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Mutations affecting development of the midline and general body shape during zebrafish embryogenesis

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SUMMARY

Tissues of the dorsal midline of vertebrate embryos, such as notochord and floor plate, have been implicated in inductive interactions that pattern the neural tube and somites. In our screen for embryonic visible mutations in the zebrafish we found 113 mutations in more than 27 genes with altered body shape, often with additional defects in CNS development. We concentrated on a subgroup of mutations in ten genes (the midline-group) that cause defective development of the floor plate. By using floor plate markers, such as the signaling molecule sonic hedgehog, we show that the *schmalspur* (*sur*) gene is needed for early floor plate development, similar to *one-eyed-pinhead* (*oep*) and the previously described *cyclops* (*cyc*) gene. In contrast to *oep* and *cyc*, *sur* embryos show deletions of ventral CNS tissue restricted to the mid- and hindbrain, whereas the forebrain appears largely unaffected. In the underlying mesendodermal tissue of the head, *sur* is needed only for development of the posterior prechordal plate, whereas *oep* and *cyc* are required for both anterior and posterior prechordal plate development. Our analysis of *sur* mutants suggests that defects within the posterior prechordal plate may cause aberrant development of ventral CNS structures in the mid- and hindbrain. Later development of the floor plate is affected in mutant *chameleon*, *you-too*, *sonic-you*, *iguana*, *detour*, *schmalhans* and *monorail* embryos; these mutants often show additional defects in tissues that are known to depend on signals from notochord and floor plate. For example, *sur*, *con* and *yot* mutants show reduction of motor neurons; median deletions of brain tissue are seen in *sur*, *con* and *yot* embryos; and *cyc*, *con*, *yot*, *igu* and *dtr* mutants often show no or abnormal formation of the optic chiasm. We also find fusions of the ventral neurocranium for all midline mutants tested, which may reveal a hitherto unrecognized function of the midline in influencing differentiation of neural crest cells at their destination. As a working hypothesis, we propose that midline-group genes may act to maintain proper structure and inductive function of zebrafish midline tissues.

Key words: neurogenesis, neuronal development, regionalisation, body shape, floor plate, neurocranium, neural induction, zebrafish, *Danio rerio*

INTRODUCTION

Formation of the vertebrate neural plate, and generation of cell diversity within it, are thought to depend on patterning influences of the mesoderm. Mesendodermal tissue of the prechordal plate in the head and notochord in the trunk play a key role in these inductive events (reviewed by Nieuwkoop, 1989). Transplantations and ablation experiments of the notochord, principally in developing chick, frog and mouse embryos, showed that a contact-dependent signal passes from the notochord to the overlying neuroectoderm, where it induces the floor plate, a specialized stripe of large cuboidal cells in the ventral neural tube, and neighboring motor neurons (van Straaten et al., 1989; Yamada et al., 1991, 1993; Plazcek et al., 1993). These experiments showed that cell fate along the dorsal-ventral axis of the neural tube can be controlled by midline tissues, such as notochord and/or floor plate (reviewed by Plazcek et al., 1993; Ruiz i Altaba and Jessel, 1993; Smith, 1994). Similarly, notochord-derived signals also influence patterning of somites (Bumcrot and McMahon, 1995).

A prime candidate for the inductive signal is the secreted sonic hedgehog (shh) molecule, the product of one of several vertebrate relatives of the *Drosophila* segmentation gene *hedgehog*. Notochord and floor plate of several vertebrate

embryos, including zebrafish, express *shh* in a very similar way, suggesting that the inductive mechanism involving *shh* is highly conserved; moreover, *shh* is capable of inducing floor plate when ectopically expressed *in vivo* (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994). Different concentrations of an N-terminal proteolytic fragment of shh may mediate both the contact-dependent induction of floor plate, the contact-independent induction of motor neurons, and patterning of the somites, limbs and gut (Marti et al., 1995; Roelink et al., 1995; Roberts et al., 1995; reviewed by Fietz et al., 1994; Concordet and Ingham, 1995; Bumcrot and McMahon, 1995).

In zebrafish, the analysis of mutants provides further *in vivo* evidence for inductive properties of the notochord. Mutations in *floating head* (*flh*) inactivate the zebrafish homologue of the homeobox gene *Xnot*, which is expressed in the notochord, and cause absence of the notochord and strong reduction of the floor plate (Talbot et al., 1995; Halpern et al., 1995; Odenthal et al., 1996). In transplantation experiments into wild-type hosts, *flh* mutant cells are able to form floor plate, but not notochord. These experiments show that the floor plate defect in *flh* is due to the absence of notochord (Halpern et al., 1995). Similar experiments demonstrated that a subset of somite cells depend on a signal from the notochord for their formation (Halpern et al., 1993).

Once induced, the floor plate can guide commissural axons towards and away from the ventral midline of the nervous system. Many molecules are now known that are expressed in the floor plate, but data on their functional requirement in vivo are still sparse (reviewed by Colamarino and Tessier-Lavigne, 1995). Zebrafish embryos mutant for the *cyclops* gene (*cyc*) lack a floor plate and ventral forebrain tissue, and show anteriorly fused eyes (cyclopia; Hatta et al., 1991). Expression of *shh* and of the zebrafish HNF-3β homologue, *axial*, are absent in the ventral neural tube, but present in the notochord of *cyclops* embryos (Krauss et al., 1993; Strähle et al., 1993). Disorganization of axons near the floor plate, abnormal trajectories of commissural inter neurons and cyclopia are likely to be the consequences of the observed midline defect in *cyc* mutants (Hatta, 1992; Bernhardt et al., 1992; Allende and Weinberg, 1994; Patel et al., 1994; Macdonald et al., 1994). Reduction of the prechordal plate and notochord of *cyclops* embryos might cause the observed defects in the midline of the overlying CNS (Hatta et al., 1994; Thisse et al., 1994; R. M. Warga et al., unpublished data).

In addition to cyclopia and absence of a floor plate, *cyc* mutant embryos also show a downward-curved body axis, or 'curly tail' (Hatta, 1992). Abnormal neural tube development is also observed in several mouse mutants associated with altered body shape. In *curly tail* mouse embryos, closure of the neural folds is disturbed, leading in some cases to spina bifida or exencephaly (Copp et al., 1990; van Straaten et al., 1994), defects that are also known as congenital and probably multifactorial diseases in humans (see discussion in Copp, 1994).

In our screen for mutants affecting embryonic development of the zebrafish, we have recovered many mutants with upward or downward curved tails. We present a classification of these mutants based on complementation tests and phenotypic analysis. We examine in more detail a group of mutations that, like the *cyclops* mutation in the zebrafish or the *curly tail* mutation in the mouse, cause abnormal curvature of the body and defects in development of the neural tube. As a working hypothesis, we suggest that these genes maintain proper structure and inductive functions of the zebrafish midline.

MATERIALS AND METHODS

Maintenance of fish, embryo collection and staging

Fish were raised and kept under standard laboratory conditions at about 27°C, as described by Brand et al. (1995). Mutant carriers were identified by random intercrosses, and outcrossed to wild-type fish to maintain the stock. To obtain mutant embryos, two heterozygous carriers were mated. Typically, the eggs were spawned synchronously at dawn, embryos were collected, sorted, observed and fixed after different times of development at 28.5°C. In addition, morphological features were used to determine the age of the embryos (Kimmel et al., 1995). Occasionally, 0.2 mM phenylthiourea (PTU) was added to prevent melanization. For photography, live embryos were mounted in methyl cellulose (Westerfield, 1994).

Immunocytochemistry

Whole-mount detection with antibodies is described by Schulte-Merker et al. (1992). The following antibodies were used: anti-acetylated tubulin (Sigma, 1:1000); anti-Isl (Korzh et al., 1993; 1:500); anti-Fkd2 (R. M. Warga et al., unpublished data; 1:1000); mAbs 3A10 (Furley et al., 1990; 1:3), Znp1, Zn5 (Trevarrow et al., 1990; 1:1000). Secondary antibodies from a Vectastain elite kit were used at 1:300 for detection.

In situ hybridization

Digoxigenin-labeled RNA probes were prepared using a Boehringer kit, and hybridized and detected with an anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer; Schulte-Merker et al., 1992).

Other procedures

Acridine orange staining is described by Brand et al. (1996); for cartilage staining, see Piotrowski et al. (1996) and Schilling et al. (1996). For histology, Durcupan (Fluka) embedded embryos were sectioned at 5 μm and stained with toluidin-blue/borax. Photographs were taken as slides on a Zeiss axiophot, slides were scanned, and mounted as composites using Adobe Photoshop.

RESULTS

Mutant classification and complementation analysis

In our screen for embryonic visible mutants in the zebrafish, we found 113 mutations defining at least 27 genes that affect general body shape and/or development of the spinal cord. Intercrosses between mutants with similar phenotypes were done to determine the number of affected genes (see Haffter et al., 1996, for an overview of the screen). Table 1 summarizes the different groups of genes and the results of the complementation analysis, and gives a brief phenotypic description.

