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Mutations affecting the development of the embryonic zebrafish brain

Alexander F. Schier*, Stephan C. F. Neuhauss, Michele Harvey, Jarema Malicki, Lilianna Solnica-Krezel, Didier Y. R. Stainier†, Fried Zwartkruis‡, Salim Abdelilah, Derek L. Stemple, Zehava Rangini§, Hong Yang and Wolfgang Driever**

Cardiovascular Research Center, Massachusetts General Hospital and Harvard Medical School, 149 13th Street, Charlestown, MA 02129, USA

*Present address: Skirball Institute of Biomolecular Medicine, New York University Medical Center, 550 First Avenue, New York, NY 10016, USA
‡Present address: Laboratory for Physiological Chemistry, Utrecht University, 3584 CG Utrecht, The Netherlands
†Present address: Department of Biophysics and Biochemistry, University of California San Francisco, CA 94143, USA
§Present address: Department of Oncology, Sharett Institute, Hadassah Hospital, Jerusalem 91120, Israel
**Author for correspondence (e-mail: driever@helix.mgh.harvard.edu)

SUMMARY

In a large scale mutagenesis screen for embryonic mutants in zebrafish, we have identified 63 mutations in 24 loci affecting the morphogenesis of the zebrafish brain. The expression of marker genes and the integrity of the axonal scaffold have been studied to investigate abnormalities in regionalization, neurogenesis and axonogenesis in the brain. Mutants can be broadly classified into two groups, one affecting regionalization along the anterior-posterior or dorsal-ventral axis, and the other affecting general features of brain morphology. The first group includes one locus that is required to generate the anlage of the midbrain-hindbrain boundary region at the beginning of somitogenesis. Four loci were identified that affect dorsal-ventral patterning of the brain, including the previously described *cyclops* locus. Mutant embryos of this class show a reduction of ventral neuroectodermal structures and variable fusion of the eyes. The second group includes a large class of mutations affecting the formation of brain ventricles. Analysis of this class reveals the requirement of a functional cardiovascular system for ventricle enlargement during embryogenesis. Mutations in one locus lead to the formation of supernumerary primary neurons, a phenotype reminiscent of neurogenic mutants in Drosophila. Other mutant phenotypes described here range from abnormalities in the fasciculation and outgrowth of axons to defects in the diameter of the neural tube. The identified loci establish the genetic foundation for a further analysis of the development of the zebrafish embryonic brain.

Key words: zebrafish, brain, neuroectoderm, cyclopia, cerebellum, ventricle, neurogenesis, axonogenesis

INTRODUCTION

Embryonic development of the vertebrate brain involves several steps. First, the neural plate forms on the dorsal side of the embryo and is regionalized along the anterior-posterior and dorsal-ventral axes. The formation of the neural tube from the neural plate and the development of brain ventricles then contribute to the typical morphology of the embryonic brain. Finally, the differentiation of neurons and glia and the establishment of proper synaptic connections lead to functional circuitry in the nervous system.

Embryological studies have established that the formation and regionalization of the central nervous system result from a series of inductive interactions between different regions in the embryo (Doniach, 1992; Jessell and Dodd, 1992; Kintner, 1992; Ruiz i Altaba, 1993). Initially, signals from dorsal mesoderm neuralize and pattern the adjacent ectoderm along the anterior-posterior axis. Signals from the axial mesoderm, including the notochord, and from the nonneural ectoderm specify dorsal-ventral regions in the neuroectoderm. Local interactions within the neural plate lead to further regionalization. For instance, the region of the midbrain-hindbrain boundary seems to serve as a source of activity involved in patterning the adjacent midbrain (Alvarado-Mallart, 1993; Marin and Puelles, 1994; Bally-Cuif and Wassef, 1995; Joyner, 1996). Once established, some of these domains, e.g. the hindbrain rhombomeres, seem to behave as domains of cell-lineage restriction (Lumsden, 1990; Krumlauf et al., 1993; Keynes and Krumlauf, 1994). The embryological mechanisms involved in the subsequent formation of the neural tube and brain ventricles are less well understood. Both intrinsic and extrinsic forces seem to contribute to neurulation (Schoenwolf and Smith, 1990), whereas cerebrospinal fluid pressure appears to be an important requirement for the enlargement of the embryonic brain (Desmond and Jacobson, 1977).

In recent years several molecules have been implicated in these developmental processes. Signaling molecules like noggin (Harland, 1994), follistatin (Kessler and Melton, 1994) and chordin (Sasai et al., 1994) are candidates for neural inducers. Transcription factors like krox-20 or members of the *otx*, *emx*, *pax*, *engrailed* and *Hox* gene families and secreted factors like wnt-1 and sonic hedgehog have been suggested to

be involved in the regionalization of the brain (Finkelstein and Boncinelli, 1994; Joyner and Guillemot, 1994; Krumlauf, 1994; Smith, 1994; Stuart et al., 1994; Ingham, 1995). Adhesion molecules like N-cadherin or NCAM have been implicated in neural tube formation (Papalopulu and Kintner, 1994).

Genetic approaches provide a critical test for the postulated role of these molecules in the formation of the brain (Rossant and Hopkins, 1992; Joyner and Guillemot, 1994). Indeed, the analysis of mice with targeted mutations in *wnt-1* (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), *pax5* (Urbanek et al., 1994), *engrailed-1* and *engrailed-2* (Joyner et al., 1991; Wurst et al., 1994), *krox-20* (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993) and *Hoxa-1* (Carpenter et al., 1993; Dolle et al., 1993) have demonstrated the essential role of these loci in the regionalization of the anteroposterior axis of the embryonic brain. In contrast, in the case of *NCAM* (Cremer et al., 1994; Ono et al., 1994) and *follistatin* (Matzuk et al., 1995), mutant mouse embryos do not show defects that directly support the postulated roles of these factors.

Classical genetic studies have also led to the identification of mutations affecting murine brain development (Lyon and Searle, 1989). *Kreisler* and *swaying* (an allele of *wnt1*) mutant embryos show defects in the regionalization of the brain (Lane, 1967; Frohman et al., 1993; McKay et al., 1994), and the *reeler* mutation results in the malpositioning of neurons in the brain (Rakic and Caviness, 1995). The recent molecular isolation of the genes affected in these mutants demonstrates the potential of the classical genetic approach for the identification of essential components of brain development (Thomas et al., 1991; Cordes and Barsh, 1994; D'Arcangelo et al., 1995).

The relative simplicity of the embryonic zebrafish (*Danio rerio*) brain, and the powerful embryological and genetic methodology applicable to its analysis, promise further insights into vertebrate brain morphogenesis (Wilson and Easter, 1992; Kimmel, 1993; Driever et al., 1994). During zebrafish embryogenesis the brain rudiment is already visible at the end of gastrulation (9 hours after fertilization) as a distinctly thickened structure. Within the next 20 hours the primary organization and morphology of the zebrafish brain is established (Kimmel et al., 1995), neural primordia become regionalized (Fjose, 1994; Woo and Fraser, 1995), the neural canal and brain ventricles form (Papan and Campos-Ortega, 1994), and neuronal differentiation and axonogenesis lead to a highly stereotyped axonal scaffold (Chitnis and Kuwada, 1990; Wilson et al., 1990; Ross et al., 1992). By 28 hours postfertilization (hpf) the zebrafish brain has the typical morphology of embryonic vertebrate brains (Fig. 1). Furthermore, the neuroectodermal fate map (Woo and Fraser, 1995), the expression patterns of genes like *engrailed* (Davis et al., 1991; Hatta et al., 1991a; Ekker et al., 1992; Fjose et al., 1992) and *sonic hedgehog* (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994), and the position of axon tracts (Easter et al., 1994), are very similar to other vertebrates, supporting the notion of evolutionarily conserved mechanisms of morphogenesis in the vertebrate neuroectoderm.

Genetic studies in zebrafish have identified one mutation affecting early brain development, the *cyclops* mutation (Hatta et al., 1991b). The main features of *cyclops* mutant embryos are the absence of ventral neuroectodermal structures and fusion of the eyes. Genetic mosaics have indicated that the *cyclops* gene product is cell-autonomously required for the formation of ventrally located cells in the neuroectoderm, both in the forebrain and more posteriorly in the floor plate (Hatta et al., 1991b, 1994). These midline defects lead indirectly to aberrant axonal patterning in the medial longitudinal fascicles (Bernhardt et al., 1992; Hatta, 1992). The studies on *cyclops* have highlighted the potential of a combined embryological and genetic approach in zebrafish to study vertebrate brain development.

In a large-scale screen for mutations affecting zebrafish embryogenesis, we have identified more than 60 mutations affecting the morphology of the embryonic zebrafish brain. Mutant phenotypes range from defects in the regionalization of the dorsal-ventral and anterior-posterior axis of the neuroectoderm to abnormalities in the formation of neurons, fasciculation of axons and integrity of brain ventricles.

