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# Midline signaling in the primordium of the zebrafish anterior central nervous system

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ABSTRACT **In all vertebrates the brain develops from the enlarged anterior part of the neural plate. However, in the zebrafish mutant *cyclops*, the girth of the central nervous system (CNS) is nearly uniform along its length. Changes in expression patterns of homeobox genes and neuronal markers reveal a massive deletion of the ventral forebrain, particularly the diencephalon, as well as its precursor region in the neural plate. The deletion is due to a nonautonomous action of the mutation: very few wild-type cells transplanted to the midline of a mutant embryo can rescue the forebrain phenotype, including cyclopia. Establishment of forebrain ventral positional coordinates may thus require inductive signaling by forebrain midline cells whose specification depends upon the *cyclops* gene product.**

The developing floor plate of the central nervous system (CNS) ventral midline may participate in a signaling cascade that establishes dorsoventral patterning of the neural primordium. Floor-plate specification may require signals from underlying axial mesoderm (1–3), and, in turn, the floor plate may signal development of surrounding ventral CNS structures (4–6). However, a floor plate is not recognized in the anteriormost part of the CNS, the forebrain, where patterning is the most complex and least understood. Here we report that the zebrafish mutation *cyclops* serves to connect patterning in the forebrain with that along the rest of the neuraxis.

*cyclops* autonomously deletes the floor plate (7). In the spinal cord, the deletion is confined to the floor plate, a one-cell wide median row of cells, and to a subset of cells located immediately to either side of this midline row (8). The phenotype is more severe in the anterior hindbrain and in the midbrain, where the deletion involves not only the midline but also substantial numbers of ventral CNS neurons (9). The forebrain phenotype is the most severe (7). The paired lateral eyes, deriving from the diencephalic walls, fuse together in the ventral midline in *cyclops* mutants, and the overall volume of the forebrain is markedly reduced. We now show that, like the more posterior deficiencies, the forebrain deficiencies are largely ventral ones. Moreover, mosaic analysis reveals that, except for cells in the CNS midline, the missing territory is due to a nonautonomous action of the mutation.

## MATERIALS AND METHODS

**Histology and Microscopy.** Immunohistology and *in situ* hybridization procedures were as described (9–11). Frozen sections (16 μm) were taken after *in situ* hybridization reactions in whole mounts. For tracing axons, a Bio-Rad MRC-600 confocal laser microscope was used to collect successive images (81 for Fig. 2*C* or 45 for Fig. 2*D*) of immunolabeled wholemounts at 1.3-μm intervals, and the reconstructions were made as stereopairs by using Micro Voxel software.

**Mosaic Analysis.** Cells from wild-type donors entirely labeled with lineage tracer dye were transplanted into the embryonic shields of 5- to 6-hr host embryos obtained by crossing *cyclops* heterozygotes, and results were analyzed at 26 hr (7, 9). One-fourth of the hosts were expected to be mutant, and in cases of complete forebrain and eye rescue, hosts could still be identified as being mutant by the missing floor plate and disturbed axonal pathways in the posterior CNS (7, 9).

## RESULTS

Beginning near the end of gastrulation, the homeobox gene *pax6* is expressed within the forebrain primordium (10, 12, 13) and provides a useful marker for learning how *cyclops* perturbs forebrain patterning (Fig. 1 *A* and *B*). In wild types, two bilateral domains of expression within the neural plate are separated by a midline area in which expression is absent. In *cyclops* mutants, the domains are fused together to encompass the midline.

The difference persists as the neural plate forms a tube and becomes regionalized along the neuraxis. The wild-type *pax6* expression domains (Fig. 1*C*) now include the eyes and the dorsolateral forebrain walls; the midline nonexpressing area is now positioned ventrally. In *cyclops* (Fig. 1*D*), two partial retinal rudiments develop, and although they are shifted ventrally and fused medially, they express *pax6* as usual. The ventral forebrain region that normally separates the two eyes never develops. The missing ventral region at this stage corresponds to the missing medial region at the neural plate stage. In contrast to this prominent dorsoventral change in *pax6* expression, the anterior and posterior boundaries of expression in the CNS seem unchanged in *cyclops*.

We observed (Fig. 1 *E* and *F*) a similar ventral midline fusion at the optic stalk of the normally bilateral expression domains of the related gene *pax2* (11, 12, 14). As for *pax6*, the extent of *pax2* expression along the anterioposterior axis seems unaffected by the mutation.