The most frequently observed phenotype is a sickle-shaped body, or 'curly tail down' (Fig. 1A,B); wild-type embryos are straight (Fig. 1C). This phenotype is first detectable during the pharyngula period [24-48 hours of development (h)], when the embryo has not yet hatched, but persists if the embryo is artificially hatched. Other body shape changes are bending of the axis upwards (*balloonhead*, Fig. 1D), spiraling of the body upwards (*curly up*, Fig. 1E), curvature of the body sideways

Table 1. Mutations affecting midline development and body shape

Gene	Symbol	Alleles	CNS Phenotype	Other Phenotype	Major description	Other references
Group 1: Mutations affecting CNS development, with curly tails downwards						
Group 1A: Midline group mutants, curly tails						
cyclops	*cyc*	*tf219, te262c, b16, b213, b229, m101, m294*	No floorplate, red. diencephalon, medial brain tissue reduced, eyes fused, abn. retinotectal projection	Reduced prechordal plate, curled down	a	c,d,h
one-eyed -pinhead	*oep*	*tz257, m134*	No floorplate, anterior forebrain reduced, eyes fused	No prechordal plate, tail curled down	b,c	d
schmalspur	*sur*	*ty68b*	No floorplate, medial mid- and hindbrain absent, eyes turned in	Reduced prechordal plate, curled down, braincase fused	d	
chameleon	*con*	*tf18b, tu214, ty60, tm15a, th6*	Partial floorplate, reduced ventral CNS, nucleus VI. absent, eyes turned-in, retinotectal projection	Curled down, no myoseptum, no aorta, braincase fused, red. motility	d	e,h,i,j
detour	*dtr*	*ts269, te370a, tm276b*	Partial floorplate, nucleus VI. absent, some comissures missing, eyes turned-in, abnormal retinotectal projection	Curled down, braincase fused	d	h
iguana	*igu*	*tm79a, ts294e*	Floorplate irregular, eyes turned-in, abnormal retinotectal projection	Curled down, posterior myoseptum, braincase fused	d	h
you-too	*yot*	*ty17a, ty119*	Partial floorplate, eyes turned-in, abnormal retinotectal projection	Curled down, no myoseptum, no aorta, braincase fused, red. motility	e	d,h,i,j
sonic-you	*syu*	*tq252*	Partial floorplate	No myoseptum, no aorta, red. pectoral fins, motility	e	d,j,i,k
schmalhans	*smh*	*tn222*	Indistinct floorplate	Curled down	d	
monorail	*mol*	*tv53*	Indistinct floorplate	Curled down	d	
Group 1B: Mutants lacking the midbrain-hindbrain boundary, with curly tails						
acerebellar	*ace*	*ti282a*	No midbrain-hindbrain boundary, no cerebellum, retinotectal projection	Curled down, red. circulation, small ear	f	l,i,m
spiel-ohne-grenzen	*spg*	*m216, m308*	No midbrain-hindbrain boundary, no cerebellum	Curled down, red. circulation, small ear	c	
Group 1C: Mutants with CNS degeneration and curly tails						
sense	*sen*	*tm28a*	CNS degeneration	Curled down	d	
schnitter	*snt*	*tg226c*	CNS degeneration	Curled down	d	
Group 2 : Mutants without CNS defects, with curly tails downwards						
Group 2A: Mutants with curly tails only						
spirale	*spi*	34 alleles, please see appendix of Haffter et al.	-	Curled down, no swimbladder	d	
sickle	*sic*	*ty71, tm123b*	-	Curled down, no swimbladder	d	
haken	*Hkn*	*ta211*	-	Curled down, dominant	d	
unresolved		21 mutations, please see appendix of Haffter et al.	-	Curled down	d	
Group 2B: Mutants with kidney cysts and curly tails						
locke	*lok*	*tm138a, tj8, ts277, tl215, to237b*	-	Curled down, kidney cyst	d	
unresolved		*tf206, tf214a, tg238a, tm304, tp202, tz214a, tz288, tp49d*	-	Curled down, kidney cyst	d	
Group 2C: Mutants with curly tails and other defects						
tiger	*tig*	*ta23*	-	Curled down, abnormal melanophore migration	d	g
wirbel	*wir*	*ty44a, tm15d*	-	Curled down, immotile	d	j
th242d		*th242d*	-	Curled down, short body	d	
Group 3: Mutants with tails curled upwards or sideways						
curly up	*cup*	*ty30, tp85a, tc321, tg226d*	-	Curled up	d	
saltimbanqui	*stb*	*tb241b*	-	Curled up	d	
pirueta	*pir*	*tq213b*	-	Curled up	d	
aquabat	*aqb*	*to260b*	-	Curled up, degeneration	d	
saltarin	*slt*	*ty63*	-	Curled up	d	
vicious cycle	*vic*	*tg211c*	-	Curled sideways	d	
Group 4: Mutants with an S-shaped body						
sinus	*sin*	*tr281, tw215, ts292b, ty130d*	-	Sinusoidal body	d	
cosinus	*cos*	*tw216*	-	Sinusoidal body	d	
Group 5: Other spinal chord defects						
balloonhead	*bad*	*tp71d*	Trunk neurocoel	Bent up	d	
spinestein	*sps*	*to2e, th279b*	Tail neurocoel	Viable, short adults	d	
choker	*cho*	*tm26*	Hindbrain defect	Melanophore pattern, myoseptum reduced	g	
unresolved		*tz227c, tg310a*	Trunk neurocoel	Bent up	d	

Mutations are grouped according to similarities in phenotype, and complementation behavior was typically tested within each group.
We kept mutants affecting body shape, if the embryos either showed a very specific additional aberration at an early stage, or if the embryos did not show general degeneration after 5 days of development.
References: a, Hatta et al., 1992; b, Hammerschmidt et al., 1996; c, Schier et al., 1996; d, this paper; e, van Eeden et al., 1996a; f, Brand et al., 1996; g, Kelsh et al., 1996; h, Karlstrom et al., 1996; i, Chen et al., 1996; j, Granato et al., 1996; k, van Eeden et al., 1996b; l, Whitfield et al., 1996; m, Trowe et al., 1996.

(*vicious cycle*), and an S-shaped body (*sinus*, Fig. 1F). The cause of the observed body shape changes is not known.

The ‘curly tail down’ group

The 98 ‘curly tail down’ mutants define more than 20 genes, and often show additional defects. Group 1 mutants also show CNS defects, whereas group 2 mutants have a normal CNS. Group 2 includes mutants for *spirale*, with 34 alleles the most frequently hit gene in our screen, and *sickle* (together with 21 unresolved mutations, where complementation testing is not finished), which aside from their curved shape lack a swim bladder, but are otherwise normal after 5 days of development**.** Mutant larvae for *locke* and eight unresolved single mutations show in addition an inflated pronephros and pronephric duct

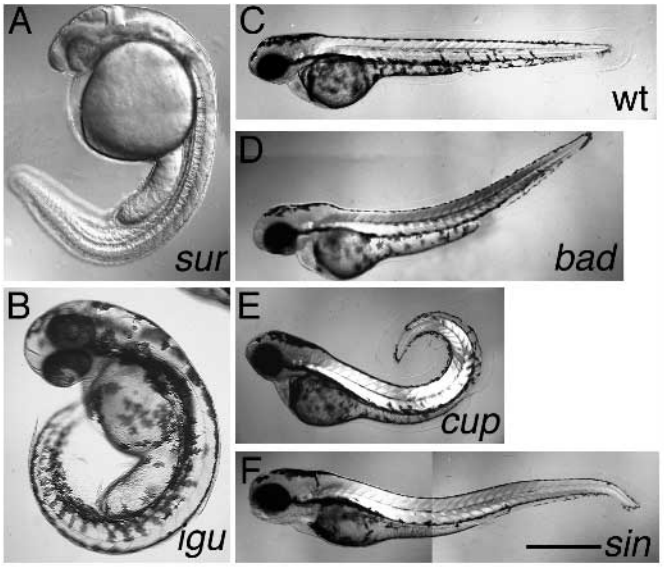

Fig. 1. Examples of body shape mutants. (A) 32 h *schmalspur* embryo, with a curly tail down. (B) *iguana* mutant embryo. (C) Straight sibling wild-type larva, at about 50 h. (D) *balloonhead* at about 50 h. (E) *curly up* on day 4 of development. (F) *sinus* on day 4. Scale bar, 0.8 mm (A,B, 0.4 mm).

(Table 1). Mutant embryos for *wirbel* are almost completely immobile, and have reduced xanthophore pigmentation. In antibody-stained specimens, major axonal organization, sensory neurons and motor neurons of *wir* embryos are morphologically normal (not shown); physiological aspects of neuronal function might be affected in this mutant.