MATERIALS AND METHODS

Mutations were induced by the mutagen N-ethyl-N-nitrosurea (ENU) and recovered in an F_2 screen as described (Solnica-Krezel et al., 1994; Driever et al., 1996). 2383 embryonic and early larval lethal mutations were identified, 63 of which are described here. All embryos were maintained at 28.5°C and staged according to Kimmel et al. (1995).

Phenotypic analysis

Embryos were initially observed under a dissecting microscope. For further analysis and photographic documentation, embryos were dechorionated, anesthetized in 0.02% 3-aminobenzoic acid methyl ester (Sigma), embedded in 2% methylcellulose and photographed either under a dissecting microscope or using differential interference contrast (DIC/Nomarski) optics on a Zeiss Axiophot microscope (Westerfield, 1994).

Whole-mount in situ hybridization was performed with digoxigenin-labeled RNA probes (Oxtoby and Jowett, 1993). Expression patterns were documented after clearing in benzylbenzoate/benzyl alcohol (2:1) and mounting in Permount (Fischer Scientific). The following probes have been used: *krox-20* (Oxtoby and Jowett, 1993); *pax[zf-b]* (Krauss et al., 1991a; Püschel et al., 1992b); *pax6* (Krauss et al., 1991b,c; Püschel et al., 1992a); *hlx1* (Fjose et al., 1994); *wnt1* (Krauss et al., 1992a; Kelly et al., 1993); *dlx2* (Akimenko et al., 1994); *engrailed-2* (Ekker et al., 1992; Fjose et al., 1992); *sonic hedgehog* (Krauss et al., 1993); *rtk1* (Xu et al., 1994); *islet1* (Inoue et al., 1994). Antisense RNA probes were used to analyze the following structures: *krox20*, rhombomeres 3 and 5; *pax[zf-b]*, midbrain-hindbrain boundary region, optic stalk, otic vesicles, pronephros, a subset of commissural interneurons; *pax6*, optic vesicles, diencephalon, rhombencephalon (anterior border in middle of rhombomere 1), spinal cord; *hlx1* at 28-32 hpf, thin stripes adjacent to interrhombomeric boundaries, tegmentum, tectum, part of dorsal thalamus, part of ventral thalamus; *wnt1*, dorsal neuroectoderm, anterior portion of midbrain-hindbrain boundary region; *dlx2*, pharyngeal arch primordia, subregion of telencephalon and diencephalon, pectoral fin bud; *engrailed-2*, midbrain-hindbrain boundary region, muscle pioneers, jaw muscle precursors; *sonic hedgehog*, ventral neuroectoderm including hypothalamus and floor plate; *rtk1*, rhombomeres 1, 3, and 5; *islet1*, primary neurons including a subset of motorneurons, Rohon-Beard neurons, a subset of interneurons, trigeminal ganglion neurons, acoustic nerve ganglion neurons, epiphysial neurons, polster.

Immunocytochemistry with monoclonal antibodies anti-acetylated α-tubulin (Piperno and Fuller, 1985) and *3A10* (Furley et al., 1990; Hatta, 1992) was performed after fixation for 1-2 hours at room tem-

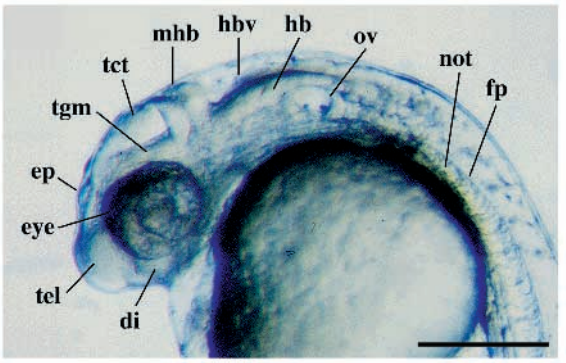

Fig. 1. Morphology of the embryonic zebrafish brain at 28 hours after fertilization. The following structures can be identified in living zebrafish embryos under dissecting stereomicroscopes: tel, telencephalon; di, diencephalon with the ventrally located hypothalamus and the epiphysis (ep); tgm, tegmentum; tct, tectum and tectal ventricle; mhb, midbrain-hindbrain boundary. Based on cell fate studies in amniotes and gene expression analysis, it is likely that the cerebellum is one of the derivatives of the region of the midbrain-hindbrain boundary. hb, hindbrain; hbv, hindbrain ventricle; eye with lens; ov, otic vesicle with two otoliths. The otic vesicle is located lateral to rhombomere 5. not, notochord. The notochord extends anteriorly to the level of the otic vesicle. fp,floor plate. The floor plate extends anteriorly into the caudal diencephalon. Here and in all other figures anterior is to the left and dorsal is up, except where indicated. Scale bar, 250 μm.

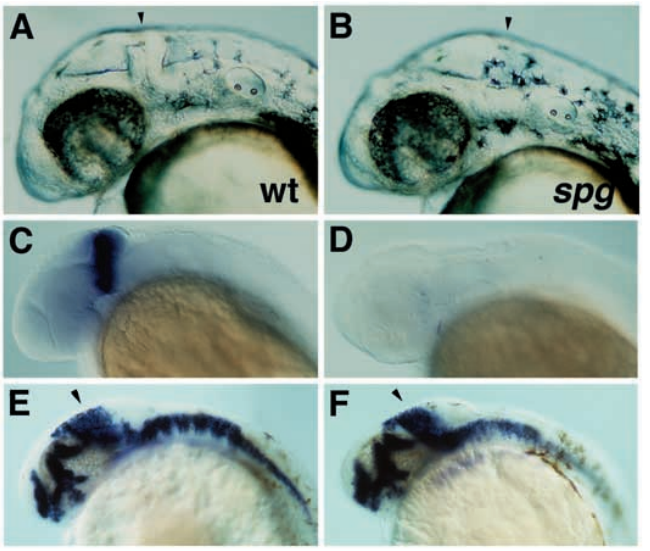

Fig. 2. Phenotype of *spiel ohne grenzen (spg)* mutants on day 2 of development. (A,B) DIC image of wild-type (A) and *spg*^{m216} mutant (B) embryos at 30 hpf. Arrowhead indicates the position of midbrain-hindbrain boundary. (C,D) Expression of *engrailed-2* in wild-type (C) and strong *spg*^{m216} mutant (D) embryos at 28 hpf. Weaker *spg*^{m216} mutants retain a small dorsal patch of *engrailed-2* expression. (E,F) Expression of *dlx2* and *hlx1* in wild-type (E) and *spg*^{m216} mutant (F) embryos at 28 hpf. Arrowhead indicates position of the prospective tectum.

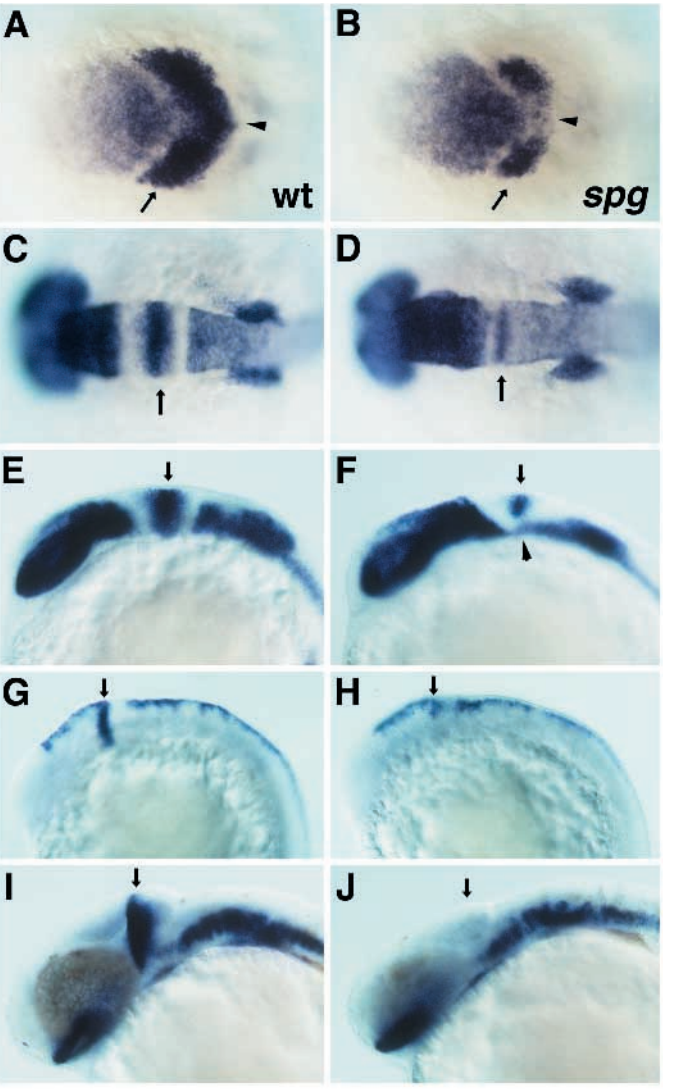

Fig. 3. Phenotype of *spiel ohne grenzen (spg)* mutants during somitogenesis. (A,B) Expression of *pax[zf-b]* (arrow) in the region of the midbrain and presumptive midbrain-hindbrain boundary region and *pax6* (anterior to *pax[zf-b]* stripe) in the forebrain of wild-type (A) and *spg*^{m216} mutant (B) embryos at the 1-somite stage; dorsal view. Note the reduced medial expression domain of *pax[zf-b]* (arrowhead). (C,D) Expression of *pax[zf-b]* at the midbrain-hindbrain boundary (arrow) and *pax6* (forebrain, eye anlage and hindbrain) in wild-type (C) and *spg*^{m216} mutant (D) embryos at the 10-somites stage; dorsal view. (E,F) Lateral view of embryos in C and D, respectively. Note the absence of ventral *pax[zf-b]* expression at the midbrain-hindbrain boundary and the shift of the *pax6* expression domains in forebrain and hindbrain with respect to each other (arrowhead). (G,H) Expression of *wnt1* in wild-type (G) and *spg*^{m216} mutant (H) embryos at the 14-somites stage. Note the reduction of *wnt1* expression at the midbrain-hindbrain boundary (arrow). (I,J) Expression of *pax[zf-b]* (optic stalk, midbrain-hindbrain boundary (arrow), hindbrain) in wild-type (G) and *spg*^{m216} mutant (H) embryos at 26.5 hpf.