These findings suggest that *cyclops* alters the forebrain fate map, deleting ventral fates that normally derive from the midline region of the neural plate and, presumably as a consequence, shifting dorsal fates to a more ventral position. The ventral deletion largely or entirely accounts for the marked overall reduction in the volume of the *cyclops* forebrain.

Study of groupings of neurons and their axonal pathways supports the interpretation that the deletion is specific to ventral tissue and furthermore suggests that, along the neuraxis, *cyclops* most severely effects the diencephalon (Fig. 2). Normally (17, 18), bilaterally paired large neuronal clusters are present in the telencephalon and ventral diencephalon (Fig. 2*E*). *cyclops* deletes the ventral diencephalic

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Abbreviation: CNS, central nervous system.

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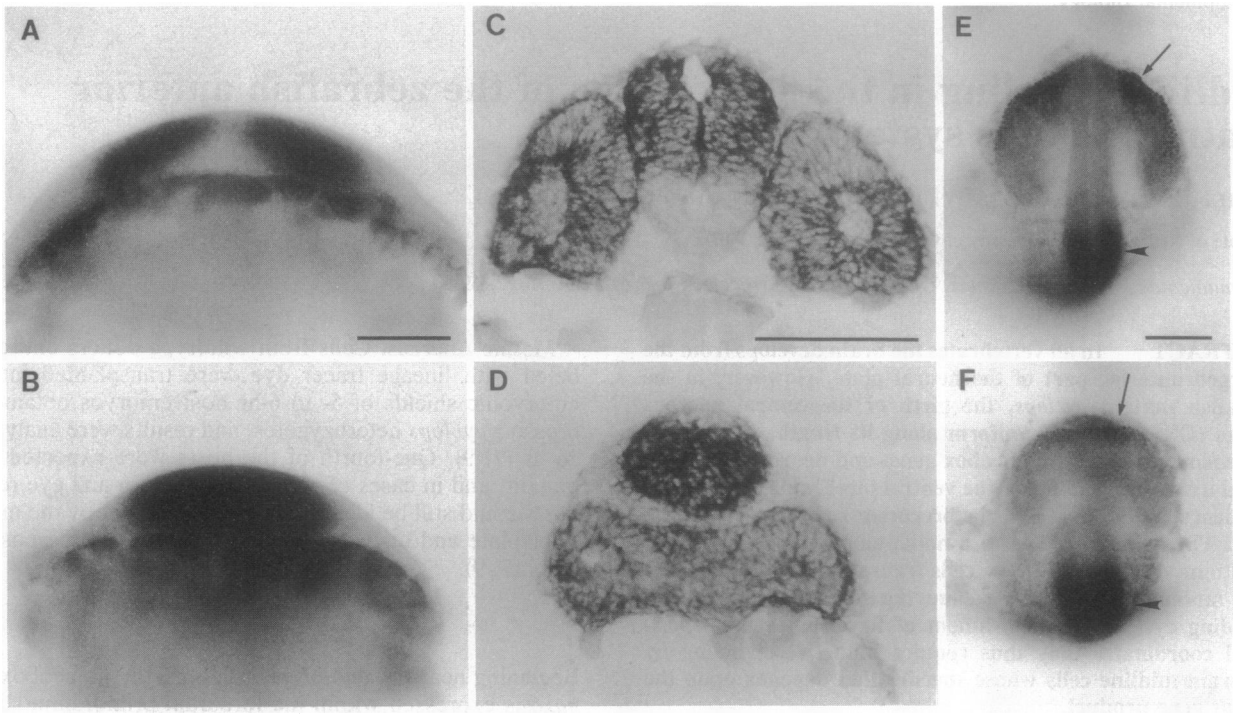


