Neural degeneration mutants in the zebrafish, *Danio rerio*

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SUMMARY

Forty zebrafish mutants with localized or general neural degeneration are described. The onset and duration of degeneration and the distribution of ectopically dying cells are specific characteristics of each mutant. Mutants are classified into four groups by these parameters. Class I: late focal neural degeneration mutants. These 18 mutants have restricted cell death mainly in the tectum and the dorsal hindbrain after 36 hours. The degeneration does not spread and disappears at later stages of development. Class II: early focal neural degeneration mutants. Ten mutants in this class exhibit transient restricted degeneration affecting mainly the diencephalon, the hindbrain and the spinal cord at 20 hours. The midbrain is less affected. The degeneration shifts to the dorsal diencephalon and the tectum at 36 hours. Class III: late spreading neural degeneration mutants. The

8 mutants in this class display a degeneration that is first seen in the tectum and subsequently spreads throughout the nervous system from 36 hours on. Class IV: early general neural degeneration mutants. This class of four mutants already shows overall cell degeneration in the nervous system at the 15-somite stage.

Three of the class I mutants show a change in the pattern of gene expression in the anlage of a brain structure prior to the onset of degeneration. These results suggest that focal cell death may be a useful clue for the detection of early patterning defects of the vertebrate nervous system in regions devoid of visible landmarks.

Key words: neural degeneration, apoptosis, zebrafish, neural plate patterning, nervous system development, tectum, retina

INTRODUCTION

Development of the vertebrate nervous system has been divided into four broad stages: first, induction of the neuro-epithelium and formation of the neural plate, a structure characteristic of vertebrates, at the end of gastrulation; second, regionalization or patterning of the neural plate; third, determination and proliferation of neural precursors within this plate; and fourth, survival and terminal differentiation of neurons. Recent analysis of these processes in vertebrates has been aided by the cloning of homologous genes known to be involved in invertebrate nervous system development. In some cases, functions of cloned genes can be assessed by generating gene knock-out mice (reviewed by Joyner and Guillemot, 1994). The limitation to this reverse genetics approach is that the gene of interest must first be cloned.

To gain further insights into the cellular and molecular mechanisms regulating development of the nervous system in vertebrates, we have taken the alternative forward genetic approach in the fish (for a review, see Solnica-Kretzel et al., 1994; Mullins et al., 1994). By this method we have isolated embryonic lethal mutations including some affecting the four

stages of neurogenesis. To isolate mutants systematically, it is necessary to set proper criteria with which one can distinguish mutations affecting each stage of neurogenesis. Patterning defects in the neural tube appear to be good criteria for the identification of such mutants for the first two stages of neural development (for neural induction see Hammerschmidt et al., 1996; Mullins et al., 1996; for the neural tube patterning see Mullins et al., 1996; Odenthal et al., 1996; Heisenberg et al., 1996; Brand et al., 1996a,b; Jiang et al., 1996). However, patterning defects affecting discrete areas of the brain are likely to be missed by simple morphological observations alone, because a large proportion of the vertebrate embryonic nervous system is devoid of good morphological landmarks.

Mutants affecting motility, ear and retinotectal axonal projection (see Granato et al., 1996; Whitfield et al., 1996; Trowe et al., 1996 and Karlstrom et al., 1996) might have defects in the two latter stages of neural development in parts of the neurons in the nervous system. However, there must exist mutants having defects in other parts of the nervous system which are phenotypically invisible by simple observations. How can these mutants be detected?

It has been suggested that cell differentiation and cell death

might be tightly coupled events, especially in the nervous system, and a cell that does not reach the correct stage of differentiation by a certain time in development would then undergo cell death (Raff, 1992). In *Drosophila*, a number of the mutations affecting neuronal cell determination, proliferation, survival and terminal differentiation, are known to display abnormal cell death. For instance, loss of function of the *achaete-scute* complex and *daughterless*, causing neural hypoplasia, a phenotype common to proneural mutants, results in massive cell death in the neural primordium (White, 1980; Brand and Campos-Ortega, 1988). Loss of function of one of five genes (*Bar, eyeless, eye absent, lozenge* and *sine oculis*) (Fristrom, 1969; Bonini et al., 1993; Cheyette et al., 1994) causes a small eye phenotype in *Drosophila*. Four of these five mutants show abnormal cell death in the eye disc. Moreover, defects in neural plate pattern formation might lead to abnormal cell death, as mislocated cells because of defective morphogenesis may die. We, therefore, assumed that abnormal cell death might be a useful indicator of mutations affecting neural plate patterning, as well as of differentiation of neural precursors, survival and terminal differentiation of neurons in vertebrates.

We describe programmed cell death in normal zebrafish development and the phenotypes of 40 mutants that exhibit abnormal cell death in the nervous system. We classify these mutants into four groups based on their temporal and spatial pattern of cell death. In situ analysis of these mutants detects alterations in pattern formation of the nervous system. These results suggest that localized cell death may be a useful marker for identifying mutations affecting the development of the nervous system.

MATERIALS AND METHODS

Fish maintenance and breeding

Fish maintenance and breeding were done as described in Brand et al. (1996a) and Mullins et al. (1994). Identification, isolation of mutants and complementation analysis is described in Haffter et al. (1996a).

Screening for neural degeneration

In the initial screening, neural degeneration was identified by the refractile appearance of cells in live embryos using a dissecting microscope at the pharyngula stage, hatching stage and on day 5 post-fertilization. For further characterization, mutant embryos were stained with the vital dye, acridine orange (AO, acridinium chloride hemi-[zinc chloride], Sigma) (Abrams et al., 1993). Embryos were dechorionated and placed in 5 µg/ml of AO (Sigma) in E3 medium (see Haffter et al., 1996a). After 30 minutes of staining embryos were washed with E3 medium and viewed using the green filter with an Axiophot fluorescence microscope (Zeiss, Germany). Pictures were taken on Kodak Ektachrome Panther 1600X film. For sequential observation and for embryos older than 48 hours, 50 µg/ml (for practical use, 1/10 concentration of AO saturated solution) of AO solution was injected into the yolk; this amount corresponds to a sphere with approximately 1/10th of the diameter of the yolk. The amount of injected AO was optimized by comparing TUNEL-stained (Gavrieli et al., 1992) cell numbers of AO uninjected and injected embryos to get maximum brightness of AO stain without killing cells artificially.

Histological sections

Embryos were fixed in 4% formaldehyde in PBS for 24 hours at 4°C, and embedded in Epon. Parasagittal sections (10 µm) were prepared and stained with toluidine blue to stain apoptotic cells (Abrams et al., 1993).

In situ hybridization

In situ hybridization was done as described in Hammerschmidt et al. (1996).