perature in 4% paraformaldehyde as described (Solnica-Krezel and Driever, 1994).

For methacrylate sections, embryos were fixed in paraformaldehyde (4%, overnight), dehydrated in ethanol and embedded in JB-4 resin (Polyscience Inc.). 5 μm sections were cut on a Leica 2065 microtome.

Embryos were photographed on 160 ASA Ektachrome Tungsten Film. Images from slides were scanned on a Kodak Professional

RFS2035 Plus Film Scanner. Figures were assembled using Adobe Photoshop 3.0 software (Adobe Corporation).

Mutations affecting the formation of pharyngeal arches, *mother superior*^{m188}, *quadro*^{m271}, *little richard*^{m433}, *mont blanc*^{m610} (Neuhauss et al., 1996) or the size of the ear, *quadro*^{m271}, *m471, m574, helter skelter*^{m504}, *golas*^{m618} (Malicki et al., 1996b) were also analyzed for defects in hindbrain patterning by morphological and gene expression analysis using the following markers: *hlx1* and *dlx2* (for mutants affecting pharyngeal arches and/or ear), *rtk1* (for ear mutants), and *krox20* and *pax[zf-b]* (for pharyngeal arch mutants). No clear abnormalities could be identified in the rhombencephalon of mutant embryos.

Genetic analysis

In order to test allelism of isolated mutations, complementation analysis among members of the phenotypically defined groups of mutations was performed. Complementation between two mutations was tested by crossing identified heterozygous parents of each mutation and screening their offspring for the mutant phenotype. A minimum of 30 embryos per complementation cross was analyzed. All mutations segregate as mendelian recessive loci. Limited complementation has been performed with mutations of similar phenotypes isolated in Tübingen. The following loci have been identified in both screens: *oep, cyc, boz, snk, sly, gup, bal*. *spg*^{m216} was found to complement the midbrain-hindbrain boundary mutants *noi* and *ace* that were isolated in Tübingen.

RESULTS

Identification of mutations affecting the embryonic brain

In a systematic F_2 screen for mutations affecting zebrafish development, the morphology of the brain of living zebrafish embryos was examined at days 1, 2 and 3 of development with dissecting stereo microscopes. At these stages, the size and shape of telencephalon, diencephalon, tectum, tegmentum, midbrain-hindbrain boundary, hindbrain and brain ventricles can be scored (Fig. 1). More subtle features (e.g. sub regions of the hindbrain or particular neurons) are not identifiable at this level of analysis. Among 2383 embryonic and larval lethal mutations identified, we have isolated 63 mutations constituting 24 loci that lead to abnormal brain morphology by 28 hours postfertilization (hpf). An additional 50 mutations lead to CNS degeneration during somitogenesis and are described in an accompanying paper (Abdelilah et al., 1996).

Here we describe the genetic and phenotypic characterization of mutations affecting brain morphogenesis. Mutant embryos were analyzed using dissecting microscopes and compound microscopes with Nomarski interference contrast illumination and with molecular markers. Mutations with similar phenotypes were tested for complementation (see Table 1). The general features of identified brain mutants are described in Table 1. Mutants can be broadly classified into two groups, one affected in regionalization along the anterior-posterior or dorsal-ventral axis of the neuroectoderm, and the other affected in general morphological features of the brain. These phenotypes are described in detail below.

Mutations affecting anterior-posterior patterning

The midbrain-hindbrain boundary (MHB) region consists of the posteriormost midbrain and the anteriormost hindbrain region, also including the cerebellum (Fig. 1). We have identified one locus, *spiel ohne grenzen* (*spg*^{m216} and *spg*^{m308}), that is required for the formation of this region. Morphological inspection (Fig. 2B) and the aberrant expression of *pax[zf-b]* (a member of the pax-2/5/8 family; Fig. 3J) and *engrailed-2* (Fig. 2D) at the MHB indicate that a large portion of the MHB region is deleted in *spg* mutants at 28 hpf. Phenotypes range from the absence of the ventral portion to a complete deletion of this region. Both the adjacent prospective tectum and posterior hindbrain are present, as judged from both morphological observations, as well as the expression patterns of *hlx1* (Fig. 2F) and *krox20* (data not shown); however, more subtle defects are visible. *Hlx1* expression in the hindbrain of *spg* mutants appears less distinct than in wild type (Fig. 2F), and the otic vesicles are reduced in some mutant embryos.

Gene expression, fate mapping and transplantation studies indicate that the anlage of the MHB region is established during the end of gastrulation and at the beginning of somitogenesis (Hatta et al., 1991a; Krauss et al., 1991a; Püschel et al., 1992b; Alvarado-Mallart, 1993; Oxtoby and Jowett, 1993; Marin and Puelles, 1994; Woo and Fraser, 1995). To determine when the *spg* defect becomes apparent, embryos were analyzed for the expression of *pax[zf-b]* and *pax6* (expressed in forebrain and hindbrain) at the beginning, middle and end of somitogenesis (Fig. 3). Already at the beginning of somitogenesis (1-somite stage), *pax[zf-b]* but not *pax6* shows an aberrant expression pattern in *spg*^{m216} mutant embryos (Fig. 3B). *Pax[zf-b]* expression in the MHB anlage is limited in its anterior-posterior extent and reduced in the medial (future ventral) region of the neural plate. Other domains of *pax[zf-b]* expression are not affected. At the 10-somite stage, the *pax[zf-b]* expression domain is severely restricted and absent ventrally (Fig. 3D,F). Concomitantly, the expression domains of *pax6* in the forebrain and hindbrain are shifted closer to each other, nearly touching ventrally. Furthermore, the rostral boundary of *pax6* expression in the hindbrain is affected, suggesting that the *spg* phenotype extends into rhombomere 1. Consistent with reduced *pax[zf-b]* expression, the domains of *wnt1* (Fig. 3H) and *engrailed-2* (data not shown) are also severely reduced in the MHB region at mid-somitogenesis. By 26 hpf *pax[zf-b]* expression in the MHB region is lost in most mutant embryos (Fig. 3J). We conclude that *spg* is required for the development of the anlage of the MHB region as early as at the beginning of somitogenesis.

Mutations affecting dorsal-ventral patterning

We have identified mutations in four loci that affect the dorsal-ventral patterning of the brain. Single alleles of the *one-eyed-pinhead* (*oep*^{m134}; Schier et al., unpublished data; Strähle et al., unpublished data), *uncle freddy* (*unf*^{m768}) and *bozozok* (*boz*^{m168}) loci and three alleles of the previously described *cyclops* locus (*cyc*^{m101}, *cyc*^{m122}, *cyc*^{m294}; Hatta et al., 1991b) have been isolated. At 28 hpf all mutants show variable fusion of the eyes, and ventral neuroectodermal structures like the hypothalamus and floor plate are reduced (Fig. 4). *oep*^{m134} embryos have one, often smaller eye (cyclopia). *cyc*^{m294} and *cyc*^{m122} behave like the previously identified *cyc*^{b16} allele and show partial fusion of the two eyes (synopthalmia), whereas *cyc*^{m101} seems to be a weaker allele, often resulting in eyes that are closer to each other antero-ventrally, but not fused. *boz*^{m168} and *unf*^{m768} show rather variable defects, ranging from synopthalmia to normal eyes. The reduction of ventral neuroecto-