Curly-tail mutants in group 1 show additional defects in CNS development. Mutant embryos for *acerebellar* and *spiel-ohne-grenzen* lack the cerebellum and the boundary between mid- and hindbrain, and are described elsewhere (Brand et al., 1996; Schier et al., 1996). Mutations in *sense* and *schnitter* cause degeneration of the tectum and the retina, as seen in acridine orange-stained specimens (not shown). An indistinctly formed neurocoel and slight or no body shape changes are observed in a small subgroup of complementing mutations that have not been characterized further (Group 5, Table 1).

The 'midline group'

We focused on one subgroup of curly tail down mutants with morphological defects in the spinal cord (14 mutants, 10 genes). Mutant embryos of this group have no or abnormal development of the floor plate and neurocoel in the spinal cord and brain, and abnormal positioning of the eye (Figs 2, 3), but each mutant has additional unique characteristics. Several

Table 2. Phenotypes of midline group mutants

Gene	Eyes (1)	Prechordal plate (fkd2)	Floor plate, morphology	Floor plate, *shh* expression	Median brain defect	Motor neurons	Optic chiasm (Zn5)	Myoseptum, morphology	Neurocranium (Alcian blue)
cyclops	Anterior cyclopia	Overall Reduced (2)	Absent (4)	Absent (5)	Yes	disorganized 2°	Ipsilateral/ normal	Normal	n.d.
one-eyed-pinhead	Anterior cyclopia	Overall Reduced	Absent	Absent (6)	Yes (6)	n.d.	n.d.	Normal	n.d.
schmalspur	Posterior cyclopia	Present anteriorly, absent posteriorly	Absent	Absent to patchy	Yes	Abnormal outgrowth of 2°	Normal	Normal	Ventral fusion
chameleon	Posterior cyclopia	Normal	Reduced	Normal	Yes	Abnormal outgrowth of 1° and 2°	Abnormal outgrowth, Ipsilateral/ normal	Absent	Ventral fusion (7)
you-too	Posterior cyclopia	Normal	Reduced	Normal	Yes	Abnormal outgrowth of 1° and 2°	Ipsilateral/ normal	Absent	Ventral fusion
iguana	Posterior cyclopia	Normal (3)	Reduced	Normal	Normal	1° n.d., 2° normal	Ipsilateral/ normal	Absent in posterior segments	Ventral fusion
detour	Posterior cyclopia	Normal	Reduced	Normal	Normal	1° n.d., 2° normal	Ipsilateral/ normal	Normal	Ventral fusion
sonic-you	Normal	n.d.	Reduced	Normal	n.d.	Abnormal outgrowth of 1° , 2° n.d.	n.d.	Absent	n.d.
schmalhans	Normal	Normal (3)	Reduced	Normal	Normal	1° n.d., 2° normal	Normal	Normal	n.d.
monorail	Normal	Normal (3)	Reduced	Normal	Normal	1° n.d., 2° normal	Normal	Normal	n.d.

The phenotype of the strongest available allele is listed for each gene in the midline group.
(1) Anterior cyclopia: complete or partial anterior fusion, 'posterior cyclopia': eyes are closer together posteriorly without fusion.
(2) Thisse et al. (1994); R. M. Warga et al., unpublished data.
(3) Based on morphology.
(4) Hatta et al. (1991).
(5) Krauss et al. (1993).
(6) Hammerschmidt et al. (1996).
(7) Seen in alleles of medium or weak strength.

mutants have defects in dorsal mesendoderm, and all are defective in the ventromedian neurocranium. Since defective development of the midline is common to mutants for all of these genes, we will refer to them as the 'midline group' (Table 1, Group 1A). A summary of the phenotypes of midline group mutants is given in Table 2.

cyc, *oep* and *sur* mutations affect early midline development

Mutations in the previously described *cyclops* gene (Hatta, 1992; Hatta et al., 1994) and in *one-eyed-pinhead* (Hammerschmidt et al., 1996; Schier et al., 1996; this paper) lead to partial or complete fusion of the eyes anteriorly (cyclopia),

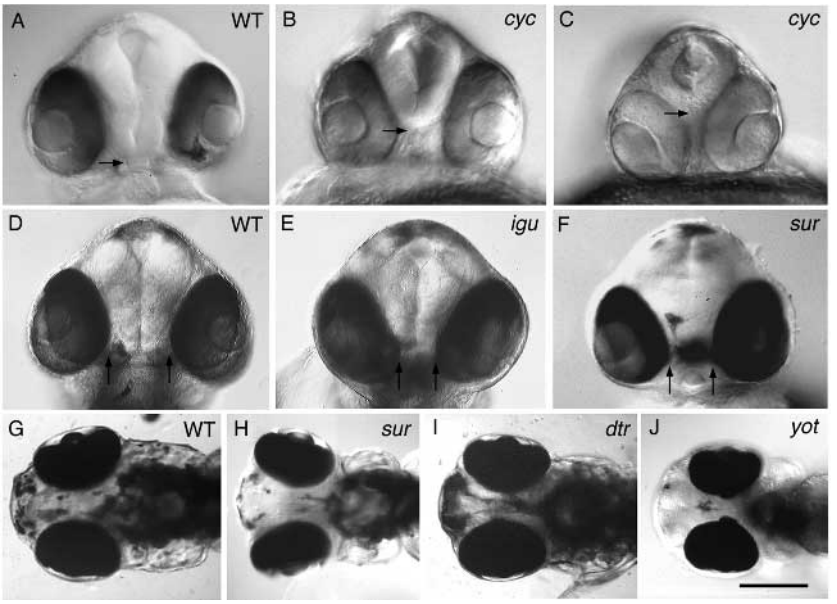

Fig. 2. Head and eye phenotypes of midline group mutants. Frontal views of live embryos are shown to illustrate the altered eye position; arrows in A-C point to the ventral limit of the forebrain. (A) Wild type at 36 h. (B) Strong *cyc*^{tf219} mutant embryo. (C) Weak *cyc*^{te262c} mutant embryo; notice the gradual loss of ventral forebrain material, and the closer position of the eyes, compared to the embryo in B. (D) Wild type at 48 h; arrows (in D-F) point to the medial surface of the eye. (E) Sibling mutant embryo for *iguana*; the distance between the eyes is reduced. (F) A similar reduction is seen in the *schmalspur* mutant embryo. (G) Dorsal view of a wild-type embryo on day 5; notice the position of the eyes. (H) Notice the altered eye position in the *schmalspur* embryo on day 5. (I,J) Eyes that are similarly turned-in posteriorly are seen in *detour* and *you-too* mutants. Genotypes are indicated. Scale bar, 180 μm (A-F), 200 μm (G-J).