To examine subtle changes in brain structure, zebrafish *achaete-scute* homologues, *Zash 1a* and *1b* (Allende and Weinberg, 1994) were employed. We used a zebrafish fork head domain gene, *fkd-3* (J. Odenthal et al., unpublished data), which is expressed at specific sites of the diencephalon, the midbrain and the hindbrain, to check tectal degeneration mutants. The cranial neural crest was examined by analysing the expression of *dlx2* (Akimenko et al., 1994) in mutants that display jaw defects. Mutants with small eyes were examined with *pax2* (Krauss et al., 1992), *pax6* (Püschel et al., 1992; Macdonald et al., 1994), *rtk1* and *rtk2* probes (Macdonald et al., 1994; Xu et al. 1994). The olfactory epithelium and otic vesicles were stained with the *dlx3* probe (Ekker et al., 1992; Akimenko et al., 1994). Mutants that show abnormal tactile response were examined for the spinal neurons, primary motoneurons and sensory Rohon-Beard neurons by staining with *isl-1*, a LIM domain homeodomain gene (Korzh et al., 1993; Inoue et al., 1994).

RESULTS

The aim of our large-scale screen was to isolate mutations leading to localized pattern formation defects. Localized defects in neuronal development were found in 235 mutants (see Hammerschmidt et al., 1996a,b; Odenthal et al., 1996; Heisenberg et al, 1996; Brand et al., 1996a,b; Jiang et al., 1996; Piotrowski et al., 1996; Schilling et al., 1996; Granato et al., 1996; Whitfield et al., 1996; Trowe et al., 1996 and Karlstrom et al., 1996). Of these mutants, some also displayed abnormal cell death at the site of the localized defect (see below and Fig. 6). An additional 660 mutants causing abnormal general or localized neural degeneration were identified during the initial screen, and in those mutants cell degeneration is not present in any other tissue. The high frequency of the abnormal cell death phenotype and the apparent lack of visible specific defects in most of them did not allow us to keep more than 40 as samples of this category of mutants.

We will describe the phenotype of these 40 neural degeneration mutants (NED mutants). For each mutation, we define the onset of cell death, its location and its extent. From this we propose that NED mutants fall into 4 categories. We further test whether a critical early patterning defect may underlie the neural degeneration through the analysis of gene expression patterns by in situ hybridization at the 5-somite stage, before the onset of any visible ectopic cell death.

Programmed cell death in normal zebrafish development

Programmed cell death occurs naturally during normal development in the fish. Although dying cells can be observed by Nomarski optics, staining of them allows better detection and precise observation. In fixed embryos, apoptotic cells are stained by toluidine blue (Abrams et al., 1993) or by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method (Gavrieli et al., 1992), which labels fragmented DNA that is a characteristic of apoptotic cells. Since dead cells are engulfed by macrophages,

most of them disappear in a few hours (by time lapse study; D. A. Kane and M. Furutani-Seiki, unpublished observation). To detect such a dynamic biological process, laborious methods using fixed embryos are not ideal. Thus, we employed a stain that enables sequential observation of single, living embryos. Acridine orange is a vital dye reported to stain apoptotic cells but not necrotic cells in *Drosophila* (Abrams et al., 1993). When acridine orange is injected into the zebrafish yolk, it is taken up by dying cells throughout the embryo. Since the number of apoptotic cells stained by AO is slightly variable among individual embryos, following descriptions are based on the representative stainings.

Apoptotic cells are first detected at the tail bud stage (Fig. 1A-D), and are mainly seen in cells of the epiblast (Fig. 1C,D,M,N). Apoptosis is rarely seen in cells of the hypoblast, for example, in the mesodermal polster. At around the 10-somite stage, the number of dying cells in the neural plate reaches its peak and then declines (Fig. 1E-J). The majority of apoptotic cells are seen in the neuroectoderm including the optic vesicles (Fig. 1O), but few apoptotic cells are seen in the mesodermal tissue, notochord and somites (Fig. 1P,Q). After the 20-somite stage very few AO stained apoptotic cells are seen (Figs 1K,L, 2C). No further localized cell death is seen in the nervous system before hatching.

Classification of the neural degeneration mutants

Neural degeneration mutants were tentatively classified into four categories, although each mutant exhibits individual characteristics. Since we did not keep all the NED mutants but only a small fraction of them, complementation test were only performed in a small number of cases and no noncomplementation groups could be identified. The four mutants described as a representative of each class complement each other. All mutations are zygotic recessive.

Class I: late focal neural degeneration (Fig. 2). These mutants show restricted neural cell death, starting between 36 hours post-fertilization (h) and day 5, which does not spread into the spinal cord. Class II: early focal neural degeneration (Fig. 3). These mutants have an onset of focal cell death around the 20-somite stage and result in a smaller head by 36 hours. Degeneration shifts to other parts of the brain by 48 hours. Class III: late spreading neural degeneration (Fig. 4). These mutants show regional onset of degeneration beginning at 36-60 hours. This neural degeneration

spreads to other parts of the nervous system. Class IV: early general neural degeneration (Fig. 5). These mutants show early general neural cell degeneration at around the 15-somite stage and complete degeneration of the head by 24 hours.

For each class of mutants we describe a representative. We then comment on interesting features of other members of the class.