Table 1. Mutations affecting the morphology of the embryonic zebrafish brain

Locus	Alleles	Brain phenotype at 30 hours	Other phenotypes	References	tu
Group I: Anterior-posterior					
spiel ohne grenzen (spg)	*m216, m308*	Midbrain-hindbrain boundary region reduced	Ventral curvature, 1 otolith (low expressivity)		nt
Group II: Dorsal-ventral					
cyclops (cyc)	*m101, m122, m294*	Eye fusion, ventral deficiencies including floor plate	Prechordal plate, curved body	a, e, h	t
one-eyed-pinhead (oep)	*m134*	Strong eye fusion, ventral deficiencies including floor plate	Prechordal plate, curved body	b, c, e	t
bozozok (boz)	*m168*	Very variable eye fusion and ventral deficiencies including floor plate	Notochord, prechordal plate	c, d, e	t
uncle freddy (unf)	*m768*	Very variable eye fusion and ventral deficiencies including floor plate	Curved body	c, d	
Group III: Brain and notochord					
sleepy (sly)	*m86, m91, m99, m152, m253, m388, m466, m515, m516, m707*	Brain irregularly shaped, hindbrain ventricle enlarged	Notochord fails to vacuolate eye defects	d	t
bashful (bal)	*m102, m113, m190, m255, m268, m277, m290, m296, m430, m473, m373*	Brain irregularly shaped, hindbrain ventricle enlarged	Notochord fails to vacuolate eye defects	d	t
grumpy (gup)	*m135, m189, m217, m726, m753*	Brain irregularly shaped, hindbrain ventricle enlarged	Notochord fails to vacuolate eye defects	d	t
Group IV: Ventricles					
fullbrain (ful)	*m133, m157*	Ventricles reduced	Heart, circulation, delayed, reduced touch response		t
zonderzen (zon)	*m163, m670*	Ventricles reduced (transient)	Heart, circulation, delayed, reduced touch response, variable reduction or absence of pectoral fins, recover on d2		
glaca (glc)	*m309*	Ventricles reduced	Heart, circulation, delayed, no touch touch response		
white snake (wis)	*m427*	Ventricles reduced	Heart, circulation, delayed, no touch response		t
kuehler kopf (kuk)	*m484*	Ventricles reduced	Heart, circulation, very reduced touch response		
landfill (lnf)	*m528, m551*	Ventricles reduced	Heart, circulation, delayed, reduced touch response		
logelei (log)	*m628, m673*	Ventricles reduced	Heart, circulation, delayed, reduced touch response		
turned down (twn)	*m359*	Ventricles reduced	Heart, circulation, curved ventrally, delayed, reduced touch response, pigmentation		
eraserhead (esa)	*m725*	Ventricles reduced (variable)	Heart, circulation, delayed, reduced touch response		
snakehead (snk)	*m115, m273, m523*	Ventricles severely reduced, unstructured morphology, thin neural rod	Heart, circulation, body pigmentation delayed, no touch response, turbid yolk, ear undifferentiated		t
Group V: Ventricles and pigmented epithelium					
oko meduzy (ome)	*m98, m289, m298, m320*	Ventricles reduced	Pigmented epithelium, circulation, heart	e	
nagie oko (nok)	*m227, m520*	Ventricles severely reduced	Pigmented epithelium, circulation, heart	e	
heart and soul (has)	*m129, m567, m781*	Ventricles reduced	Pigmented epithelium, circulation, heart	e, f	
Group VI: Neurogenesis					
mind bomb (mib)	*m132, m178*	Supernumerary primary neurons, irregular hindbrain, reduced hindbrain vetricle	Reduced circulation, irregular touch response, notochord, tail, ear, less melanocytes, somite borders less distinct	c, g	t
Miscellaneous:					
flachland (fll)	*m517*	Hindbrain neural tube thinner, reduced ventricles	Heart, circulation, slight delay, ear rounder and smaller with 1 otolith	g	
turned on (tun)	*m357*	Hindbrain ventricle slightly reduced, day 5 head tilted dorsally at level of hindbrain	Curved dorsally		

Detailed phenotypic aspects are described in (a) Hatta et al. (1991); (b) Schier et al. (unpublished) and Strähle et al., (unpublished). Other phenotypic aspects are described in (c) Solnica-Krezel et al. (1996); (d) Stemple et al. (1996); (e) Malicki et al. (1996a); (f) Stainier et al. (1996); (g) Malicki et al. (1996b); (h) Thisse et al. (1994).

t: allelic to Tübingen mutant (tu), name unified; nt: complements all Tübingen loci with similar phenotype.

Mutants within groups but not between groups were tested for complementation, with the exception of *turned down*^{m359}, which was not tested against any other loci.

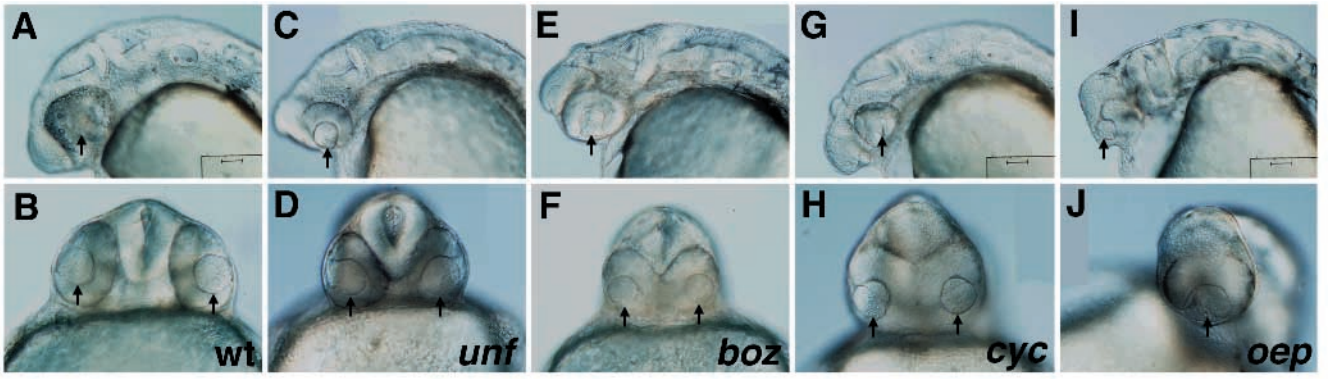

Fig. 4. Phenotypes of mutations affecting the formation of ventral neuroectoderm on day 1 of development. Lateral (A,C,E,G,I) and anterior-ventral (B,D,F,H,J) views of wild-type (A,B), *uncle freddy (unf)*^{m768} (C,D), *bozozok (boz)*^{m168} (E,F), *cyclops (cyc)*^{m122} (G,H) and *one-eyed-pinhead (oep)*^{m134} (I,J) mutant embryos at 28 hpf. Arrows indicate the position of the lens.

derm in more severely affected *unf*^{m768} embryos is reflected in the partial loss of *sonic hedgehog* expression (Fig. 5B). In addition, the axonal scaffold at the midline of *unf*^{m768} mutant embryos is severely disrupted. The axons of the medial longitudinal fascicles are disorganized and fused at the ventral hindbrain midline (Fig. 5D). These defects are very similar to the defects described for *cyclops* mutant embryos (Hatta, 1992).

Mutations affecting notochord and brain

Mutants at the three loci *sleepy* (*sly*, 10 alleles), *bashful* (*bal*, 11 alleles) and *grumpy* (*gup*, 5 alleles) have similar notochord (Stemple et al., 1996) and brain phenotypes (Fig. 6). The entire brain is abnormally shaped and folded, and the hindbrain ventricle is enlarged. The eyes of mutant embryos are slightly smaller and tilted ventrally. Despite these gross morphological malformations, the general anterior-posterior and dorsal-ventral patterning appears normal (Fig. 6M-P). Detailed inspection of *hlx1* (Fig. 6Q-T) and *sonic hedgehog* (Fig. 6I-L) expression at 29 hpf reveals subtle defects that might reflect the abnormal architecture of mutant brains. The *sonic hedgehog* domain seems more irregular in *sly*^{m86}, *bal*^{m190} and *gup*^{m189}, and there is an expansion in the region of the midbrain-forebrain boundary. *Hlx1* expression in the hindbrain is less distinct. Most strikingly, while the axonal scaffold is present, fewer axons and non fasciculated axons are present in mutants at these loci (Fig. 6U-X).