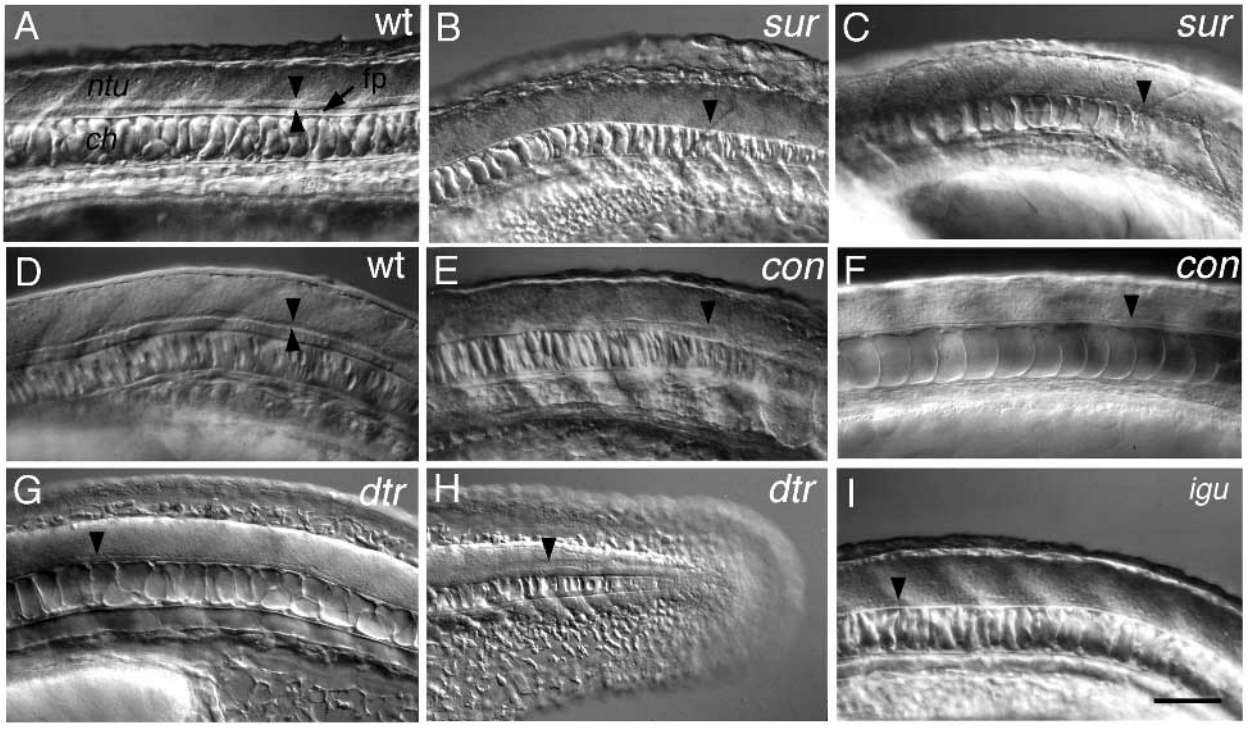

Fig. 3. Floor plate development in living mutants of the midline group. Lateral views of embryos during the pharyngula period, at about the level of the yolk tube. Arrowheads outline the expected position of the neurocoel. The arrow points to the floor plate (fp). ntu, neural tube, ch, notochord. (A) Wild-type spinal chord; (B,C) *schmalspur* at 28 h and at 42 h. No floor plate or neurocoel is visible. (D,E) Wild type and mutant *con* embryo at the 20-somite stage. Only patches of floor plate are visible in the mutant. (F) *con* at 30 h. The floor plate and neurocoel are not visible and the remainder of the neural tube shows abnormal cellular morphology. (G,H) *dtr* embryo at 28 h, at the level of the tail, and the tail tip. Notice the partially formed floor plate, which is more pronounced in the tail tip. (I) *iguana* embryo, showing a partially formed floor plate and neurocoel. Genotypes are indicated. Scale bar, 62 μm.

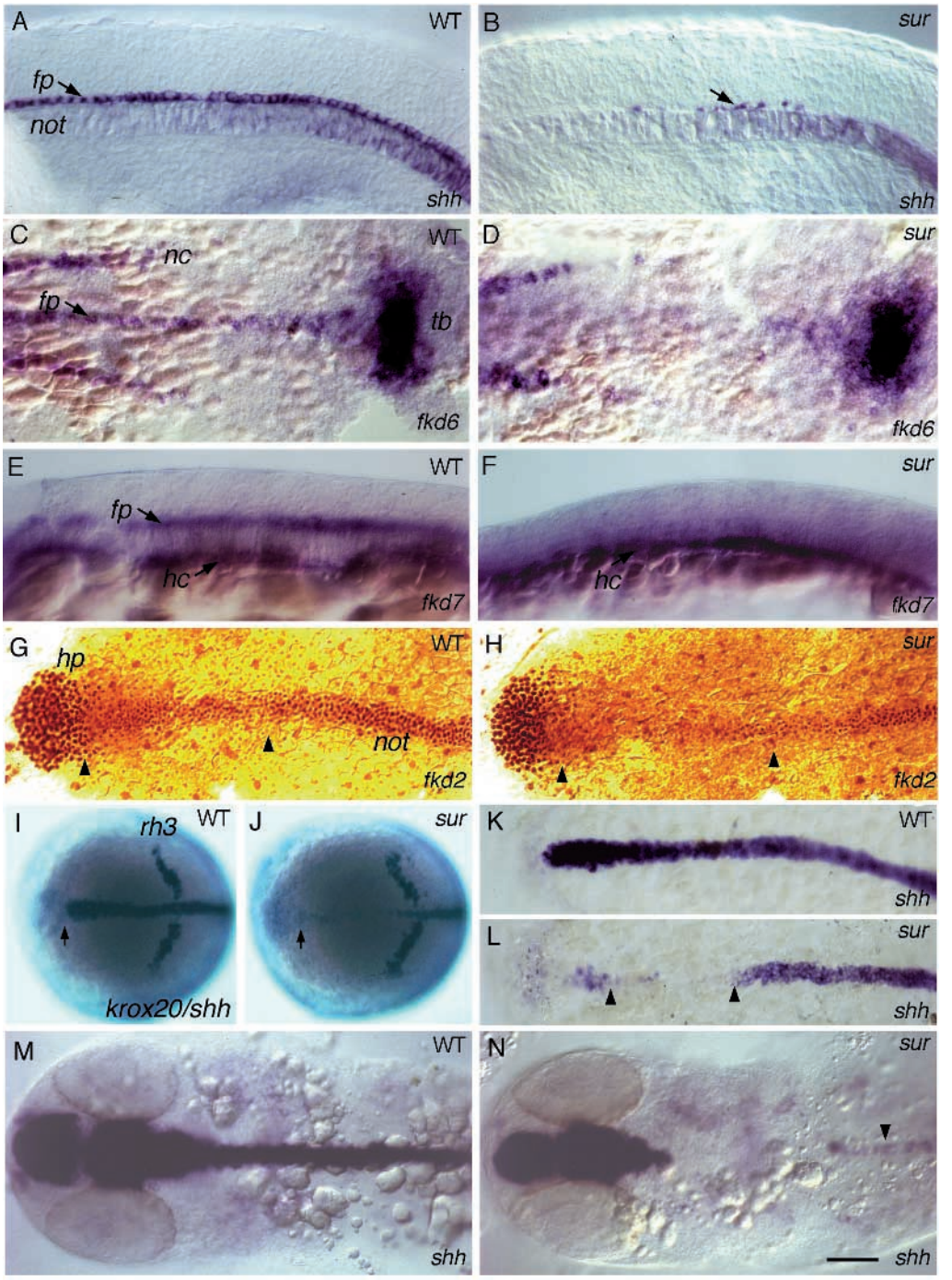

Fig. 4. Expression of floor plate markers in *schmalspur*. (A) 24 h wild type, stained for *shh* RNA. Strong staining is seen in a single line of cells, the floor plate (fp); weaker staining is seen in the notochord (not). (B) Mutant sibling of the wild type in A; the arrow points to a patch of undifferentiated floor plate of representative size. (C,D) Wild-type and mutant embryos stained for *fkd6* RNA. Expression in floor plate precursors is lacking. nc, neural crest cells. (E,F) Wild-type and mutant embryos stained for *fkd7*. Staining in the floor plate is eliminated, but persists in the hypochord (hc). (G,H) Anti-Fkd2 staining at the tailbud stage. Notice the strong reduction of staining in the posterior prechordal plate (between the arrowheads) in the mutant, but the normal staining in the polster (hp). The notochord (not) also has slightly fewer nuclei. (I,J) *shh* and *krox20* expression at the tailbud stage. *shh* in the cephalic neural plate is eliminated anterior to the rhombomere 3 stripe (rh3) of *krox 20*. (K,L) *shh* expression at the 8-somite stage. The defect of *shh* expression is seen as a gap, and as a slightly reduced expression. Posterior staining is in the notochord at this stage; anterior expression in the forebrain is gradually developing. (M,N) *shh* expression at 26 h. Normal expression in the forebrain is seen. Arrowhead points to the notochord. Genotypes and markers are indicated in the upper and lower right corners. Scale bar, 72 μm.

indicative of ventral brain defects, and to absence of the floor plate. We recovered two additional *cyc* alleles: cyc^{tf219} is slightly weaker than the original strong cyc^{b16} allele. cyc^{te262} is a weak allele that leads to only slightly turned-in eyes, and to an interrupted floor plate in the trunk, but a normal floor plate in the tail (Fig. 2B,C). In contrast to *cyc* and *oep* mutants, the eyes of *schmalspur (sur)* mutant embryos are turned inwards *posteriorly* (Fig. 2F,H), but they also lack a morphologically visible floor plate and neurocoel (Fig. 3B,C). A variable, but small amount of floor plate-like cells is sometimes seen in the tip of the tail (similar to *dtr*, Fig. 3H), indicating that the only known *sur* allele (sur^{ty68b}) may not be a complete lack-of-function allele. Depending on the genetic background, the notochord is sometimes less well vacuolated, indicative of defects in the dorsal mesoderm (see below).