In situ hybridization analysis of neural degeneration mutants

We have postulated that some mutants with defects in neural pattern formation might display restricted neural degeneration. Since there are relatively few landmarks for visual inspection in the nervous system despite its complex structure, we used in situ hybridization with various probes to detect subtle changes in neural patterning. For that, we used zebrafish *achaete-scute* homologues, *Zash 1a* and *1b* (Allende and Weinberg, 1994) for the following reasons. Firstly, the probes are expressed from
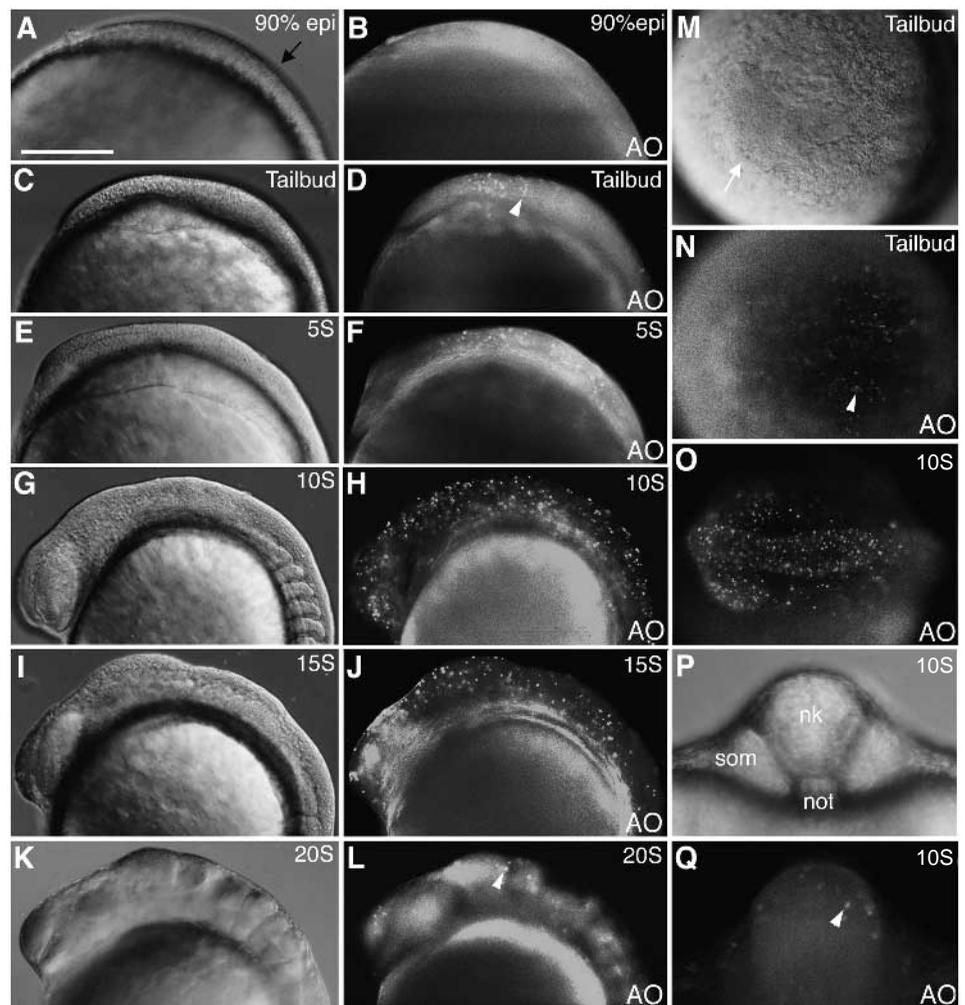

Fig. 1. Acridine orange (AO) stains apoptotic cells during zebrafish embryonic development. (A-L) Lateral view; (M-O) dorsal view; (P,Q) optical transverse section. (A,B) Apoptotic cells are not seen at 90% epiboly in the thickening neural anlagen (arrow). (C,D,M,N) Apoptotic cells (arrowheads in D,N) are first detected at the tail bud stage in the epiblast, but not in the hypoblast. In the polster (arrow in M) no apoptotic cells are detected. (E-J,O) Number of apoptotic cells peaks at 15-somite stage. (P,Q) Apoptotic cells (arrowhead) are mainly seen in the neural keel (nk) rather than somite (som) and notochord (not). (K,L) Few apoptotic cells (arrowhead) are seen after the 20-somite stage. Scale bar, approximately 100 μm.
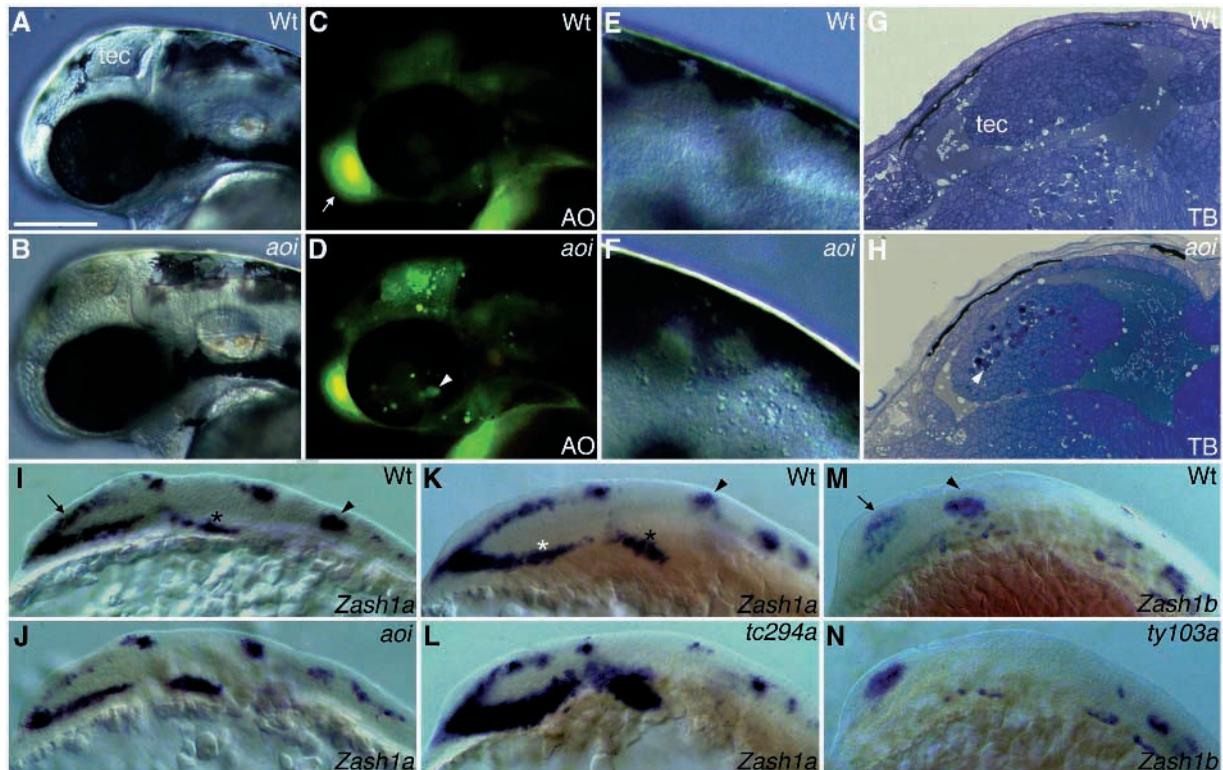
Fig. 2. Class I mutant phenotypes. (A,C,E,G,I,K,M) Wild type; (B,D,F,H,J) *aoi*, (L) *tc294a* and (N) *ty103a.* (A-H) at 36 h; (I-L) at the 10-somite stage and (M,N) at the 15-somite stage. (C,D) Acridine orange (AO)-stained embryos of (A,B) respectively. (G,H) Parasagittal section of the tectum stained with toluidine blue. In situ hybridization with *Zash 1a* probe (I-L) and with *Zash 1b* probe (M,N). Apoptotic cells are indicated by white arrowheads. In *aoi* mutants apoptotic cells are present in the tectum (D,H arrowhead), cerebellar fold, dorsal hindbrain (F) and the neural retina (D arrowhead). Normal olfactory epithelium is stained with acridine orange (C, arrow). (I,J) The dorsal forebrain expression (arrow) and rhombomere 2 expression of *Zash 1a* (arrowhead) are reduced in *aoi* mutants whereas ventral midbrain expression (black star) is increased; (K,L) the ventral forebrain expression (white star), tegmentum expression (black star) are increased whereas the midbrain-hindbrain boundary expression of *Zash 1a* (arrowhead) is reduced in *tc294a* embryos; (M,N) the telencephalic expression of *Zash 1b* (arrow) is increased in contrast to the absence of its midbrain expression (arrowhead) in *ty103a.* Scale bar, approximately 100 μm in A-D and I-N; 200 μm in E and F; 300 μm in G and H.