Mutations affecting ventricle formation

The ventricles of the brain start to inflate at about 17 hpf (Papan and Campos-Ortega, 1994; Kimmel et al., 1995). Parallel to the onset of circulation at about 24 hpf, ventricles enlarge further and by 28 hpf the inflated ventricles contribute to the characteristic morphology of the embryonic brain (Fig. 1). We have identified a large number of mutations that lead to reduced brain ventricles, ranging from a slight reduction to a complete absence of ventricle inflation. The weakest mutants, with a partial reduction of ventricle size, include heart mutations (Stainier et al., 1996) like *silent heart* (non-beating heart, Figs 7F, 8B,F), *cloche* (no endocardium), or *bonnie and clyde* (cardia bifida). All these mutants lack circulation and show a reduction in ventricle enlargement. Mutations in the loci *fullbrain* (Figs 7E, 8C,G), *glaca* (Fig. 7B), *white snake* (Fig. 7C), *landfill* (Fig. 7J), *logelei* (Fig. 7L), *turned down* (Fig. 7I) and *eraserhead* (Fig. 7K) result in more severely reduced ventricles (Table 1). Circulation in the head is absent in these mutants. In addition, development appears generally delayed, and tactile sensitivity is impaired. *snakehead* (*snk*, 3 alleles) mutants show the most severe form of ventricle phenotype (Figs 7D, 8D,H). The brain appears flat, unstructured and reduced in diameter. No ventricles are present at 30 hpf, only a very thin neural canal (Fig. 8D,H). In contrast to the ventricle mutants described above, the ventricle phenotype of *snakehead* mutant embryos is not only stronger but also readily identifiable before 24 hpf (data not shown), i.e. before the onset of circulation. Marker analysis indicates that the brain is normally patterned (Fig. 8I-L) and an axonal scaffold develops (data not shown).

zonderzen mutant embryos (Fig. 7G) develop normal circulation at the end of day 1 or on day 2 of development. Despite the initial reduction of ventricles, the brain goes on to develop normally. In all other ventricle mutants circula-

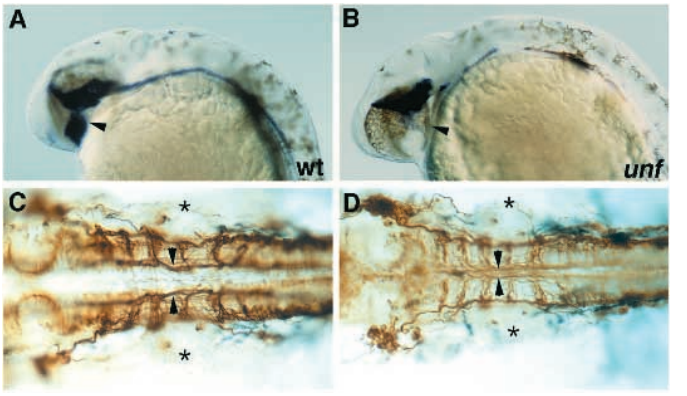

Fig. 5. Phenotypic analysis of *uncle freddy (unf)*^{m768} mutants. (A,B) Expression of *sonic hedgehog* in hypothalamus (arrowhead) and floor plate in wild-type (A) and *unf*^{m768} mutant (B) embryos at 29 hpf. (C,D) Medial longitudinal fascicles (arrows), lateral longitudinal fascicles and commissures in hindbrain stained with antibody against acetylated tubulin in wild-type (C) and *unf*^{m768} mutant (D) embryos at 29 hpf. Note the partial fusion of the medial longitudinal fascicles at the midline of the *unf*^{m768} mutant embryo. Asterisks indicate the position of the otic vesicles.

tion is permanently affected, and the brain is smaller and starts to degenerate after two or three days of development (Fig. 8N-P).

Mutations affecting neurogenesis

We have identified one locus, *mind bomb* (*mib*^{m132} and *mib*^{m178}), that affects neurogenesis in the entire nervous system (Fig. 9). Analysis of *islet1* expression, a marker for a subset of primary neurons (Korzh et al., 1993; Inoue et al., 1994) reveals a dramatic increase in the number of cells expressing this gene in *mib* mutant embryos (Fig. 9D,F). The anterior-posterior and dorsal-ventral position of these dense neuronal clusters is normal, indicating that the supernumerary *islet-1* positive cells do not form at ectopic sites but are localized in the regions of normal primary neuron formation (Fig. 9D,F). To determine if supernumerary cells differentiate as primary neurons or have

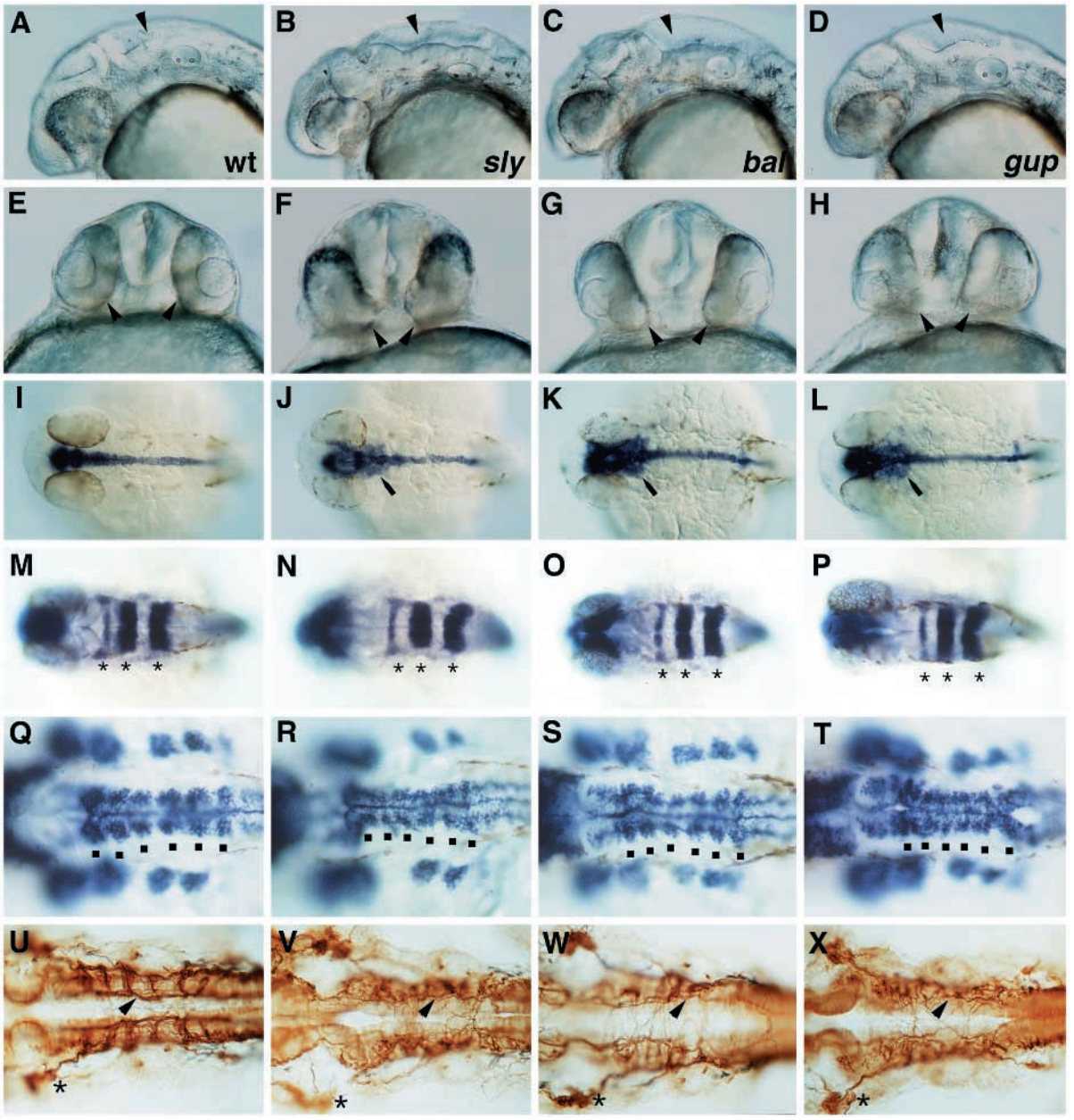

Fig. 6. Phenotypes of mutations affecting notochord and brain. (A,E,I,M,Q,U) wild type; (B,F,J,N,R,V) *sleepy (sly)*^{m86}; (C,G,K,O,S,W) *bashful (bal)*^{m190}; (D,H,L,P,T,X) *grumpy (gup)*^{m189}. Lateral (A,B,C,D) and anterior-ventral (E,F,G,H) view of embryos at 28 hpf. Note the enlarged hindbrain ventricles and the irregular morphology of the hindbrain (arrowhead in A-D) and the position of the eyes (arrowheads in E-H). (I,J,K,L) Expression of *sonic hedgehog* at 29 hpf; dorsal view. Note the lateral expansion posterior to the eye in mutant embryos (arrow in J,K,L). (M,N,O,P) Expression of *rtk1* in rhombomeres 1, 3 and 5 (asterisks). (Q,R,S,T) Expression orf *dlx2* in pharyngeal arch primordia and *hlx1* in hindbrain at 29 hpf; dorsal view. Note the slightly irregular expression domains of *hlx1* adjacent to rhombomere boundaries (dots) in mutant embryos. (U,V,W,X) Medial longitudinal fascicles (arrowhead in the region of rhombomere 5), lateral longitudinal fascicles and commissures in hindbrain stained with antibody against *acetylated tubulin* at 29 hpf. Dorsal view. Asterisks highlight the position of the trigeminal ganglion.

an aberrant fate, we analyzed the formation of Mauthner neurons in *mib* mutants (Fig. 9H). In wild-type embryos single Mauthner cells lie within the fourth rhombomere on either side of the midline. In contrast, multiple Mauthner cells differentiate at the same position in *mib* mutants, suggesting that *mib* is required to ensure the formation of the correct number of primary neurons during neurogenesis.