Mutants with partial floor plate development

Mutations in *chameleon (con)*, *you-too (yot)*, *detour (dtr)* and *iguana (igu)* lead to inward-turning of the eyes posteriorly, but not to fusion (Fig. 2), and to abnormal floor plate development (Fig. 3; Table 2). Abnormal floor plate development without altered eye position is observed in *schmalhans (smh)* and *monorail (mol)* mutants, which are tentatively included in this group. In *con*, *yot* and *sonic-you (syu)* mutant embryos, patches

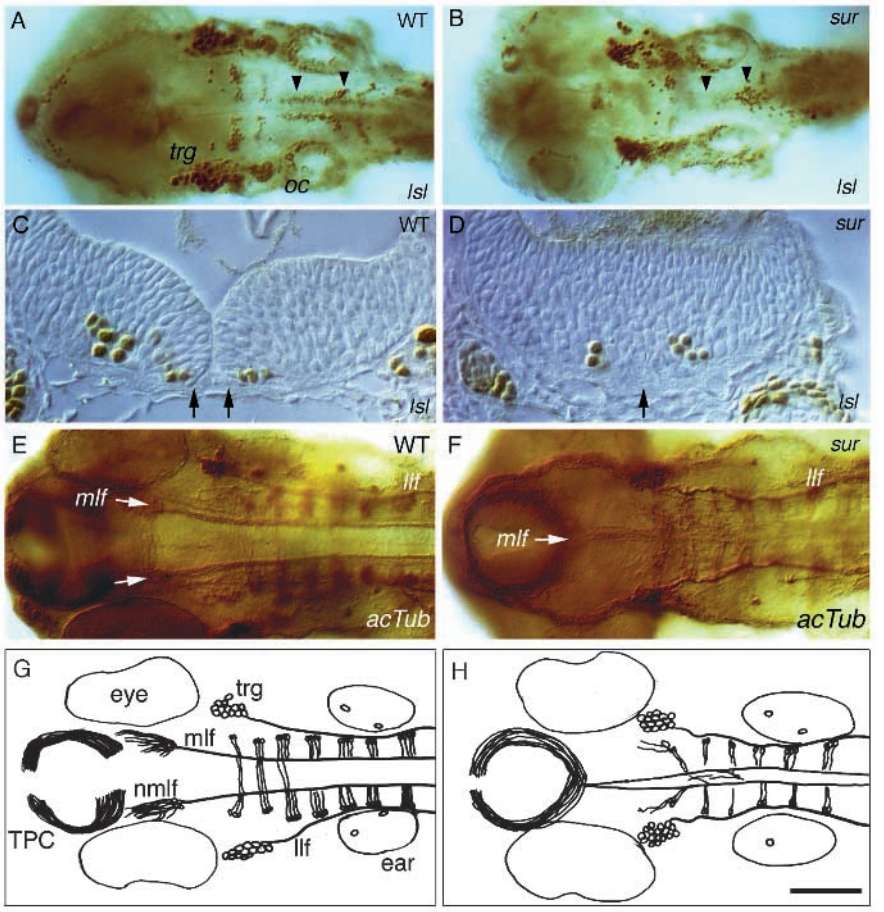

Fig. 5. Brain defects in *schmalspur*. (A,B) Isl antibody staining at 32 h. Arrowheads point to median clusters in the brain that are absent or fused in *sur* mutant embryos. (C,D) Transverse section through an Isl-stained embryo, at the level of rhombomere 5. Arrows point to the floor plate in the wild type, and the corresponding position in the mutant. Notice the absence of the fossa rhomboidea. (E,F) Acetylated tubulin staining at 30 h. (G,H) Schematic drawing of the embryos in E and F. Notice the fusion of the mlf and the tract of the posterior commissure (TPC). trg, trigeminal ganglion; oc, otic cyst; llf, lateral longitudinal fascicle; mlf, medial longitudinal fascicle. Scale bar, 72 μm (C,D 36 μm).

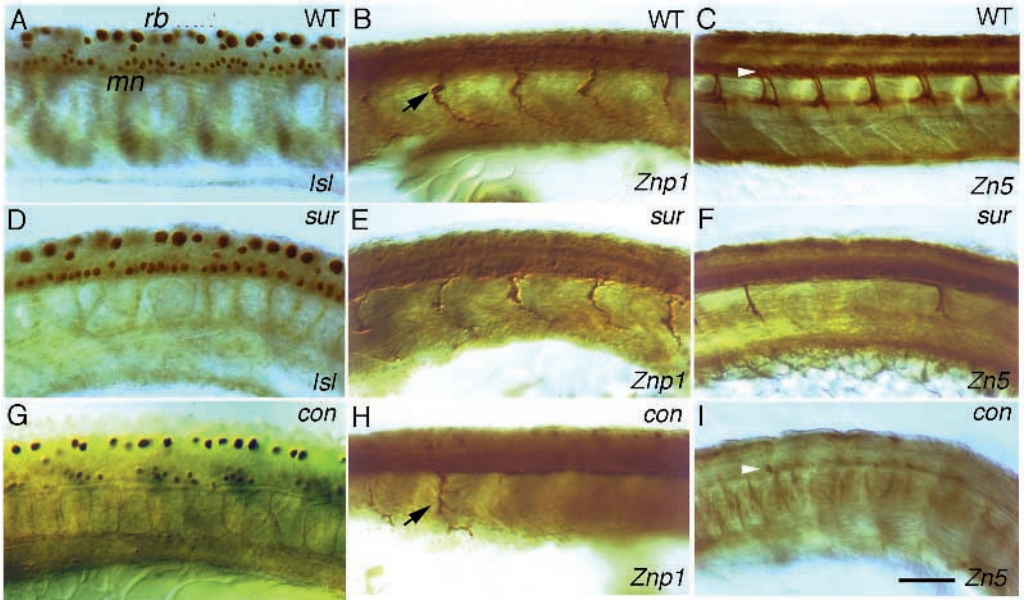

Fig. 6. Motor neuron development in *sur* and *con*. (A-C) Wild type, (D-F) *sur* mutants, (G-I) *con* mutants. (A,D,G) Isl-stained embryos at 30 h, (B,E,H) Znp1-stained embryos at the same age. (C,F,I) Zn5-stained embryos at 48 hours of development. rb, Rohon-Beard neurons, mn, motor neurons. Arrows in B and H point to the position of the horizontal myoseptum, which is contacted by the wild-type axons, and is missing in con mutants. Note the less well organized appearance of the axon. In neighboring segments, axons grow along the neural tube, instead of projecting into the periphery. Arrowhead in C points to the thick layer of secondary motor neuron cell bodies which is missing in *con*. mutants (I). Here, the arrow points to an isolated Zn5-positive cell body. Scale bar, 82 μm.

of floor plate form during late somitogenesis stages (19 h, Fig. 3E), and a partially formed floor plate is present at 24 h. In *con* and *yot* mutants, the floor plate is no longer detected around 30 h, when the whole neural tube appears thinner and the arrangement of cells becomes irregular (Fig. 3F). The similarity between the *con*, *yot* and *syu* phenotypes is also evident from their common lack of a dorsal aorta (Chen et al., 1996) and of the horizontal myoseptum (van Eeden et al., 1996b). Similar

partial development of the floor plate is also observed for homozygous *dtr* (Fig. 3G,H), *igu* (Fig. 3I), *smh* and *mol* (not shown) mutant embryos. In these mutants, the floor plate appears thinner and less well differentiated, and the neurocoel above it is collapsed or absent (Fig. 3G-I). Thickness and cellular morphology of the remainder of the neural tube are not affected in these embryos. An irregularly formed floor plate and neurocoel is often seen in the tail tip of *dtr* embryos (Fig. 3H).

Early markers of floor plate development are affected in *sur* mutants

To examine formation of the floor plate further, we looked at expression of molecular markers in mutants of the midline group (Table 2). In wild-type embryos at 24 hours of development, expression of *sonic hedgehog* (*shh*) marks the floor plate, and more weakly the posterior notochord (Fig. 4A; Krauss et al., 1993). In mutant *sur* and *cyc* embryos at this age, *shh* expression is found only in small islands of poorly differentiated cells in the position of the floor plate; expression in the notochord and pectoral fin bud is unaffected (Fig. 4B; Krauss et al., 1993). *fkd6* is a *forkhead*-domain gene expressed in precursors of floor plate, neural crest and in the tailbud (J. Odenthal, unpublished data; Fig. 4C). In *sur* embryos at the 3-somite stage, only a small remnant of *fkd6* expression in the floor plate precursors is observed close to the tailbud, while expression in the remainder of the midline is absent (Fig. 4D). Expression of *fkd7* occurs in floor plate and hypochord cells, which form two single rows of cells dorsal and ventral to the notochord (J. Odenthal, unpublished data; Fig. 4E). Floor plate expression of *fkd7* is abolished, whereas hypochord expression is unaffected in *sur* mutant embryos (Fig. 4F). We conclude that floor plate development is inhibited at an early stage in *sur* mutant embryos. We also examined expression of *shh*, *fkd6* and *fkd7* in *con*, *yot*, *igu*, *dtr*, *smh* and *mol* mutants, and did not observe a defect in the floorplate.

Defective prechordal plate and notochord in *schmalspur* mutants

Previous studies showed that the axial mesoderm of the head (prechordal plate), trunk and tail (notochord) can influence patterning in the overlying neural plate. We therefore used an antibody to Fkd2 protein (R. Warga et al., unpublished data) as a marker of axial mesoderm to follow its development in *sur* embryos. Expression of Fkd2 is strongly reduced in the posterior prechordal plate in *sur* mutant embryos, whereas the anterior part, which includes the polster, is relatively normal (Fig. 4G,H, between arrowheads). Similarly, expression of *goosecoid* (Stachel et al., 1993; Schulte-Merker et al., 1994) in the anterior part was normal (not shown). In the overlying neural plate of wild-type embryos, *shh* is expressed by a continuous stripe of cells at the cephalic midline (Fig. 4I). In *sur* mutant embryos at the tailbud stage double-stained for *shh* and *krox-20* RNA, *shh* is absent from the area overlying the posterior prechordal plate (Fig. 4J). At the 8-somite stage, an anterior patch of expression is observed in *sur* mutants (Fig. 4K,L). By 26 hours of development, *shh* expression is normal in the forebrain and posterior notochord, but is absent or reduced to small patches in the remainder of the CNS midline (Fig. 4M,N). Thus, regionalized defects in the posterior prechordal plate in *sur* embryos correlate with corresponding regional defects in the overlying neural plate posterior to the forebrain.