FIG. 1. *cyclops* alters the patterns of *pax6* (*A–D*; *in situ* hybridization) and *pax2* (*E* and *F*; immunolabeling) expression in the forebrain. (*Upper*) Wild-type embryos. (*Lower*) Matching homozygous *cyclops* mutants ($cyc^{b16}$; ref. 7). (*A*) Frontal view of whole mount at 11 hr postfertilization at 28.5°C showing the forebrain region of the wild-type neural plate. Two bilateral domains of *pax6* expression are separated by a median area free of expression. (*B*) In *cyclops*, a single domain spans the midline. (*C*) Transverse section at 24 hr of the wild-type forebrain and eyes. *pax6* expression is present in the retinas and along about two-thirds of the dorsolateral walls of the diencephalon. Expression is absent in a large ventral region and in the roof plate. (*D*) In *cyclops*, the area normally devoid of *pax6* in the ventral diencephalon is entirely missing, and the retinas are fused under the diencephalon. The roof plate remains free of expression. (*E*) Dorsal view of the head of a wild-type whole mount at 18 hr immunolabeled with anti-Pax2 antibody. Anterior brain is at the top. Cell nuclei are labeled in bilateral patches around the anterior edges of the optic stalks (arrow) and in a band at the presumptive midbrain–hindbrain junction area (arrowhead). (*F*) In *cyclops*, the two domains of labeling in the optic stalks are weaker and are fused ventromedially (arrow). There is no obvious change in labeling at the midbrain–hindbrain junction (arrowhead). (Bar = 100 μm.)

clusters but not the telencephalic clusters or an unpaired dorsal diencephalic cluster (Fig. 2 *A* and *B*). As reported previously (9), some neurons of the ventral midbrain are still present in mutants, although they form a single midline cluster rather than a bilateral pair. Only in the ventral diencephalon are neuronal groups altogether missing.

In a corresponding fashion, *cyclops* most severely disrupts axonal pathways of the ventral diencephalon (Fig. 2 *C* and *D*). The mutation shortens but does not otherwise disturb the telencephalic anterior commissure. The telencephalon and diencephalon normally communicate by the supraoptic tracts, and in *cyclops* these tracts are either absent or they form an abnormal looping commissure. The ventral diencephalic postoptic commissure and the tracts that normally connect the ventral diencephalon and midbrain appear to be completely eliminated. Pathways originating in the ventral midbrain, the posterior commissure, and the medial longitudinal fascicle are only variably absent, and the tract of the postoptic commissure, in part originating from a dorsal midbrain nucleus (NPC), is always present.

We used mosaic analysis to learn to what extent the massive deletion of ventral forebrain is due to the direct

Table 1. Wild-type (wt) cells rescue the *cyclops* brain and eye

| Location of transplanted wt cells | Total cases | Brain regions rescued | | | |
|---|---|---|---|---|---|
| | | None | Forebrain* | Forebrain* and midbrain | Midbrain |
| In midline | | | | | |
| Forebrain alone | 4 | 2 | 2 | 0 | 0 |
| Forebrain and periphery† | 7 | 0 | 4 | 3 | 0 |
| Midbrain alone | 3 | 0 | 0 | 1 | 2 |
| Midbrain and periphery† | 3 | 0 | 0 | 3 | 0 |
| Periphery alone† | 18 | 17 | 0 | 1 | 0 |
| Not in midline | | | | | |
| Forebrain‡ | 7 | 7 | 0 | 0 | 0 |
| Midbrain‡ | 21 | 21 | 0 | 0 | 0 |
| Periphery alone† | 7 | 7 | 0 | 0 | 0 |
| Total | 70 | 54 | 6 | 8 | 2 |

*In every case with forebrain rescue, the eye phenotype was also at least partially rescued.
†Periphery means outside of the CNS and includes prechordal plate and hatching gland.
‡In some cases labeled cells were also present in the head periphery.