early neurulation in a reasonably fine pattern covering most of the brain architecture. Secondly, *Zash* genes are postulated to be involved in proliferation and differentiation of neurons and their expression would be expected to be perturbed by genes regulating neural induction, regionalization and patterning (for other probes used, see Materials and Methods). Some of the class I NED mutants show changes in a gene expression pattern. Detailed in situ hybridization results for the mutants are described below, together with their phenotypes.

Class I: late focal neural degeneration

The class I mutants contain 17 mutants which are characterized by having restricted and transient cell death. These mutants can be identified either by degeneration of the tectum, small eyes or both between 36 hours and day 5. Some mutants have dorsal hindbrain degeneration as well. Only *tw202e* mutant embryos display degeneration in the tegmentum on day 5 (see Table 1). All class I mutants eventually have small eyes due to retinal degeneration (see below). The restricted degeneration is transient leaving the head small and distorted. In some mutants, the head appears flat, similar to *flathead (fla)* mutants (Schilling et al., 1996). These mutants also show a jaw phenotype later on day 5 (see Piotrowski et al., 1996; Schilling et al., 1996). The olfactory epithelium and the lateral line

appear normal in these mutants. These mutants do not show abnormal cell death in the spinal cord and none exhibit motility defects.

aoi (aoi^{tr222d}) mutants are first identifiable by the onset of tectal (Fig. 2A,B), cerebellar and dorsal hindbrain (Fig. 2E,F) degeneration at 36 hours. Direct observation of *aoi* mutants with Nomarski optics, acridine orange and toluidine blue staining show similar patterns of cell death, thus indicating apoptotic degeneration of the neuroepithelium (Fig. 2C,D,G,H). Apoptosis in *aoi* does not spread but disappears by 60 hours resulting in a small and distorted head. Histological examination of *aoi* shows degeneration of the tectum (Fig. 2G,H) and of the neural retina (data not shown). *aoi* mutant larvae do not develop a swimbladder and die by day 10. Subtle but consistent changes of gene expression patterns are observed in *aoi*. At 15 hours, the dorsal telencephalic and rhombomere 2 stripes of *Zash 1a* are reduced (Fig. 2I,J) whereas the ventral midbrain stripe is increased. Alteration of these expression patterns of *Zash 1a* partly correlates with the prospective degenerative region. These results show that *aoi* brain patterning is changed before any visible degeneration.

tc294a and *ty103a* mutants can be first identified by a tectal and cerebellar degeneration at 36 hours (similar to that of *aoi*, data not shown). The mutants have small eyes likely due to
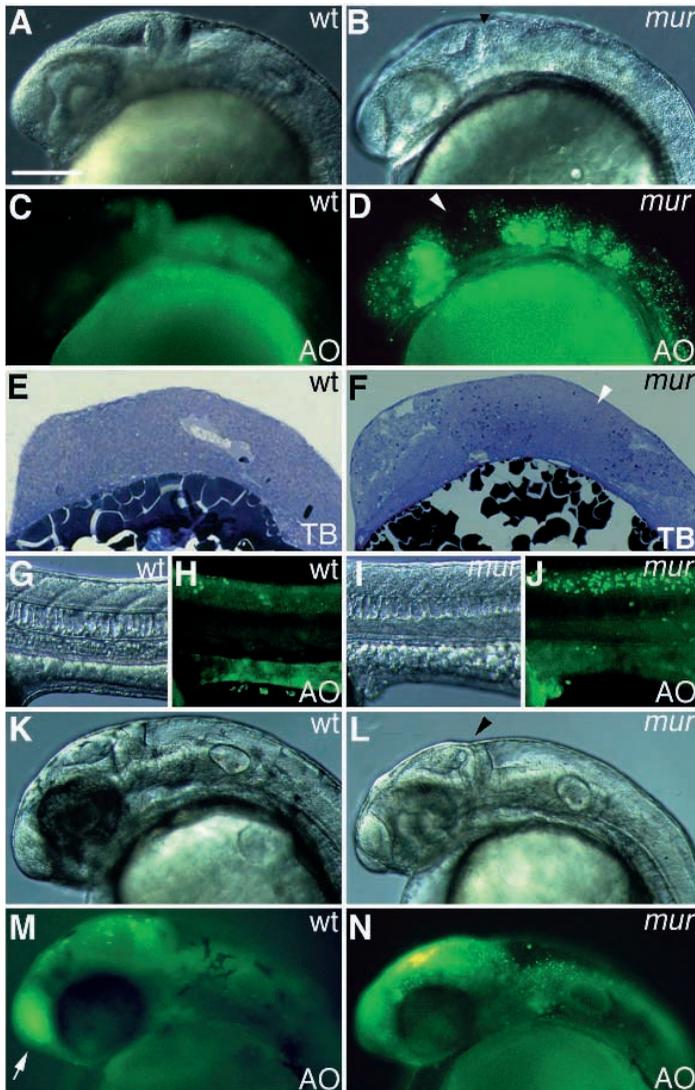
Fig. 3. Class II mutant phenotypes. (A,C,E,G,H,K,M) Wild type embryos; (B,D,F,I,J,L,N) *mur* embryos. (A-J) at 24 hours, (K-N) at 36 hours. (C,D,H,J,M,N) Acridine orange (AO)-stained embryos of A,B,G,I,K,L, respectively. (E,F) Histological structure of the brain, as shown in parasagittal section. (A,B,K,L) Pointed midbrain-hindbrain boundary (black arrowhead) is seen in *mur* embryos; (C,D) apoptotic cells are seen in parts of the forebrain, hindbrain and the spinal cord (H,J). The midbrain (white arrowhead in D) is less affected. (E,F) Toluidine blue stained apoptotic neuroepithelium is not seen in the midbrain (white arrowhead) in *mur* embryos; (K,L) small and less pigmented head of *mur* embryos; (M,N) apoptotic cells are seen in dorsal diencephalon and midbrain. Apoptotic cells in the hindbrain and the spinal cord are reduced. Acridine orange stained olfactory epithelium (white arrow in M) is reduced in *mur* embryos. Scale bar, 100 µm.