Median deletions of brain tissue in *sur*, *con* and *yot* mutants

To further characterize the brain defects of midline group mutants, we examined them with neuron-specific antibodies (Table 2). Formation of early differentiating neurons in the brain can be visualized with an antibody recognizing several *Isl* proteins (Korzh et al., 1993; Inoue et al., 1994). In the mid- and hindbrain of 32 h wild-type embryos, bilaterally symmetric clusters of neurons can be observed (Fig. 5A) that are absent or fused in *sur* mutant embryos (Fig. 5B, arrowheads). Similarly, the nucleus of the abducens nerve, normally located close to the midline in rhombomeres 5 and 6 (Trevarrow et al., 1990), is absent in Zn5-stained preparations of *sur*, *con* and *yot* mutants, but is present in *dtr*, *smh* and *mol* mutants (not shown). In cross sections of *sur* embryos stained with *Isl* antibody, no floor plate is observed in the hindbrain of mutant embryos, and the fossa rhomboidea, or median cleft, in the hindbrain is absent (Fig. 5C,D). To learn how the observed changes at the midline affect formation of the major axonal tracts, we examined *sur* mutant embryos stained with an antibody to acetylated tubulin. Instead of forming two separate fascicles along each side of the floor plate, the medial longitudinal fascicle (mlf) is fused in the mid- and hindbrain of *sur* mutant embryos; organization within the bundle is less tight (Fig. 5E-H). The bilateral trochlear nerves in the midbrain of *sur* embryos stained with Zn5 are similarly fused (not shown). Formation of the ventral forebrain nuclei (Isl staining) and commissures (acetylated tubulin staining) are not affected in *sur* mutant embryos (not shown). In summary, loss of median mid- and hindbrain tissue is observed in *sur* mutant embryos and, more weakly, in the hindbrain of *con* and *yot*, but not of *dtr*, *igu* and *smh* mutant embryos (Table 2); the other mutants were not examined.

Abnormal motor neuron development in *sur*, *con* and *yot* mutants

Signals from the notochord and floor plate are well known to influence the induction and patterning of spinal cord motor neurons. We therefore examined the formation of primary (early arising) and secondary (later arising) motor neurons in most midline mutants. We found defects in *sur*, *con*, *yot* and *syu*, but no defects in *igu*, *dtr*, *smh* and *mol* mutants (Table 2); *oep* was not examined.

In wild-type embryos at the pharyngula stage stained with an antibody to Isl proteins, small, ventrally located motor neuronal nuclei can be distinguished from large, dorsally located nuclei of the Rohon-Beard sensory cells (Fig. 6A). Segmentally arranged axons of identified primary motor neurons are labeled by the Znp1 antibody (Fig. 6B). Axons and cell bodies of secondary motor neurons, located close to the floor plate, can be stained with the Zn5 antibody (Fig. 6C). In *sur* mutant embryos, we observe a normal number and location of Isl-stained nuclei and Znp1-stained axons of primary motor neurons (Fig. 6D,E), whereas only few or no axons of secondary motor neurons are observed (Fig. 6F). In cross sections of the spinal cord of Isl-stained embryos, floor plate and neurocoel are not detectable (not shown). In *con* mutant embryos, Isl-stained primary motor neurons at the 20-somite stage appear normal (not shown), but at 28 hours of development, a reduced number of ventrally located motor neurons (primary or secondary) is seen, whereas the dorsally located

Rohon-Beard sensory neurons are unaffected (Fig. 6G). Consistent with this observation, outgrowth of Znp1-stained primary motor neurons is absent, or reduced and disordered, in most segments of *con* mutant embryos; often, axons appear to grow along the neural tube instead, or show abnormal morphology (Fig. 6H). Fig 6I shows that in addition, only isolated cell bodies (arrowhead) and no outgrowth of secondary motor neurons can be observed in *con* mutant embryos stained with Zn5. Reduced or aberrant outgrowth of primary and secondary motor neurons is also observed in embryos mutant for *yot* (van Eeden et al., 1996b; and not shown). In summary, outgrowth of secondary motor neurons is aberrant in *sur*, whereas both primary and secondary motor neurons are affected in *con* and *yot* (Table 2).

Early commissural axons project normally across the midline in *sur* mutants

The floor plate is well known to attract or repel axonal growth of spinal cord interneurons. We therefore examined mutants of the midline group with 3A10 antibody, which recognizes a small number of early arising identified interneurons in the hindbrain and spinal cord (Hatta, 1992). In *cyc* mutants stained with 3A10, contralateral projections of the Mauthner and RoL2 interneurons in the hindbrain are mildly abnormal (Hatta, 1992). In 30 h *sur*, *con* and *dtr* mutant embryos, contralateral projections of the Mauthner and RoL2 interneurons in the hindbrain, and of the CoPA interneurons in the spinal cord, are not affected (not shown). Thus even though floor plate development is disrupted early on, other factors can guide early commissural interneurons across the midline in *sur*, *con* and *dtr* mutant embryos.

In contrast to early commissural interneurons, formation of the later established optic nerve chiasm is more strongly affected in some midline group mutants, as seen in stained embryos during the early larval stage (48 h; Table 2). In 48 h wild-type embryos stained with Zn5, retinal ganglion cell axons project via the optic nerve into the contralateral optic tectum (Fig. 7A). Outgrowth of the optic nerve from the eye is often absent or abnormal in *con* mutant embryos (Fig. 7C). Abnormal projections into the ipsilateral tectum, indicative of abnormal midline function, are seen in about half of the embryos mutant for *yot*, *dtr* (Fig. 7D,E) and *igu*, and are sometimes seen when an optic nerve has formed in *con* mutants. Not surprisingly, optic nerve formation is normal in *sur* mutant embryos, where midline formation in the forebrain seems not affected (Fig. 7F), and in curly tail mutants without CNS defects (we tested *spirale*, *sickle* and *locke*). *yot*, *dtr and igu* mutations were identified independently in the screen for defective retinotectal projection; in *dtr* mutants at least, other brain commissures are affected as well, again suggesting a more general midline defect (Karlstrom et al., 1996).

Defective development of the brain correlates with defects in the ventral neurocranium

The lower jaw is hanging down in larvae mutant for any of the midline group genes (except for *smh* and *mol*). To examine this defect further, we stained the cartilaginous skeleton of the jaw and neurocranium of 5-day old larvae with alcian blue (Fig. 8). In all mutants of the midline group we examined, we observe defects in the ventral neurocranium, whereas the viscerocranium is usually unaffected, except in cases of generally retarded development (e.g. for strong *con* and *yot* alleles). In wild-type larvae, the ventral neurocranium consists of the posterior parachordals and the trabeculae, which fuse anteriorly to form the ethmoid plate (Fig. 7A). In midline mutants, parachordals and trabeculae partially fuse along the midline (Fig. 7B,C), as was confirmed in histological sections (not shown). Importantly, formation of the ethmoid plate is not affected in *sur* mutant embryos (Fig. 8B, arrows), whereas it is affected in the other mutants. This observation points to an interesting correlation between localized defects in the midline and the ventral neurocranium, which is formed from neural crest and somitic cells that originate outside the midline (see Discussion).

DISCUSSION

In several vertebrates, the study of embryonic midline tissue, mainly notochord and floor plate, has provided experimental, genetic and molecular evidence for important patterning functions of these tissues in development of the neural tube and somites. Ectopic notochord induces floor plate and motor neuron development, whereas ablation leads to their loss (reviewed by Plazcek et al., 1993; Ruiz i Altaba and Jessel, 1993; Smith, 1994). Similarly, signals from notochord and neural tube induce differentiation of sclerotome in neighboring somites (Bumcrot and McMahon, 1995). A key player in these inductive events is the secreted sonic hedgehog (shh) protein, which is expressed in notochord and floor plate of several species at the right time and place (Concordet and Ingham, 1995; Fietz et al., 1994). In zebrafish, analysis of notochord-less mutants like *floating head*, *doc* and *no tail*, for which we also found alleles in our screen, provides evidence for inductive functions of the notochord in floor plate and motor neuron development (Halpern et al., 1995; Talbot et al., 1995; Odenthal et al., 1996). Components specifically involved in transducing, receiving or processing inductive signals might be affected in mutants with normal notochords that show defects in target tissues, like the neural tube (this paper) and/or the somites (van Eeden et al., 1996b).