Miscellaneous

Two mutations do not fall in any of the above categories and are described here separately.

turned on^{m357} shows a weak reduction of the hindbrain ventricle at 30 hpf and a slight dorsal curvature of the tail (Fig. 10B). By day 6 of development the head is tilted backwards at the level of the hindbrain and the tail is curved onto itself (Fig. 10D). *Hlx1* and *dlx2* expression at 29hpf and *rtk1* expression at 14-somites appear normal (data not shown).

flachland^{m517} mutant embryos are characterized by a flat hindbrain region at 30 hpf (Fig. 11B). This phenotype is already manifest at the 9-somite stage by a thin and flat neural rod in the hindbrain region (Fig. 11D). *Krox20* and *pax[zf-b]* are expressed in rhombomeres 3 and 5 and in the MHB region, but the neural rod is severely reduced in diameter, particularly at the level of the hindbrain (Fig. 11F).

DISCUSSION

In a large scale screen for mutations affecting zebrafish embryogenesis, we have identified mutations in 24 loci that affect the morphogenesis of the zebrafish brain. The isolated mutations provide genetic entry points into diverse processes like anterior-posterior and dorsal-ventral patterning, ventricle formation, neurogenesis and axonogenesis. No mutations were isolated that impair the general induction of neuroectoderm or early neurulation.

There are several explanations for the identification of a relatively small number of mutations affecting brain morphogenesis. It is important to consider firstly, that our screen has not reached saturation. 10 of the 24 loci described here are represented by a single allele. Secondly, members of large gene families often have partially overlapping or redundant expression patterns and functions. Consequently, their exact role can only be uncovered in double or triple mutants (Rudnicki et al., 1993). Thirdly, neural inducers, patterning molecules and components required for neurulation might be maternally provided or haploinsufficient. Finally, our visual screen was designed to find rather drastic defects. Mutants with more subtle phenotypes might be isolated in future screens using molecular markers or behavioral assays. In the following we will discuss the potential role of identified loci with respect to vertebrate brain morphogenesis.

Formation of the midbrain-hindbrain boundary region

Fate map studies performed in amniotes have shown that the region of the midbrain-hindbrain boundary (MHB), i.e. the posterior region of the mesencephalon and the anteriormost portion of the metencephalon, gives rise to, among other structures, the cerebellum and isthmic nuclei (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990). This brain territory is also defined by the local expression of *pax[zf-b]* at the MHB, *wnt1* in the anterior portion of the MHB region, and

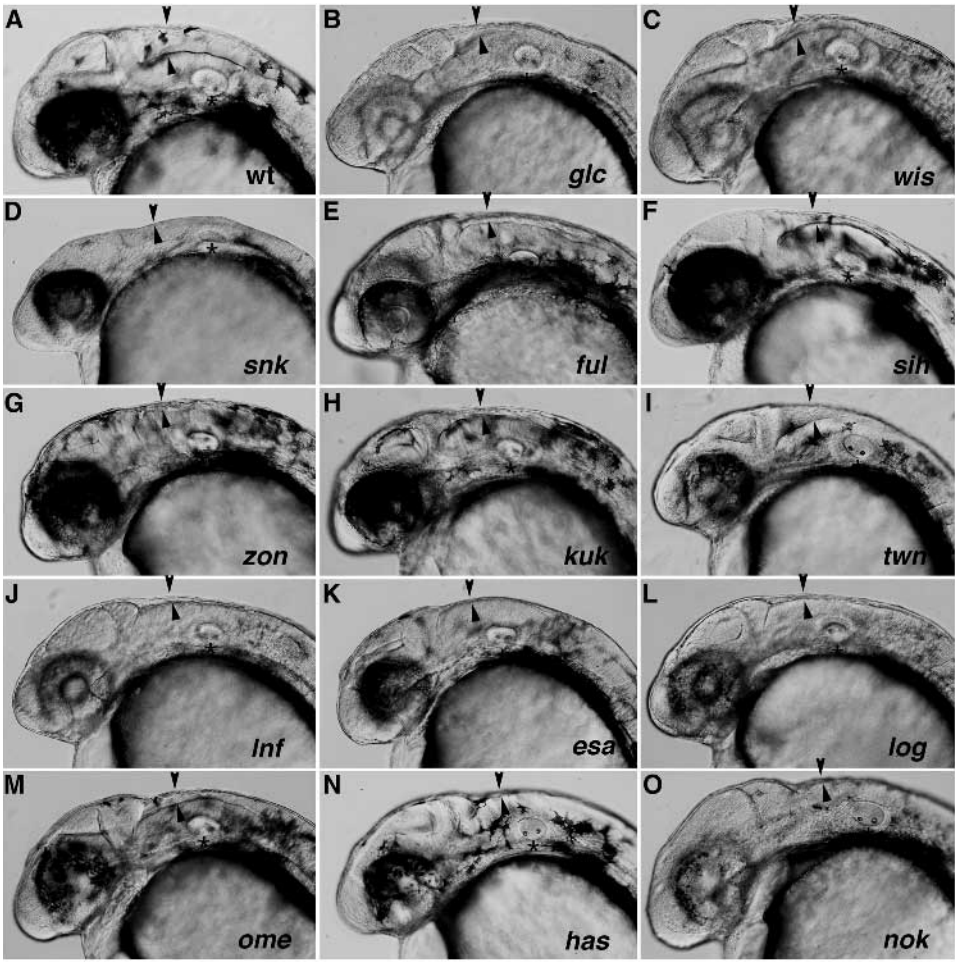

Fig. 7. Phenotypes of mutations affecting ventricle enlargement. (A) Wild type; (B) *glaca (glc)*^{m309}; (C) *white snake (wis)*^{m427}; (D) *snakehead (snk)*^{m273}; (E) *fullbrain (ful)*^{m133}; (F) *silent heart (sih)*^{b109}; (G) *zonderzen (zon)*^{m163}; (H) *kuehler kopf (kuk)*^{m484}; (I) *turned down (twn)*^{m359}; (J) *landfill (lnf)*^{m551}; (K) *eraserhead (esa)*^{m725}; (L) *logelei (log)*^{m628}/*log*^{m673} transheterozygous; (M) *oko meduzy (ome)*^{m98}; (N) *heart and soul (has)*^{m129}; (O) *nagie oko (nok)*^{m227} (Malicki et al., 1995a) between 30 and 33 hpf. Arrowheads outline the border of the hindbrain ventricle anterior to the otic vesicle (asterisk).

engrailed in the MHB region and fading towards the rostral mesencephalon (Wilkinson et al., 1987; Davis et al., 1991; Krauss et al., 1991a,b; Püschel et al., 1992b). Whereas it is not known how mid/hindbrain pattern is regulated in the fish, grafting and rotation experiments in the chick indicate that the MHB region is specified by the 10- to 14-somites stage and can induce ectopic *engrailed* expression and tectum formation in host tissue (Alvarado-Mallart, 1993; Bally-Cuif and Wassef, 1994, 1995; Marin and Puelles, 1994; Joyner, 1996).

We have isolated one locus, *spiel ohne grenzen (spg)*, that is required for the proper formation of the MHB region as early as at the beginning of somitogenesis. *spg* mutant embryos consistently lack most of the MHB region, but the adjacent prospective tectum and posterior hindbrain region seem intact, although we cannot exclude more subtle defects. The early deficit suggests that *spg* is required for the initial establishment of the MHB region. Alternatively, *spg* might be one of the first factors required for the maintenance of this brain region. Absence of the *spg* gene product might lead to defects in growth, survival or specification of cells in the anlage of the MHB. Since the *pax[zf-b]*-expressing region seems to be partially deleted in *spg*, and since there is no obvious cell death detectable in this region at 1- and 10-somite stages (unpublished results), we suggest that a specification and/or proliferation defect is responsible for the observed phenotype in *spg* mutants.

It is noteworthy that the injection of antibodies against the *pax[zf-b]* protein into zebrafish embryos causes a downregulation of *pax[zf-b]* transcripts and MHB defects similar to *spg* (Krauss et al., 1992b). It is conceivable that the *spg* phenotype might be in part a direct consequence of the reduced expression of *pax[zf-b]* in the MHB region (also see (Urbanek et al., 1994; Torres et al., 1995).

The *spg* phenotype is similar to the MHB region deficits observed in mouse embryos mutant for *wnt-1* or *engrailed-1* (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Wurst et al., 1994). Similar to *spg*, *en-1* and *wnt-1* mutant phenotypes are already apparent during early somitogenesis. Studies of *engrailed* expression in *wnt-1* mutant embryos suggest that the MHB anlage is initially established normally (1- to 4-somites stage) but then progressively deleted (McMahon et al., 1992). Similarly, the expression of *engrailed-2* in *en-1* mutant animals is already perturbed by the 10-somites stage and is restricted to a dorsal patch in the mid-hindbrain region by E9.5 (Wurst et al., 1994). The direct comparison of *wnt-1*, *en-1* and *spg* mutants is complicated by the variability of phenotypes. Strong *wnt-1* phenotypes involve the deletion of both the MHB region and tectum), a defect that is stronger than the *spg* phenotype. Weaker *wnt-1* phenotypes, however, are mainly restricted to deficits in the cerebellum, a phenotype more reminiscent of *spg*. As for *spg*, it is not clear if *wnt-1* and *en-1* are required for the survival, growth or specification of the MHB region. The observation that the ectopic expression of *wnt1* has a strong mitogenic effect in the CNS might point towards a role of *wnt-1* in cell proliferation (Dickinson et al., 1994). Cell lineage and transplantation studies should determine the primary defects in *spg* mutants.