Fig. 4. Class III mutant phenotypes. (A,B,E,G) Wild type; (C,D,F,H) *yug* embryos. (A-F) at 36 hours, (G,H) at 60 hours. (B,D) Acridine orange staining of A and C, respectively. (E,F) Parasagittal section of the brain. (A,C) The brain appears normal except for the presence of degenerating cells in *yug* embryos. (B,D) Apoptotic cells exist throughout the brain including the neural retina (arrowhead); acridine orange (AO)-staining of the olfactory epithelium (arrow) is reduced in *yug*; toluidine blue stained apoptotic cells are seen in the tectum in *yug*. The apoptotic cells are in a clump (arrow). Viable cells in the tectum are less well stained in *yug* embryos compared to wild type. The neuroepithelium is not differentiated properly (E,F). The head and eyes are small in *yug* embryos (G,H). Scale bar, 100 µm.

retinal degeneration. *tc294a* and *ty103a* mutants have a flat head and a jaw defect on day 5 (see Schilling et al., 1996). *Zash 1a* expression is altered in *tc294a* at the 5-somite stage (Fig. 2K,L). Expression in both the ventral forebrain and midbrain shows an increase, whereas expression in the presumptive midbrain-hindbrain boundary is decreased. The telencephalic expression of *Zash 1b* is increased whereas its midbrain expression is absent in *ty103a* (Fig. 2M,N). Therefore, *tc294a*
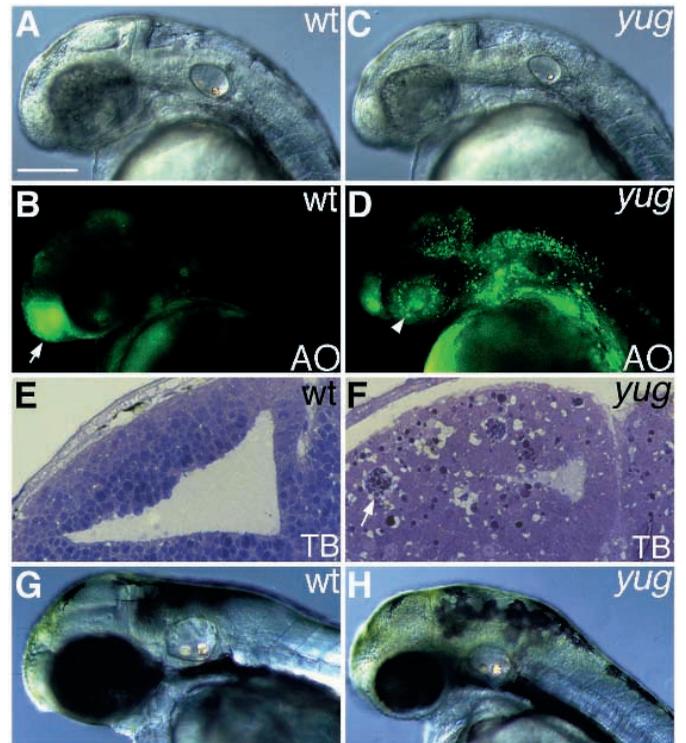
and *ty103a* may have a forebrain and midbrain patterning defect before the onset of neural degeneration.

Class II: early focal neural degeneration

Eleven mutants belong to this class (Table 1). *murasaki (mur[th257])* mutants can be first sorted at the 20-somite stage by diffuse cell degeneration in the nervous system. By 24 hours, both the fourth ventricle and tectum are developed, despite degeneration of the neuroepithelium (Fig. 3A,B). However, the midbrain-hindbrain boundary appears more pointed than in wild type (Fig. 3A,B,K,L). This phenotype is frequently observed in class II mutants. Both acridine orange staining and a histological examination of *mur* show restricted degeneration of neuroepithelium (Fig. 3C-F). The midbrain is less affected. Neuroepithelial cells of the spinal cord are also degenerating (Fig. 3G-J). By 36 hours, *mur* mutants are less pigmented and have a smaller head and eyes (Fig. 3K,L). The density of apoptotic cells is now reduced in the brain, with the exception of the tectum and dorsal hindbrain (Fig. 3M,N). In the spinal cord, there are almost no apoptotic cells left. *mur* mutant embryos hatch normally. By day 3 the olfactory epithelium is reduced in *mur* mutants (data not shown). The mutants show

Table 1. Phenotypes of NED mutants

Gene name	Alleles	Onset	Lesion	Head	Eye	Jaw	Olf	Lat	Tac	Co	Major description
Class I											
	tc294a	36 h	tc+c	flat	s	r	nd	nd	w	w	b
	te322	36 h	tc+c	flat	s	r	nd	nd	w	w	b
recover (rec)	te356a	d 4	tc+c+dh	w	s	w	nd	nd	w	w	a
	tg279	36 h	tc+c	flat	s	r	nd	nd	w	w	b
sturgeon (stu)	tg419	36 h	tc+c	ham	s	r	nd	nd	w	w	c
	tj229g	36 h	tc+c	flat	s	r	nd	nd	w	w	b
	tl17e	48 h	tc+c+dh	dist	s	w	w	w	w	w	a
	tp230	36 h	tc+c	flat	s	r	nd	nd	w	w	b
	tq235b	36 h	tc+c	flat	s	r	nd	nd	w	w	b
aoi (aoi)	tr222d	36 h	tc+c+dh	dist	s	w	w	w	w	w	a
	ts276f	36 h	tc+c	dist	s	w	w	w	w	w	a
	tw202	d 5	tg	dist	s	w	w	w	w	w	a
vanille (van)	ty6d	36 h	tc+c	flat	s	r	nd	nd	w	w	d
	ty17e	48 h	tc+c	dist	s	w	w	w	w	w	a
	ty85a	36 h	tc+c	dist	s	w	w	w	w	w	a
	ty103a	36 h	tc+c	flat	s	r	nd	nd	w	w	b
bleached (blc)	3 alleles*	d 4	tc+c	dist	s	w	nd	nd	w	w	d
braindead (brd)	9 alleles†	72 h	tc+c	dist	s	w	w	w	w	w	e
Class II											
	tc234e	20 s	d+h+sc	s, pm	s	n	r	r (n+s)	w	w	a
	te356b	25 s	d+h+sc	s, pm	s	n	w	r (s)	cr	uc	a
murasaki (mur)	th257	20 s	d+h+sc	s, pm	s	n	r	w	w	uc	a
	tl43f	20 s	d+h+sc	s, pm	s	n	n	r (n)	cr	uc	a
	tm79c	25 s	d+h+sc	s, pm	s	n	r	w	w	w	a
	to15d	20 s	All	s, pm	s	n	w	r (n+s)	r	uc	a
	ts40	25 s	All	s, pm	s	n	w	w	w	w	a
	tw3a	25 s	All	s, pm	s	n	w	r (s)	n		a
	ty19a	20 s	d+h+sc	s, pm	s	n	w	w	cr	uc	a
	tw17a	20 s	All	s, pm	s	n	s	w	r	uc	a
Class III											
	ta53b	48 h	tc+c (sp)	s	s	–	–	–	w	w	a
yugiri (yug)	tc1	36 h	tc+c (sp)	s	s	r	w	r (n+s)	w	uc	a
	tc11a	30 h	tc+c (sp)	s	s	r	w	w	w	uc	a
	tc31	36 h	tc+c (sp)	s	s	w	w	w	w	uc	a
	tc233b	36 h	tc+c (sp)	s	w	w	r	w	r	uc	d
delayed fade (dfd)	tj41a	36 h	tc+c (sp)	s	w	w	w	w	w	uc	e
	tm81	60 h	tc+c (sp)	s	s	w	w	w	w	uc	a
	tt227d	36 h	tc+c (sp)	s	w	r	w	w	w	uc	a
Class IV											
	tj20a	15 s	All	s	s	–	–	–	r	uc	a
	tj20c	20 s	All	s	s	–	–	–	r	uc	a
awayuki (awa)	tm17	15 s	All	s	s	–	–	–	r	uc	a
	tu13	15 s	All	s	s	–	–	–	r	uc	a

