor from ventral cells that are spared in *cyclops* embryos nor an inhibitory factor secreted by cells at or near the dorsal midline.

Are axons an important source of directionality cues in the zebrafish brain? In *cyclops* embryos, nuc PC axons extended ventrally then posteriorly in all cases examined despite the lack of the tracts (PC and TPOC) they normally follow (Fig. 9). Likewise, the overwhelming majority of nuc PC growth cones extended posteriorly through the tegmentum following surgical elimination of the TPOC from the tegmentum (Chitnis et al., 1992). These observations suggest that these two tracts are not an important source of directionality cues. Instead cues independent of these two tracts are sufficient to provide directionality information to nuc PC growth cones along both the dorsal/ventral and the anterior/posterior axes. One potential source of cues are the endfeet of neuroepithelial cells (Chitnis et al., 1992).

Given that directional cues can be widely distributed, do growth cones in the zebrafish brain also utilize widely distributed directionality cues? Our results are consistent with this possibility. The fact that nuc PC growth cones extend posteriorly even when following an aberrant tract (MLF) suggests the possibility that directional cues are not limited to the appropriate pathway. In fact, recent cell transplantation experiments in the zebrafish brain are consistent with this possibility (Kanki et al., 1992). Ectopic epiphysial neurons project growth cones that nearly always extend ventrally as do normal epiphysial neurons. This occurs even when epiphysial neurons are transplanted onto or near the host PC that contains numerous axons extending dorsally. One explanation for these results is that epiphysial growth cones sense a widely avail-

able cue that is distributed as a gradient along an axis. Our observations in the zebrafish brain along with those of others in a variety of embryos suggest that two separate types of cues guide growth cones. Growth cones may reach their targets by sensing a combination of spatially restricted specific pathway cues that are devoid of directional information and more globally distributed directionality cues.

The authors thank R. Bernhardt, A. Chitnis, and S. Wilson for discussions and comments on the manuscript, R. Bernhardt for assistance with some of the immunohistology, P. Knafl for technical assistance, and D. Bay for photography. This research was supported by grants from NIH and MOD.

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