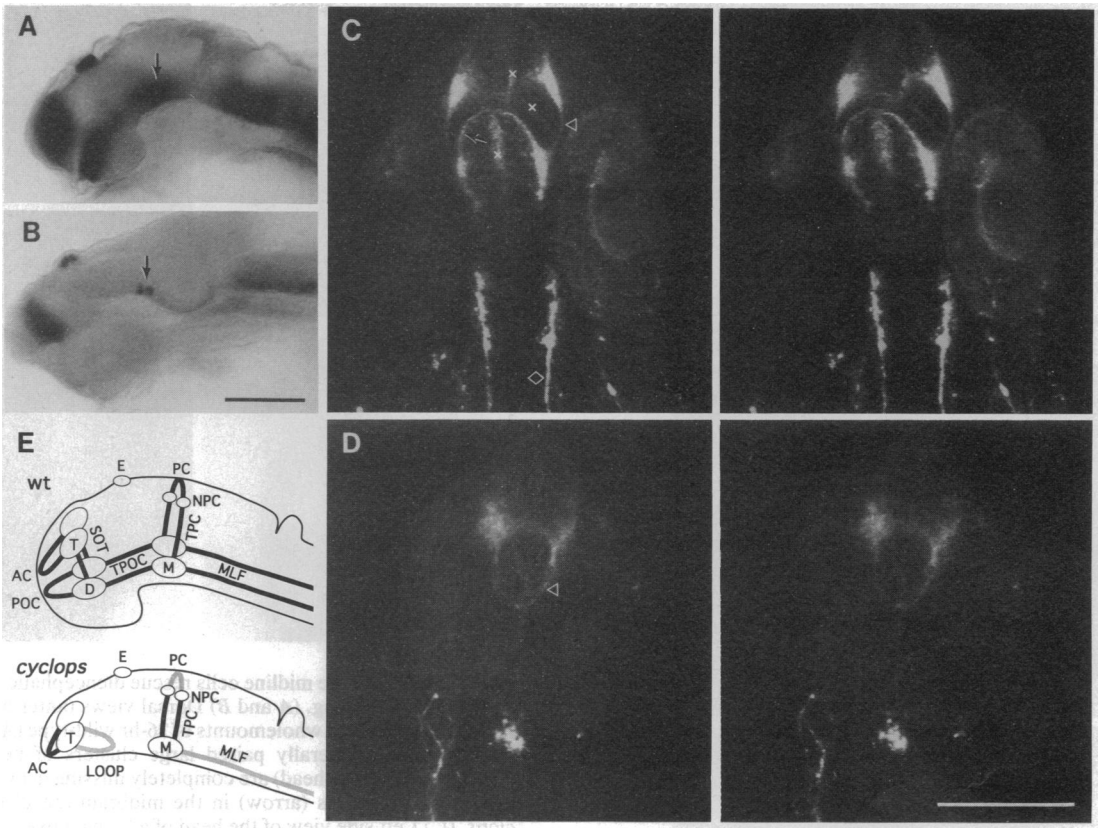


FIG. 2. *cyclops* alters neuronal and axonal organization in the anterior brain. Immunolabeled whole-mount preparations at 26 hr, with wild-type embryos above and mutants below. (*A* and *B*) Left side views (dorsal up) of whole-mounted wild-type embryo (*A*; eye removed by dissection) and mutant embryo (*B*; cyclopic eye present ventral to the forebrain) labeled with the monoclonal antibody zn-1, revealing neuronal cells (15). *cyclops* deletes the ventral diencephalic cluster and reduces the size of the midbrain cluster (arrows). The prominent telencephalic neuronal cluster (to the left) and the smaller epiphyseal cluster in the dorsal diencephalon (upper) are present in wild types and mutants alike. (*C* and *D*) Stereo-pair confocal microscopic views (from the ventral aspect, anterior brain at the top) of embryos labeled with the monoclonal antibody zn-12 (16) to show neuronal groups and axonal pathways. The postoptic commissure is prominent in the wild type (arrow in *C*) but is missing in the mutant (*D*), and the supraoptic tracts (arrowheads) connect together in an abnormal loop across the midline. In the wild type, the medial longitudinal fascicles form prominent posteriorly directed tracts (diamond) from paired midbrain neuronal groups, but these tracts are absent in this example of the mutant, and the midbrain neurons occupy a single midline cluster. x, Labeling of neuroepithelial cells, not axonal fascicles. (*E*) Left-side summary views of the neuronal and axonal deficiencies. Tracts that are variably present or absent in *cyclops* are indicated by gray lines. In *cyclops*, the anterior commissure (AC) is shortened, the supraoptic tracts (SOT) frequently (in 70% of the cases, $n = 24$) form an abnormal commissural loop posterior to the fused optic stalk. The posterior commissure (PC) was present in 40% of the mutants ($n = 24$). D, ventral diencephalic neuronal cluster; E, epiphyseal cluster; M, midbrain cluster; MLF, medial longitudinal fascicle; NPC, nucleus of the posterior commissure; POC, postoptic commissure; T, telencephalic cluster; TPC, tract of the posterior commissure; TPOC, tract of the postoptic commissure. (Bars = 100 $\mu$m.)

action of the *cyclops* mutation. Our previous studies suggested that in the more posterior CNS, the mutation appears to directly delete only the floor plate; the other aspects of the phenotype are nonautonomous, seemingly due to a missing signaling function that the floor plate normally provides (7, 9). In similar experiments we find that wild-type cells transplanted into mutant gastrulas can rescue the forebrain phenotype, including cyclopia (Fig. 3). The lateral eyes and forebrain volume are restored, and the patterning of neuronal somata and axonal pathways is wild type in appearance (Fig. 4). The rescued neurons are all genotypically mutant—that is, derived from the host embryo.