In 'Onset' column: h, hours; d, days; s, somite stage.

In 'Lesion' column: All, throughout the nervous system; c, cerebellum; d, diencephalon; db, dorsal brain; dh, dorsal hindbrain; dsc, dorsal spinal cord; h, hindbrain; sc, spinal cord; tc, tectum; tg, tegmentum; (sp), spreading.

In 'Head' column: dist, distorted head; flat, flathead; ham, no tissue anterior to eyes; pm, pointed midbrain-fold; s, small.

In 'jaw' column: n, no jaw; r, reduced ; w, wild type; –, embryos dead prior to examination.

In 'Olf' column: olf, olfactory epithelium: nd, not done.

In 'Lat' column: lat, lateral line sensory epithelium; r (s), reduced neuromast; r (n), reduced number of cells in each neuromast.

In 'Tac' column: tac, tactile response-reactivity; hy, hyper-reactive.

In 'Co' column: co, tactile response-coordination; uc, uncoordinated response.

*3 alleles: th204b, ts23, ty89

†9 alleles: tc265z, tl21, tl41, tm42z, tm46y, tp41z, tv59y, ty91, ty103z.

References: a, this paper; b, Schilling et al. (1996); c, Piotrowski et al. (1996); d, Kelsh et al. (1996); e, Trowe et al. (1996).

retarded development of jaw and gill arches and do not survive over a week.

Class II mutants often have additional variable defects in sensory organs, i.e. olfactory epithelium and the lateral line (Table 1). Mutant embryos, tc234e, tm79c and ty19a have a reduced olfactory epithelium. In the lateral line, the neuromast number is reduced in tc234e, tl43f and to15d, whereas the cell number in each neuromast is reduced in tc234e, te356b, to15d

and tw3a. All class II mutants are retarded in development. The tactile response is affected to a variable extent, probably due to the spinal cord degeneration in each mutant (Table 1). No change in gene expression pattern were found by in situ analysis of 10 class II mutants.

Class III: late spreading neural degeneration

Nine mutants belong to this class. These phenotypes are more

heterogeneous between mutants than those of class II and IV (Table 1). Prior to neural degeneration these mutants appear normal. Neural degeneration in these mutants starts from the tectum and dorsal hindbrain between 30 and 60 hours. Spreading cell death results in a distorted head by day 3. The tactile response is affected to a variable extent in all the mutants (Table 1). The olfactory epithelium and the lateral line are generally normal except in *tc1* and *tc233b*. Mutants in this class do not develops a swimbladder and die by day 7.

yugiri (*yug^{tc1}*) mutants can first be identified by tectal and spinal cord degeneration at 36 hours (Fig. 4A,C). This degeneration is due to apoptosis (Fig. 4B,D), and in this mutant, the central canal is not well formed and the tail slightly curves up with defects probably resulting from cell death in the spinal cord. By 48 hours, apoptotic regions spread throughout the nervous system and include the neural retina. Histological examination reveals that the neuroepithelium may not differentiated properly (Fig. 4E,F); however, primary neurons remain intact despite the severe degeneration in neuroepithelium (data not shown). By 60 hours, apoptotic cell numbers are reduced. Spinal cord degeneration remains visible after disappearing from the brain. In *yug* mutants, the head and eyes are small (Fig. 4G,H); additionally the number of lateral line sense organs and sensory cells in the neuromast are decreased by day 3 (data not shown). No changes in expression patterns of the set of genes are detected in 8 class III mutants (see Materials and Methods).

Class IV: early general neural degeneration

Four mutants belong to this class (Table 1). They are very similar in phenotype. *awayuki* (*awa^{tm17}*) mutant embryos can be identified by ubiquitous cell degeneration in the nervous system at around the 15-somite stage (revealed by acridine orange staining and toluidine blue staining of histological sections; Fig. 5A-F). Cell degeneration is restricted to the neural tissue. In contrast, other tissues like the notochord or somites are not degenerating (Fig. 5G,H). At 24 hours, almost all neuroepithelial tissue is degenerating. The midbrain-hindbrain boundary, the tectum and the fourth ventricle are not well formed in *awa* mutants (Fig. 5I,J). At 30 hours, the neuroectodermal tissues are completely degenerated and the mutants do not survive thereafter. Expression patterns of *Zash 1a* and *1b* are not altered in class IV mutants.