Formation of ventral neuroectoderm

Embryological studies have established that signals from the notochord induce ventral neuroectodermal structures in the overlying neural plate (Jessell and Dodd, 1992; Ruiz i Altaba and Jessell, 1993). The sonic hedgehog signaling pathway seems to be directly involved in this process, both in the more posterior neuroectoderm and in the forebrain (Smith, 1994; Ericson et al., 1995; Ingham, 1995). We have isolated four loci, including the previously identified *cyclops* locus (Hatta et al., 1991), that lead to ventral deficits in the neuroectoderm. Ventral deficits encompass the entire neuroectoderm, from a reduction of floor plate cells posteriorly, to deficiencies in the forebrain more anteriorly. These defects suggest that the four identified loci are good candidates for factors involved in the production, transmission or response to the sonic hedgehog signal.

Recent studies have suggested that sonic hedgehog emanating from the diencephalic midline also regulates the formation and partitioning of the optic primordium (Ekker et al., 1995; Macdonald et al., 1995). Loss of this signal might lead to cyclopia, the formation of a single or fused, often median eye. The eye fusions in the mutants described here might therefore result from the deletion of ventral forebrain structures i.e. a source of *sonic hedgehog*. Our observation that *sonic hedgehog* is absent in the anteriormost ventral brain region of *unf*^{m768} embryos is consistent with this view. This notion is also supported by the finding that the eye phenotype of *cyclops* mutant animals can be indirectly rescued by the presence of wild-type cells in the ventral forebrain region of mutant embryos (Hatta et al., 1994).

It is interesting to note that at least three of the identified loci (*cyclops*, *one-eyed-pinhead*^{m134}, *bozozok*^{m168}) also show defects in the formation of the prechordal plate (Thisse et al., 1994; Solnica-Krezel et al., 1996; Schier et al., unpublished data; Straehle et al., unpublished data). Studies in *Amblystoma* have suggested that during gastrulation and neurulation the eye field region is split into two domains, due to the influence of underlying prechordal plate (Adelmann, 1936). Nervous system fate maps indicate that the zebrafish neural retina also derives from a single coherent region that later bifurcates (Woo and Fraser, 1995). It is therefore possible that eye fusions and ventral defects observed in cyclopic mutants result, in part, from prechordal plate defects.

Notochord and brain

The three loci *bashful*, *sleepy* and *grumpy* affect the formation of the notochord (Stemple et al., 1996) and brain. Despite a severely aberrant brain morphology and enlarged hindbrain ventricle, primary patterning appears normal in these mutants. In contrast, the axonal scaffold is disorganized. We cannot distinguish if these defects are due to abnormal specification, proliferation or survival of neurons. Our observations support the view that correct neuronal patterning is not simply a consequence of normal regional patterning in the brain, but involves additional mechanisms. This conclusion is further supported by the *mind bomb* mutant phenotype (see below).

Interestingly, all three loci show defects in notochord differentiation. Further analysis will show whether the neural and notochord phenotypes are directly linked. This analysis might help to investigate the later functions of the notochord (and possibly the head mesoderm) in brain morphogenesis, as opposed to its early role in dorsal-ventral patterning of the neuroectoderm.

Formation of brain ventricles

The ventricles of the brain start to enlarge subsequently to the

formation of the neural tube and become fully inflated after the onset of blood circulation. Very little is known about the mechanisms leading to ventricle enlargement. Our analysis of mutant embryos indicates that mutations that permanently block circulation in the brain (e.g. mutants with a defective cardiovascular system) show an incomplete inflation of brain ventricles. Subsequently, the brain does not enlarge and degenerates. Physiological studies suggest a possible mechanism underlying these phenotypes. In the adult animal, a complex balance between the cerebrospinal fluid and blood is responsible for the proper integrity of brain ventricles. The choroid plexus, a secretory epithelium, maintains the chemical stability of the cerebrospinal fluid by bidirectional transport and secretion between blood and central nervous system. Studies on perfused isolated sheep choroid plexus have indicated that a diminished rate of perfusion, with its accompanying diminished capillary pressure, can lead to the reduction of cerebrospinal fluid secretion and pressure (Deane and Segal, 1979). Animal models with lowered systemic arterial pressure support this finding. In these studies, the extent of blood flow through the brain was reduced, resulting in a decreased rate of cerebrospinal fluid production (Carey and Vela, 1974; Weiss and Wertman, 1978). During chick embryogenesis, a positive cerebrospinal fluid pressure seems to be an important requirement for the normal enlargement of the embryonic brain (Desmond and Jacobson, 1977). Based on these observations, it is conceivable that the lack of circulation in cardiovascular mutants in zebrafish results in a reduction or absence of cerebrospinal fluid pressure, and thus impairs the inflation of brain ventricles. The possible connection of blood flow, blood pressure and ventricle inflation also suggests that an increase of blood flow or blood pressure might lead to an enlargement of ventricles. Consistent with this notion, we have found that mutants with reduced body length (*no tail*: Halpern et al., 1993; *floating head*: Talbot et al., 1995; *dopey* or *sneezy*: Stemple et al., 1996) have enlarged brain ventricles (unpublished observations). We might speculate that these mutants, due to a shorter vascular system, have increased blood flow or blood pressure, resulting in enlarged brain ventricles. At least one locus, *snakehead*, is required for ventricle formation before the onset of circulation. At the current level of analysis, we cannot exclude the possibility that some of the other identified ventricle mutations may also have a more direct role in brain morphogenesis.

Neurogenesis

During early neurogenesis, primary neurons arise as clusters in the brain and at distinct medial-lateral positions of the neural plate, forming columns of primary sensory neurons, interneurons and motorneurons. We have identified one locus, *mind bomb (mib)*, that is required for the specification of the correct number of primary neurons. The supernumerary primary neurons in *mib* embryos still arise at their correct position, but form denser and broader domains. The observed abnormalities in the *mib* nervous system are strikingly similar to the phenotype of neurogenic mutants in *Drosophila* and to defects induced in *Xenopus* embryos by the injection of an antimorphic *Delta* construct (Campos-Ortega, 1993; Chitnis et al., 1995). In both systems, interference with the *Notch-Delta* system for lateral specification leads to the overproduction of primary neurons. It is conceivable that the *mib* gene product is a component of a lateral specification system, where a cell committed to a primary neural fate forces its neighbors to remain uncommitted or to follow a different fate. The observation that supernumerary cells form only locally suggests further that these regions constitute equivalence groups or proneural fields. The pleiotropic phenotype of *mib* might indicate that similar mechanisms are used in many regions in