We investigated where wild-type cells need to be present to rescue the forebrain phenotypes (Table 1). Generally, the transplanted cells scatter and contribute to several tissues in the head, which makes critically addressing this issue difficult. In all of 14 cases in which the forebrain and eye phenotypes of mutant hosts were partially or completely restored to the wild-type condition, wild-type cells were present in head midline structures. Among these, wild-type cells occupied the ventral midline of the forebrain itself in nine mosaics. Two were of particular interest, because very few wild-type cells were present, and they were located exclusively in the ventral midline of the diencephalon. Rescue was exclusively of the forebrain. The midbrain phenotype was mutant.

The ability to rescue the forebrain may not be limited to the midline of the forebrain, for we observed rescue in five cases where wild-type cells were present in other midline locations (Table 1). The most effective of these (three cases) seems to be a combination of cells in the prechordal plate (just underneath the forebrain) and midbrain floor plate (just posterior to the forebrain). Neither of these locations is very effective alone. Wild-type cells occupying nonmidline positions never mediated rescue of any aspect of the *cyclops* phenotype.

## DISCUSSION

*cyclops* deletes ventral structures in the anterior brain, as it does in the posterior brain and spinal cord. A small number of wild-type cells transplanted into the midline of mutant hosts can recruit and pattern a large number of genetically mutant cells, rescuing the forebrain and eye phenotypes. We interpret these results, along with previous ones (7, 9), to

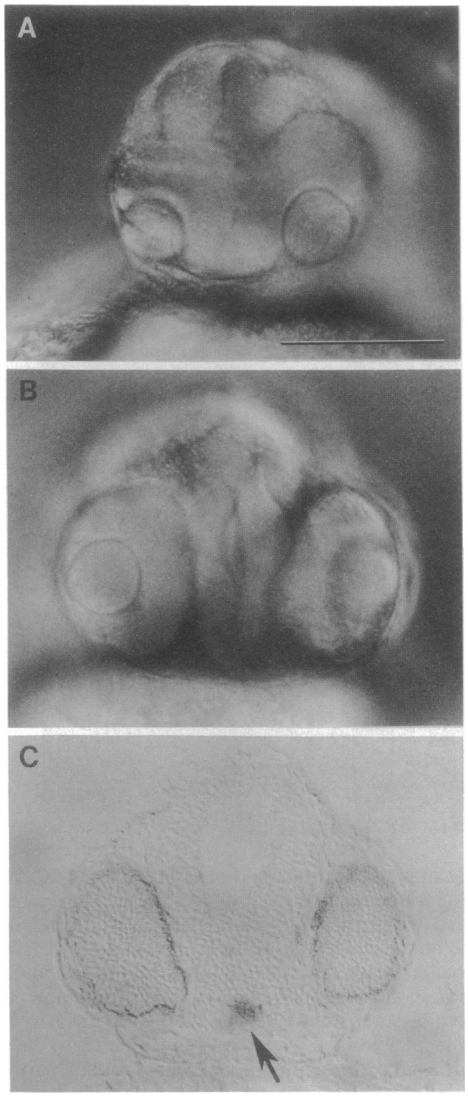


FIG. 3. Wild-type midline cells rescue cyclopia and forebrain morphogenesis in mosaics. (*A*) Face view of a mosaic embryo in which transplanted wild-type cells (not shown) were restricted to the trunk. The head is typically mutant in appearance. (*B*) Face view of a mosaic embryo in which the head morphology is essentially completely normal. However, the host was a *cyclops* mutant, as ascertained from the phenotype of the trunk and tail. A total of about 10 labeled wild-type cells (not shown) were present in the ventral midline of the forebrain and in the head periphery and hatching glands, which derive from the prechordal plate. (*C*) Transverse section through the head of another mosaic in which lateral eyes were restored (the retinal pigmented layer appears dark), and the forebrain was partially rescued by three or less biotin-labeled wild-type cells (arrow) in the midline of the ventral diencephalon. (Bar = 100 μm.)