DISCUSSION

Phenotypic consideration of mutants that have defects in the nervous system

To identify genes involved in vertebrate neurogenesis, we have isolated mutants that have nervous system defects. 256 of these mutants exhibited visible morphological abnormalities restricted to a part of the nervous system (Brand et al., 1996a,b; Granato et al., 1996; Heisenberg et al., 1996; Jiang et al., 1996; Piotrowski et al., 1996; Schilling et al., 1996; Whitfield et al., 1996). In addition, 660 mutants show either general or localized abnormal neuronal cell death, with no apparent preceding morphological defects during development. In many of these neural degeneration (NED) mutants cell degeneration spreads over the nervous system resulting in degeneration of the embryo. Therefore, most of these mutants were discarded

without further analysis. It cannot be excluded, however, that two groups of potential interest might be included in the NED mutants. Firstly, mutations in genes involved in neuronal proliferation, differentiation and survival may be expected to display a cell death phenotype, as it has been suggested that neuronal cell proliferation and differentiation are tightly coupled to cell death (Raff et al., 1993). In fact, *white tail (wit)* mutants that show hypertrophy of neurons appear to have neuronal degeneration as well (see Jiang et al., 1996). Secondly, mutations affecting pattern formation of the nervous system might be causing a cell death phenotype, as failure of patterning during embryogenesis often leads to cell death in *Drosophila* (Abrams et al., 1993). *no isthmus (noi)* mutants display restricted cell death in the region where the midbrain-hindbrain boundary is formed (see Brand et al., 1996a). As a result, *noi* mutants have no midbrain-hindbrain boundary (Fig. 6A-D). To address the possibility of cell death as a marker of defective neuronal patterning, we have analyzed in detail a fraction of NED mutants kept in the initial screen.

We tentatively classify the NED mutants into four general categories, but each phenotype examined shows slight differences. This may result from the fact that we randomly kept only 40 of 660 mutants. Neuronal degeneration was a frequent phenotype in our screen (19% of all mutants found). Assuming equal allele frequencies (2.5 per gene, see Haffter et al., 1996a), the 660 mutants are expected to define about 300 genes.

It is surprising that all the neural degeneration mutants examined display apoptotic cell death rather than necrosis. Necrosis is characterized by a swollen appearance of cells and loss of membrane integrity (Wyllie, 1981). Dying cells in the NED mutants, in contrast, appear to condense, becoming refractile in appearance. These cells are stained with acridine orange, toluidine blue and TUNEL, which stain apoptotic cells specifically (Abrams et al., 1993). During normal development, the majority of apoptotic cells are detected in neuroectodermal tissues after the end of gastrulation in the zebrafish. Cell lineage analysis also shows programmed cell death only after gastrulation in the epiblast (R. Warga, personal communication). These results indicate a close link between neuronal cell development and a suicide program.

Abnormal cell death as a clue in detecting defects in neural development

In *Drosophila*, the larval cuticle is rich in morphological features that allow the identification of mutants with subtle defects (Nüsslein-Volhard et al., 1984). In contrast, a large proportion of the vertebrate nervous system is devoid of good morphological landmarks and therefore subtle defects at early stages cannot be detected by simple optical screening. Consequently, it is necessary to employ molecular probes or antibodies to detect subtle defects.

Cell death phenotypes are associated with neural patterning mutants identified in our screen. In addition to *noi* mutants, flat head mutants that lack a substantial portion of the midbrain show midbrain degeneration. Moreover, while *aoi* mutants show no apparent morphological defect at early stages, cell death is associated with a change in gene expression pattern within the forebrain, midbrain and hindbrain. This indicates a subtle alteration in early pattern formation in these mutants. Although cell death phenotypes were common in our screen, the analysis described here shows that pattern defects are seen
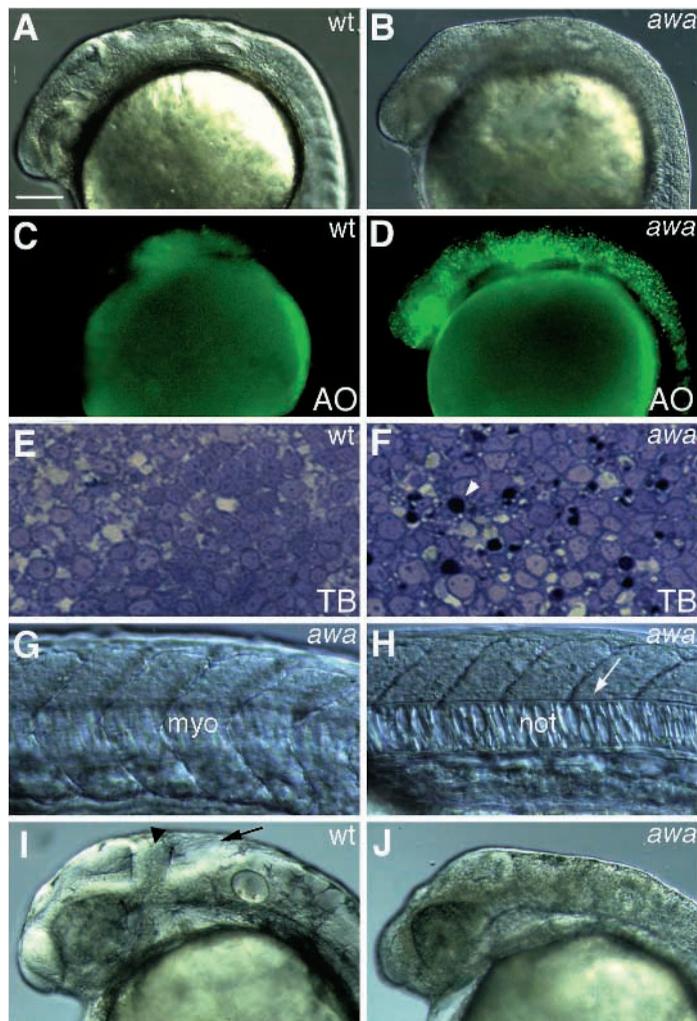
Fig. 5. Class IV mutant phenotypes. (A,C,E,I) Wild type; (B,D,F,G,H,J) *awa* embryos. (A-H) at the 25-somite stage; (I,J) at 36 hours. (C,D) Acridine orange (AO)-staining of A and B, respectively. (E,F) Parasagittal section of the brain. (A-D) General apoptosis of neuroepithelium in the nervous system of *awa*; (E,F) toluidine blue (TB)-stained apoptotic cells (arrowhead) in *awa*. The rest of the cells in the neuroepithelium appear unaffected; (G,H) myotomes (myo), floor plate cells (arrow) and vacuolated cells of notochord (not) are relatively normal in *awa* embryos; (I,J) small and distorted head of *awa* embryos. The midbrain-hindbrain boundary (arrowhead), tectum and the fourth ventricle (arrow) are not well developed in *awa* embryos. Scale bar, 100 μm.

Fig. 6. Mutants that show abnormaly localized apoptotic cells. (A,C,E,G) Wild type; (B,D) *noi*; (F,H) *dns*. (A-D) At 24 h; (E-H) at 36 hours. (A-D) Localized apoptotic cells are seen in the presumptive isthmus in *noi* (arrowhead); (E-H) restricted apoptotic cells are detected in the anterior prechodal plate in *dns* embryos (arrowhead). Scale bar, 100 μm.

only in mutations causing localized cell death that does not spread. Thus, although we analyzed only a limited sample number, localized cell death that does not spread may be a useful criterion for selecting mutations that have subtle patterning defects.