The midline group

The ‘midline group’ of mutations may represent such a collection of genes involved in the induction of floor plate and motor neurons. These mutants are found among the mutants affecting body shape isolated in our screen (Table 1; see Haffter et al., 1996, for an overview of the screen). Although mutations in these genes cause very similar phenotypes, characteristic differences can be defined; a summary of the mutant phenotypes is given in Table 2.

Mutations in three genes isolated in our screen lead to early absence of the floor plate, in spite of the presence of a notochord: *schmalspur (sur)*, *one-eyed-pinhead (oep)*, and the previously identified *cyclops* (*cyc*) gene. Early loss of midline tissue in the brain and spinal cord can be directly demonstrated in these mutants. *fkd6*-positive floor plate precursors are already absent in *sur*, and a similarly early stage of floor plate development is affected by *cyc* and *oep* mutations (Hatta, 1992; Krauss et al., 1993; Strähle et al., 1993; Hammerschmidt et al., 1996; Schier et al., 1996). These genes could therefore be directly involved in floor plate induction, as was previously

suggested for *cyc* (Hatta et al., 1991; Hatta et al., 1994), possibly in the *shh* pathway.

An alternative possibility is suggested by the observation that *oep*, *cyc* and *sur* mutants show defects of decreasing strength in the prechordal plate. The prechordal plate derives from the embryonic shield, the zebrafish equivalent of Spemann's organizer, and can be followed by expression of the *gsc* gene (Schulte-Merker et al., 1994; Stachel et al., 1993). Analysis of *fkd2* and *gsc* expression shows that the entire prechordal plate is absent in *oep* (Hammerschmidt et al., 1996) and strongly reduced in *cyc* mutants (Thisse et al., 1994; R. Warga et al., unpublished data). Reduced expression of *gsc* is already seen before (*oep*) or during (*cyc*) gastrulation, suggesting that *oep* and *cyc* may function more generally in development of the shield (Hammerschmidt et al., 1996, and discussion therein; Thisse et al., 1994). *cyc* and *sur* mutants are similar in many aspects, but differ in the degree to which the anterior prechordal plate and the overlying forebrain are affected. In stained embryos, *sur* embryos stained for *fkd2* and *gsc* exhibit a strongly reduced posterior prechordal plate, whereas the anterior part is normal. This may simply reflect a failure of the posterior part of the prechordal plate to differentiate properly. Alternatively, it is possible that *sur* also functions in formation or development of the shield, but has a weaker or regionally different requirement than *oep* and *cyc* in this process. The requirement for *sur* would, in this model, still allow the anterior prechordal plate to form normally. As a caveat, it is important to note that there is, at present, only a single allele of *sur*, which may not eliminate *sur* gene function completely. Double mutant combinations between the midline group mutants and linkage tests with known genes expressed in the floor plate might be revealing.

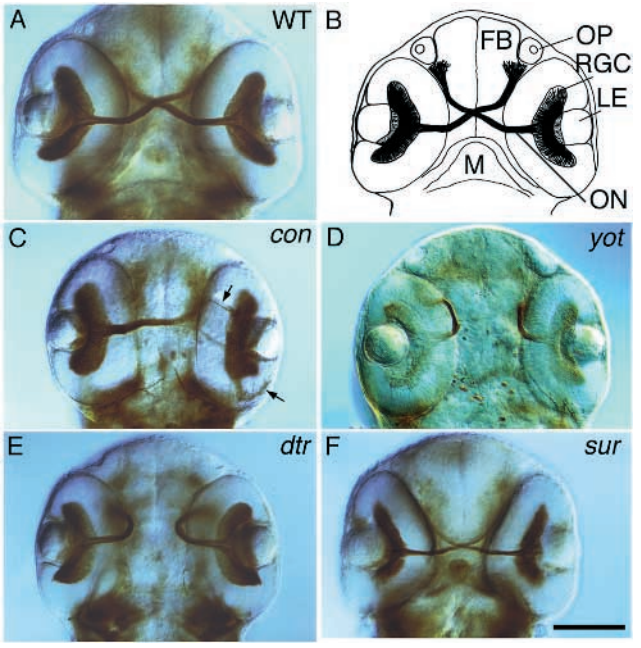

Fig. 7. Formation of the optic chiasm. Genotypes are as indicated. Shown are ventral views of Zn5-stained heads at 50 hours of development. In *con* mutant embryos, retinal ganglion cell (RGC) axons often do not leave the eye of origin (arrows). In about half of the mutant embryos, at least one, often thin, optic nerve (ON) is formed, which may grow to the contralateral or ipsilateral tectum. Ipsilateral projection is seen in *yot* and *dtr*, whereas *sur* mutants are normal. LE, lens; M, mouth; OP, olfactory pit, FB, forebrain. Scale bar, 140 μm.

Mutants with partial floor plate development

Mutations in another seven genes (*chameleon*, *you-too*, *sonic-you*, *detour*, *iguana*, *schmalhans* and *monorail)* cause a partial development of the floor plate, but the expression of the early floor plate markers *shh*, *fkd6* and *fkd7* is normal. Mutants for these genes resemble the 'strong' floor plate mutants in different ways (Table 2). Similar to *sur* embryos, *con*, *yot*, *dtr*

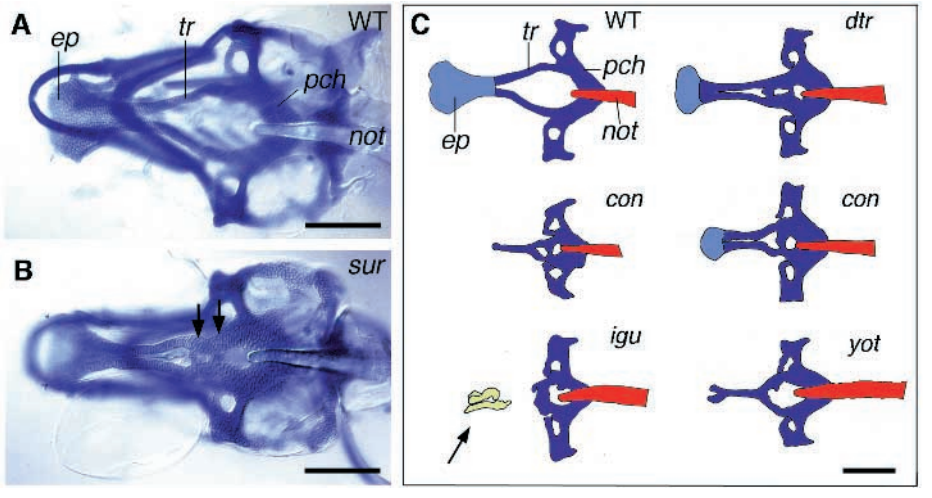

Fig. 8. Defects in the ventral neurocranium of midline group mutants. Ventral views of alcian blue-stained preparations of the chondrocranium on day 6 of development. Focus is on the neurocranium. ep, ethmoid plate; tr, trabeculae; pch, parachordals; not, notochord. (A) Wild-type. (B) *schmalspur* mutant embro. Notice the fusions (arrows) of the parachordals and trabeculae. (C) Schematic representation of fused neurocrania of different midline group mutants. The different parts are color coded; the boundary between trabeculae and parachordals is tentative. *chameleon* mutant embryos can be grouped into a phenotypic series of decreasing strength. The phenotype of the weak allele *con*^{th6d} is variable: embryos with the weakest phenotypes exhibit partially fused trabeculae and parachordals, but an almost normal ethmoid plate (*con* on the right); strongly affected embryos have severe fusions and no ethmoid plate (on the left). The phenotypes observed for other midline mutants fall into the range of this phenotypic series. *iguana* mutant embryos show a strong phenotype: the parachordals are fused, and only islands of the ethmoid plate are present (arrow). In *you-too* mutants, fusion of the trabeculae to a trabeculum communis is observed, as in the medium strength *con*^{th6d} embryos. In *detour* mutants, fusion of the posterior neurocranium occurs, and a well-delineated intertrabecular cleft extends apparently to more anterior levels than in the wild type. Sometimes the trabeculae remain split and do not fuse anteriorly in *dtr* mutants. Scale bar, 125 μm.

and *igu* mutant embryos show inward-turned eyes posteriorly. Like *cyc* (Hatta, 1992; Bernhardt et al., 1992), *sur*, *con* and *yot* mutants show absence or fusion of neuronal clusters along the CNS midline in the mid- and hindbrain.

Spinal cord motor neuron development is affected in *cyc*, *sur*, *con*, *syu* and *yot* mutants, and *con*, *syu* and *yot* mutants lack a horizontal myoseptum (see below). Since the affected tissues are known targets for inductive cues from the midline, these genes are included in the same group. Further phenotypic analysis is needed to understand why floor plate development is partial in these mutants. For instance, pathways for floor plate induction may be partially redundant, as suggested by the observation of homeogenetic mechanisms of floor plate formation (Hatta et al., 1991).