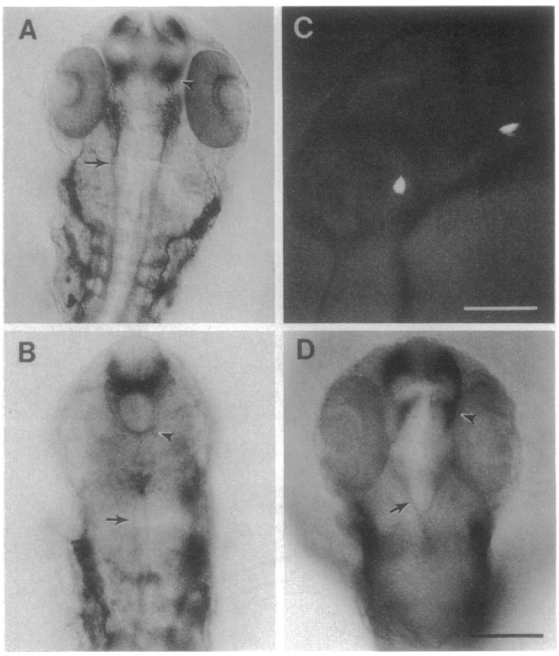


FIG. 4. Wild-type midline cells rescue diencephalic neurogenesis and axonal patterning. (*A* and *B*) Dorsal views (anterior brain at the top) of zn-12-labeled wholemounts of 26-hr wild-type (*A*) and *cyclops* (*B*). Normally bilaterally paired large clusters of neurons in the diencephalon (arrowhead) are completely missing in *cyclops*. Medial longitudinal fascicles (arrow) in the midbrain are disrupted in *cyclops*. (*C*) Left side view of the head of a living mosaic embryo, with two small clusters (*ca.* 2 cells each) of rhodamine-conjugated dextran-labeled transplanted wild-type cells. The cluster to the left is at the ventral midline of the diencephalon, just posterior to the eye; the other cluster is ventral to the anterior hindbrain. A few donor cells were also present in the hatching glands and lateral hindbrain but are not visible here. (*D*) The same mosaic embryo fixed and labeled with zn-12; the view is as in *A* and *B*. The eyes and forebrain and midbrain neuronal and axonal patterning are rescued, but not the hindbrain or spinal cord. Diencephalic neuronal clusters (arrowhead) are present; medial longitudinal fascicles (and their nuclei) are separated in the midbrain as in normal embryos but come together and fuse ventrally in the hindbrain as in mutants. (Bar = 100 μm.)

mean that the mutation directly and specifically deletes ventral midline cells at all anterior–posterior levels of the CNS and that the other changes result from a missing signal normally provided by the midline. In the forebrain, midline cells may be essential for establishing all ventral positional values, and their absence in *cyclops* can account for the dramatically altered forebrain fate map.

In the wild-type midbrain and more posterior CNS, midline signaling comes from the floor plate, but no floor plate is recognized in the wild-type forebrain (7). Nevertheless, cells at the forebrain midline seem to be equivalent to floor plate cells not only in their dorsoventral position but also in their requirement for the *cyclops*$^+$ gene product and in their ability to mediate local CNS rescue in mosaics. Forebrain midline cells sometimes accompany floor plate cells in transplantation experiments (data not shown), suggesting that the entire CNS ventral midline fate maps to a single region at or near the embryonic shield of the early gastrula. At present we know little more about these forebrain cells; finding a specific marker would allow us to determine their nature more clearly.

Along the entire neuraxis, *cyclops* most severely effects the diencephalon, apparently completely deleting its ventral components. In contrast, only the midline region of the spinal cord and posterior hindbrain is perturbed. The severity of the phenotype then seems to progressively increase through the anterior hindbrain and midbrain to the diencephalon. We propose there is a smooth, monotonic, posterior-to-anterior gradient of *cyclops* action within the brain (also see ref. 26).

The telencephalon is much less disturbed than either the diencephalon or midbrain, which would seem to be at odds with this proposal. However, fate mapping studies in avian and frog embryos suggest that the anterior end of the neural plate is diencephalic (19–21); developmentally the telencephalon would seem to be a dorsal, not an anterior, forebrain lobe. Studies of forebrain neurogenesis in zebrafish (22) and molecular expression patterns in mouse (23) support this assignment, as does the *cyclops* phenotype, since the mutation largely spares the telencephalon and all other dorsal CNS regions.