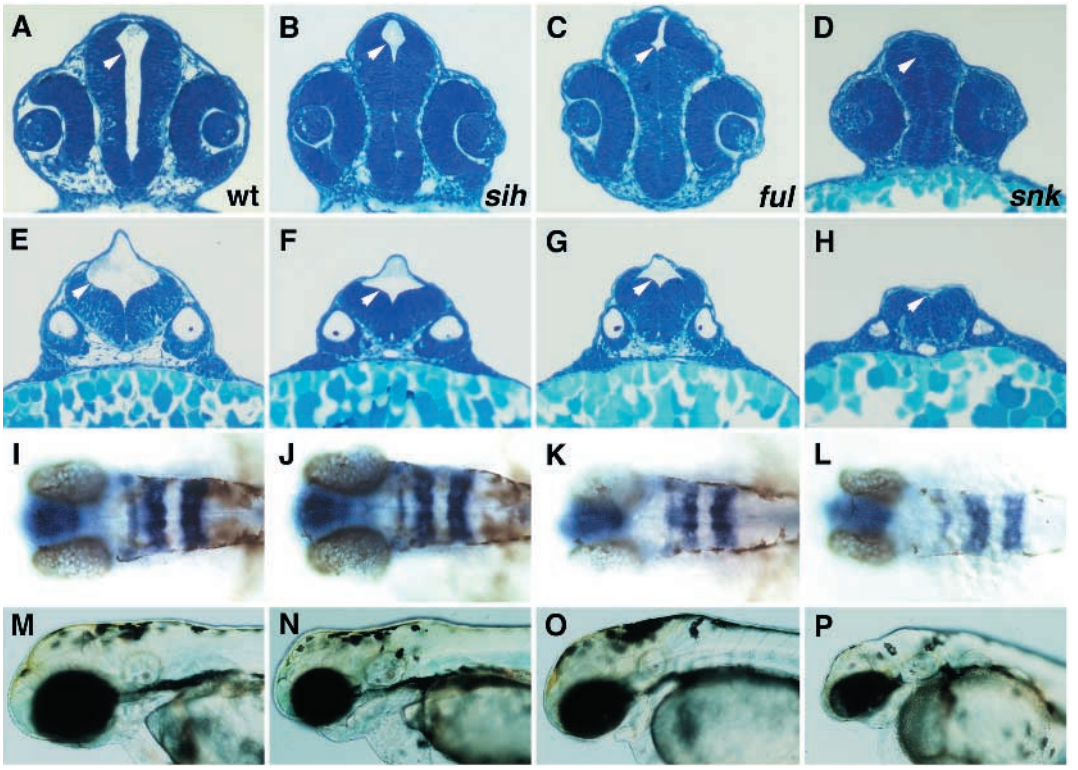

Fig. 8. Phenotypic analysis of mutations affecting ventricle enlargement. (A,E,I,M) Wild type; (B,F,J,N) *silent heart (sih)*^{b109}; (C,G,K,O) *fullbrain (ful)*^{m133}; (D,H,L,P) *snakehead (snk)*^{m273}. (A,B,C,D) Transverse sections through eye and lens at 28 hpf. (E,F,G,H) Transverse section through anterior ear and otolith at 28 hpf. Note the different degrees of ventricle (white arrowheads) reduction in *sih*, *ful* and *snk*. (I,J,K,L) Expression of *rtk1* in rhombomeres 1,3 and 5 at 31 hpf; dorsal view. (M,N,O,P) Wild-type and mutant embryos at 53 hpf. Note the differences in reduction in the size of the brain and the onset of degeneration in *sih*, *ful* and *snk*.

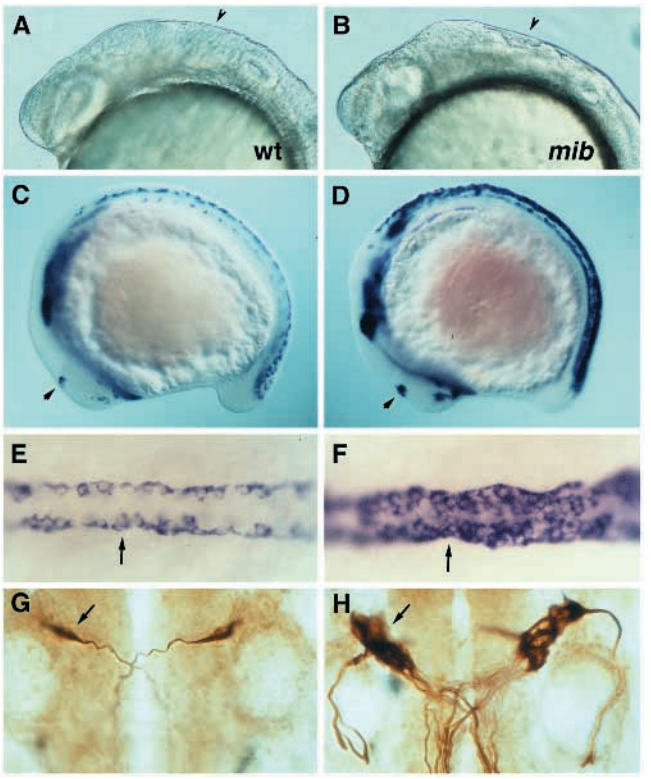

Fig. 9. Phenotypic analysis of *mind bomb (mib)* mutants. (A,C,E,G) Wild type. (B,D,F,H) *mind bomb (mib)*^{m178} mutants. (A,B) Brain in wild-type (A) and mutant (B) embryos at 25-somite stage. Note the irregularities in the hindbrain (arrowhead). (C,D) Expression of *islet1* in wild-type (C) and mutant (D) embryos at 13-somite stage. Note the dramatic increase of *islet1*-expressing cells in all regions where primary neurons are formed, including the spinal cord, epiphysis (arrow) and trigeminal ganglion. (E,F) Dorsal view of prospective Rohon-Beard cells (arrow) in dorsal spinal cord of embryos shown in C and D. (G,H) Mauthner neurons (arrow) in wild type (G) and mutant (H) embryos at 28 hpf, highlighted by *3A10* antibody. Note the abnormal projection of the most laterally located supernumerary Mauthner cell in this mutant embryo. All the other Mauthner cells project towards the midline.

the embryo (Greenwald and Rubin, 1992; Artavanis-Tsakonas et al., 1995; Conlon et al., 1995). In this model, *mib* would play a major role in the process of lateral specification and cell commitment. Alternatively, *mib* might be involved in the control of cell proliferation to prevent the overproliferation of primary neuron precursors. Blocking cell proliferation in *mib* mutants could provide a test of the latter scenario.

Prospects

The identification of developmental genes via the systematic isolation of mutants defective in morphogenetic processes has led to fundamental insights into the mechanisms that govern the development of *Drosophila* and *Caenorhabditis elegans* (Nüsslein-Volhard and Wieschaus, 1980; Horvitz and Sternberg, 1991). These mutants have not only led to the identification of key molecules, but in some cases have revealed the mechanistic logic of morphogenetic pathways. The relatively small number of isolated mutations and the diversity of

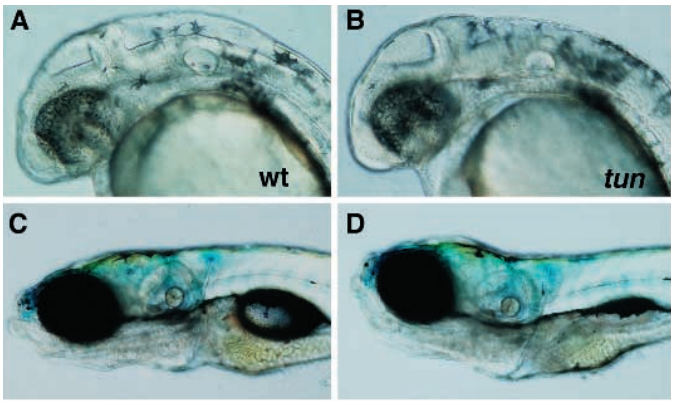

Fig. 10. Phenotype of *turned on (tun)*^{m357} mutants. (A,C) Wild-type embryo at 30 hpf (A) and on day 6 of development (C). (B,D) Mutant embryo at 30 hpf (B) and on day 6 of development (D).

associated phenotypes do not yet allow for the construction of a complex genetic pathway controlling zebrafish brain morphogenesis. Rather, we have isolated essential entry points into different aspects of brain development. First, the identified loci provide a basis for the molecular isolation of important components in brain morphogenesis. Second, detailed embryological studies in wild-type and mutant embryos promise to unravel important principles of brain development. Finally, the analysis of double mutant phenotypes, and the possibility of

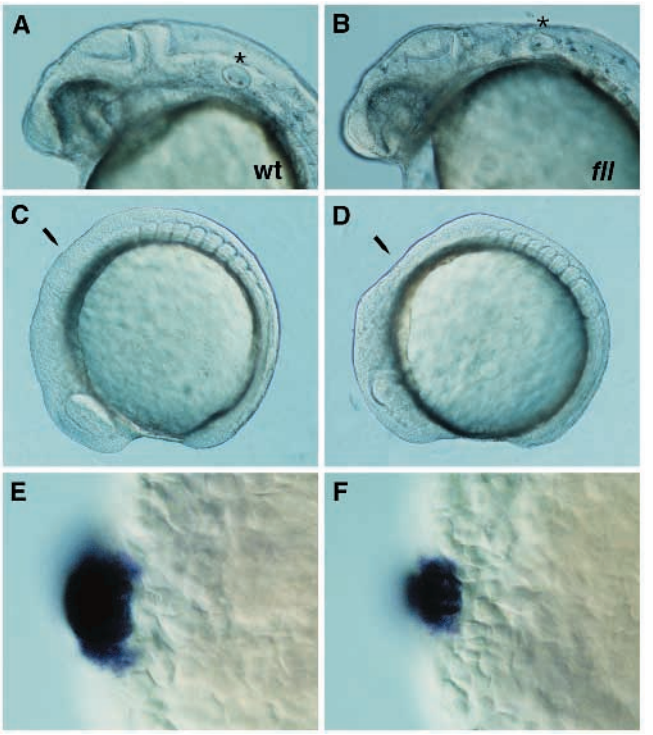

Fig. 11. Phenotype of *flachland fll*^{m517} mutants. (A,C,E) Wild-type embryos. (B,D,F) Mutant embryos. (A,B) 31 hpf; star indicates the position of the otic vesicle and rhombomere 5. (C,D) 9-somite stage; arrow indicates the hindbrain region. (E,F) Optical cross-section through the hindbrain at the level of rhombomere 5 of 14-somite embryos stained for *krox-20* and *pax[zf-b]* expression. Dorsal is to the left.

genetic modifier screens, will lead to the identification of further key components. Thus the loci described here provide the genetic framework for the further study of brain morphogenesis in zebrafish.

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Note added in proof

logelei does not complement *otter* (Jiang et al., 1996). The name of the locus will be *otter*.

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