Zebrafish midline functions in motor neuron development

Since induction of floor plate by notochord influences dorsoventral patterning in the neural tube (van Straaten et al., 1989; Yamada et al., 1991; Plazcek et al., 1993), it is expected that defective motor neuron development is seen in midline group mutants. As observed for *cyc* (Hatta, 1992), disorganized or missing secondary motor neurons are observed in *sur* mutant embryos, which could be explained by absence of the floor plate. The case of *con* and *yot* mutant embryos with partial floor plates is of interest; both reduced numbers of neurons and altered growth of axons along the neural tube were seen. Only secondary (later arising) motor neurons are affected in *sur* mutants, whereas both primary (early arising) and secondary motor neurons are affected in *con* and *yot* mutants. Additional inductive signals independent of the floor plate could therefore be disturbed in *con* and *yot* mutant embryos. In chicken, notochord can induce motor neurons without apparent floor plate differentiation (Yamada et al., 1991), and different concentrations of shh induce floor plate and motor neuron development in vitro (Marti et al., 1995; Roelink et al., 1995; Tanabe et al., 1995). Two factors point to the notochord as a possible source for such a signal in the zebrafish. Firstly, ablation of the notochord in *cyc* mutant embryos substantially increases the percentage of errors made by axons at the midline (Greenspan et al., 1993). Secondly, although *con* and *yot* mutants have a normal looking notochord, they lack cells of the horizontal myoseptum (van Eeden et al., 1996b), which depend on a signal from the notochord for their formation (Halpern et al., 1993). *con* and *yot* could therefore be components common to a signaling pathway from notochord to the neural tube and to somites. Alternatively, signaling to somites might occur via the neural tube (Münsterberg and Lassar, 1995). Transplantation experiments have so far demonstrated a requirement for wild-type *yot* function in the myoseptum, which may therefore encode a receptor, or a product downstream of it, for a signal from the notochord (van Eeden et al., 1996b). The requirement for *con* has not been tested yet, and so it remains possible that *con* function would be needed in the notochord to produce a signal required for the induction of myoseptum cells. Similarly, a notochord signal to the motor neurons, possibly *shh* itself, might be affected in *con* mutants.

Axonal growth in midline group mutants

The floor plate has been implicated in orienting commissural axon growth towards and across the midline (Colamarino and Tessier-Lavigne, 1995). As in *cyc* mutants (Hatta, 1992), we have observed fusions and disoriented growth of axonal bundles close to the midline of mid- and hindbrain of *sur* embryos. Among the four types of identified hindbrain and spinal cord neurons we could examine in *sur*, *con* and *dtr* mutants we have not observed failures to cross the midline. A number of prominent commissures exist connecting the brain hemispheres. In a subset of midline group mutants, the optic nerve projects to the wrong side in about 50% of the mutant embryos (Fig. 7; Karlstrom et al., 1996), which we take as an indication of structural or functional failure of the cephalic midline.

Typically, only a fraction of the embryos mutant for *cyc*, *con*, *yot*, *dtr* and *igu* from a given clutch show abnormal ipsilateral projection of the optic nerve. A trivial explanation for the weak effects on axonal projection is that we are looking at mutations that do not completely inactivate the respective gene product, which is likely to be true for some, but not all cases. Alternatively, these genes may be partially redundant in their function, or genes with a more limited and/or later function in midline development may be affected in these mutants. Redundancies of chemoattractive cues are likely to exist at the midline. Several secreted signaling molecules, probably with diverse functions, are expressed by floor plate cells, e.g. Steel factor, sonic hedgehog and netrin-1 (Matsui et al., 1990; Krauss et al., 1993; Kennedy et al., 1994). To explain why only a fraction of axons shows abnormal ipsilateral turns in *cyc* mutant embryos, it has previously been argued that these axons normally depend on multiple guidance cues from the midline (see Colamarino and Tessier-Lavigne, 1995). Structural heterogeneity at the midline, which is well documented in the variety of different axial domains of gene expression (for reviews, see for example Rubenstein and Puelles, 1994; Colamarino and Tessier-Lavigne, 1995), is another possibility that is well documented for the midline of the *Drosophila* embryo (Goodman and Doe, 1993). Such proposals predict that double mutant combinations between these genes might show stronger or more penetrant phenotypes.

Implications for anterior-posterior patterning

To achieve subdivision of the neural plate along the anterior-posterior (a-p) axis, two major types of mechanisms are thought to operate: regional identity might depend on signals from the underlying mesendoderm (vertical induction), or on signals spreading through the ectoderm (planar induction). Evidence for both types of mechanism has been gathered from tissue recombination experiments in frogs, chicken and mice (Slack and Tannahill, 1992; Ruiz i Altaba, 1994, and references therein). The relative importance of both mechanisms in vivo is currently not clear. Mutants that reduce parts or all of the prechordal plate could help to resolve this question.

Absence of prechordal plate in *oep* mutants, and its overall reduction in *cyc* mutants, correlates with the absence of ventroanterior forebrain markers and with anterior eye fusion (Thisse et al., 1994; Hammerschmidt et al., 1996; R. Warga et al., unpublished data). However, even in the complete absence of underlying prechordal plate, limited a-p polarity is established in the dorsal anterior neural plate, presumably by a planar path, whereas ventral markers like *shh* are absent throughout the axis (Hammerschmidt et al., 1996).

Nevertheless, subregions of the prechordal plate appear to

exert different patterning influences onto the overlying ectoderm. In *sur* mutants, the anterior prechordal plate (including the polster) is intact, whereas the posterior prechordal plate is strongly reduced or absent (Fig. 4). Presence of this isolated piece of anterior prechordal plate correlates with formation of a properly formed forebrain from the overlying neural plate. The mid- and hindbrain, on the other hand, lacks ventral structures and expression of molecular markers for this region is severely affected. Conversely, in *silberblick* mutants, a misshapen polster correlates with very localized anterior forebrain defects, and partial fusion of the eyes (Heisenberg et al., 1996, and discussion therein). Our findings are consistent with explant experiments in chick, which showed that prechordal plate cells can induce the forebrain marker *tailless* (Storey et al., 1995). Similarly, a positive signal from anterior mesendoderm can stabilize expression of *otx2* in the rostral neural plate, whereas a negative signal from posterior mesendoderm represses *otx2* posteriorly (Ang et al., 1994).

These observations may represent cases of 'vertical induction' of a-p pattern, the signal having an instructive effect. Alternatively, prechordal plate signaling may help the overlying neural plate to execute its a-p positional information derived from planar signaling. For example, subregions of the prechordal plate may induce proper dorsoventral (d-v) pattern in the overlying cephalic neural plate, as the notochord does in the trunk and tail. Consistent with this possibility, a signal emanating from the midline can partition the eye primordium into medial optic stalk and lateral retina precursors. This signal is absent in *cyc* and can be mimicked by *shh* misexpression. In its absence, medial tissue of the eye primordium is transformed to a lateral character, resulting eventually in the fused, cyclopic eyes of the mutant (Macdonald et al., 1995). Further experiments will be needed to resolve this issue.

A new midline function in development of the neurocranium?

We have observed fusions of the ventral neurocranium (otocephaly, approximation of the ears; De Beer, 1937) in all tested mutants of the midline group. Such phenotypes were not observed among the jaw mutants found in our screen, even if the embryos showed signs of retardation (Piotrowski et al., 1996; Schilling et al., 1996). Most of the midline mutants affect the ventral neurocranium along its entire length. A key observation is that in *sur* mutants, the defects are confined to the posterior neurocranium, leaving the anteriorly located ethmoid plate unaffected. This finding correlates with our other observations, which indicate that the forebrain and anterior prechordal plate in *sur* embryos are normal (see above). To test whether this correlation extends to other mutants, we looked at the neurocranium phenotype of *silberblick* mutants, which have anterior forebrain defects that lead to slight cyclopia (Heisenberg et al., 1996). As predicted, we found that the ethmoid plate in *silberblick* mutants is strongly narrowed, whereas the more posterior trabeculae are normal (not shown).

In chicken, the neurocranium is derived from neural crest cells and from somitic cells, both of which originate at more posterior levels, away from the midline. They then migrate anteriorly to their target areas; in zebrafish, this may occur similarly, but this has not been proven (Noden, 1984; Couly et al., 1993). It is also largely unknown which factors cause neural crest cells to be correctly positioned at their target locations in the neurocranium, and what initiates their differentiation (Thorogood, 1988). Interestingly, the notochord has been shown to deflect migration or inhibit differentiation of crest cells (Pettway et al., 1990), suggesting that axial mesoderm could be more generally involved. Taken together, our observations raise the exciting possibility that local, region-specific signals in the midline, either from the brain or the underlying mesendoderm, pattern the neurocranium.

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