What accounts for the *cyclops* gradient? The defects in question are nonautonomous. It could be that during normal development, correct ventral specification in the posterior brain involves a combination of signals from both the floor plate and notochord. However, the forebrain seems never to be underlain by notochord (K.H., unpublished results), so that its role in forebrain signaling would be diminished and the role of the ventral CNS midline correspondingly expanded. By supposing that midline signaling and/or response is normally expanded in the anterior brain primordium, we can satisfactorily explain why *cyclops*, which appears to directly delete the midline cells along the entire CNS, most severely perturbs the forebrain.

A well-developed forebrain, forming lobes and lateral eyes, occurs in phylogenetically ancient ostracoderm fish (24) but not in our nearest invertebrate relative *Amphioxus*, which has only a tiny cerebral vesicle. Invention of neural crest might in part account for vertebrate cephalic expansion (25). Anterior expansion of a CNS ventral midline signaling system, involving cells specified by the *cyclops* gene and increasing the size and complexity of the brain, might well also have been a key step during early evolution of vertebrates.

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1. Holtfreter, J. (1934) *Arch. Exp. Zellforsch. Besonders Gewebezuechf.* **15,** 281–301.
2. von Straaten, H. W. M., Hekking, J. W. M., Wiertz-Hoessels, E. L., Thors, F. & Drukker, J. (1988) *Anat. Embryol.* **177,** 317–324.
3. Placzek, M., Tessier-Lavigne, M., Yamada, T., Jessell, T. & Dodd, J. (1990) *Science* **250,** 985–988.
4. Hirano, S., Fuse, S. & Sohal, G. S. (1991) *Science* **251,** 310–313.
5. Yamada, T., Placzec, M., Tanaka, H., Dodd, J. & Jessell, T. M. (1991) *Cell* **64,** 635–647.
6. Ericson, J., Thor, S., Edlund, T., Jessell, T. M. & Yamada, T. (1992) *Science* **256,** 1555–1560.
7. Hatta, K., Kimmel, C. B., Ho, R. K. & Walker, C. (1991) *Nature (London)* **350,** 339–341.
8. Bernhardt, R. R., Patel, C. K., Wilson, S. W. & Kuwada, J. Y. (1992) *J. Comp. Neurol.* **326,** 263–272.
9. Hatta, K. (1992) *Neuron* **9,** 629–642.
10. Püschel, A. W., Gruss, P. & Westerfield, M. (1992) *Development* **114,** 643–651.
11. Püschel, W., Westerfield, M. & Dressler, G. R. (1992) *Mech. Dev.* **38,** 197–208.
12. Krauss, S., Johansen, T., Korzh, V. & Fjose, A. (1991) *Nature (London)* **353,** 267–270.
13. Krauss, S., Johansen, T., Korzh, V., Moens, U., Ericson, J. U. & Fjose, A. (1991) *EMBO J.* **10,** 3609–3619.
14. Krauss, S., Johansen, T., Korzh, V. & Fjose, A. (1991) *Development* **113,** 1193–1206.
15. Hanneman, E., Trevarrow, B., Kimmel, C. B. & Westerfield, M. (1988) *Development* **103,** 49–58.
16. Metcalfe, W. K., Myers, P. Z., Trevarrow, B., Bass, M. B. & Kimmel, C. B. (1990) *Development* **110,** 491–504.
17. Wilson, S. W., Ross, L. S., Parett, T. & Easter, S. S., Jr. (1990) *Development* **108,** 121–145.
18. Chitnis, A. B. & Kuwada, J. Y. (1990) *J. Neurosci.* **10,** 1892–1905.
19. Puelles, L., Domenech-Ratto, G. & Martinez de la Torre, M. (1987) *J. Morphol.* **194,** 163–171.
20. Couly, G. & Le Douarin, N. M. (1988) *Development* **103,** Suppl., 101–113.
21. Eagleson, G. W. & Harris, W. A. (1990) *J. Neurobiol.* **21,** 427–440.
22. Ross, L. S., Parrett, T. & Easter, S. S., Jr. (1992) *J. Neurosci.* **12,** 467–482.
23. Bulfone, A., Puelles, L., Porteus, M. H., Frohman, M. A., Martin, G. R. & Rubenstein, J. L. R. (1993) *J. Neurosci.* **13,** 3155–3172.
24. Forey, P. & Janvier, P. (1993) *Nature (London)* **361,** 129–134.
25. Gans, C. & Northcutt, R. G. (1983) *Science* **220,** 268–274.
26. Hatta, K. & Kimmel, C. B. (1993) *Perspect. Dev. Neurobiol.* **1,** 257–268.