Restricted cell death can only be detected when embryos are checked at the proper time, since cell death often spreads, shifts or disappears in a few hours. Cell death can often be detected by the refractile appearance of cells in live embryos; however, most of the zebrafish nervous system is too translucent for cell death to be readily seen. Acridine orange staining can facilitate visual inspection of cell death, and its ease of use makes the technique suitable for large-scale screens. Examples of
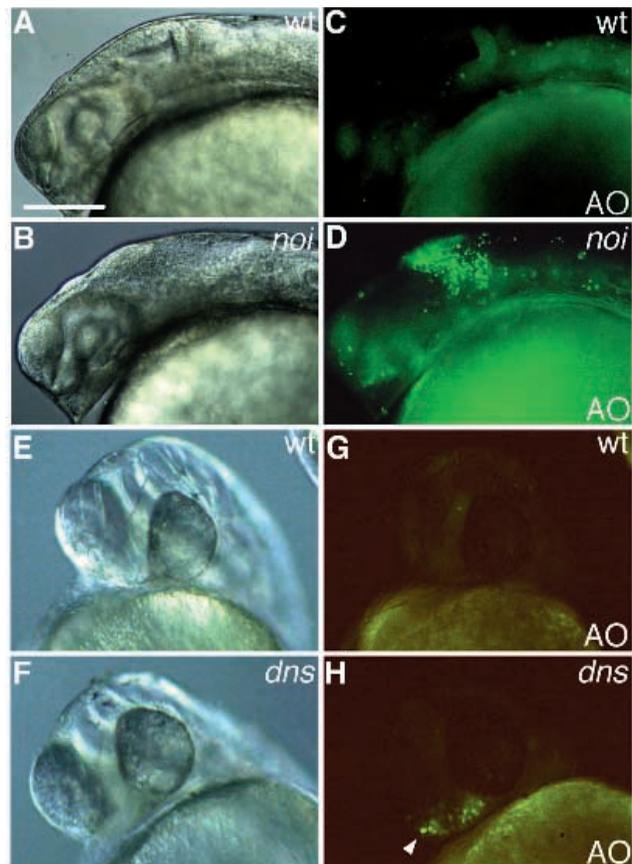
acridine orange staining of mutants found in our screen are shown in Fig. 6. *noi* mutants show apoptotic cells in the prospective isthmus (Fig. 6A-D). *dirty nose (dns)* mutants show resticted apoptosis in the anterior prechodal plate at 24 hours (Hammerschmidt et al., 1996b) (Fig. 6E-H). *masterblind (mbl)* mutants do not have optic vesicles and form an expanded epiphysis (Heisenberg et al., 1996). *mbl* mutants display apoptosis in the epiphysis at 24 hours (data not shown). *big head (bid)* mutants (Jiang et al., 1996) that have an enlarged head show a reduced number of apoptotic cells during development (preliminary observations). Taken together, our results suggest that simple acridine orange staining of embryos may facilitate isolation of mutants and phenotypic characterization of them.

A common phenotype of class I mutants: tectal degeneration

Why are restricted degenerating cells frequently found in the tectum in class I mutants? Two possible explanations can be offered. Firstly, the tectum might be a metabolically active tissue, making it prone to toxic defects. Secondly, tectal degeneration mutants may have been preferentially identified during the screen, which relied on simple visual inspections without the use of acridine orange. Cell degeneration is most easily

detected in the transparent tectum in embryos beyond the pharyngeal stage, whereas the rest of the brain, by contrast, is rather translucent.

Tectal degeneration mutants commonly have small eyes, probably due to retinal degeneration. It has been shown in both invertebrates and vertebrates that pattern formation in both the eye and the tectum depends on eye-brain interactions (Heumann and Rabinowicz, 1980; Selleck and Steller, 1991). In *Drosophila,* the relationship between retina and optic lobe, the target organ for retinal ganglion axons, has been analyzed using genetic mosaics of mutations affecting eye or optic lobe morphology. The results indicate that optic lobe development is dependent on retinal cell development (Meyerowitz and Kankel, 1978). In contrast, in *masterblind (mbl)* mutants of the zebrafish, which completely lack eyes, the tectum appears relatively normal. Furthermore, ablating the eye anlagen does not lead to tectal degeneration in the zebrafish (M. Furutani-Seiki, unpublished observation). These observations indicate that presence of the eyes is not necessary for general survival of the tectum in the zebrafish. Mosaic analysis between wild type and NED mutant embryos might provide further insights into the relationship between the eye and the tectum in vertebrates.

Alternatively, concomitant degeneration of the retina and the tectum might be caused by defects in genes expressed in both tissues. According to a regional fate map of the anterior nervous system (the forebrain, midbrain, hindbrain and neural retina) at the beginning and end of gastrulation (Woo and Fraser, 1995), prospective retina is surrounded by the midbrain field, from which the tectum originates. Defects in genes expressed in the region covering midbrain and retinal field could lead to concomitant degeneration of the tectum and midbrain. In fact, defects in the *sine oculis* gene of *Drosophila*, which is expressed in the anlagen of both the eye and the optic lobe, are known to cause degeneration of the eye and the optic lobe (Fischbach and Technau, 1984).

The class II NED mutants: genes necessary for transient survival of subsets of neuroepithelial cells?

Class II mutants show localized neural degeneration, but degeneration later shifts to the dorsal diencephalon and the tectum. Thus, different parts of the nervous system may require these genes at different times of development. None of these mutants show any changes in gene expression patterns compared to wild type (data not shown). Thus, this restricted cell death in these mutants may be caused by defects in neuroepithelial differentiation and survival rather than in neural patterning.

The class III NED mutants: indication of distinct requirements for survival of primary and secondary neurons

The zebrafish *ned-1^{b39rl}* mutation causes massive cell death in the embryonic nervous system and belongs therefore to this class (Grunwald et al., 1988). The *ned-1* mutation affects the viability of most cells in the embryonic nervous system. However, a small set of primary neurons, including Rohon-Beard sensory neurons, large hindbrain interneurons and primary motoneurons, remain unaffected. This also holds true in general for class III NED mutants. Hence, genes affected in the class III mutants are probably not required for the devel-

opment and maintenance of primary neurons. The distinct requirements of the primary neurons for their survival support the classification of primary and secondary neurons based on their soma size and time of birth (Kimmel and Westerfield, 1988).

Class IV NED mutants: defects in genes necessary for neuroepithelial cell survival?

Three possibilities could explain the ubiquitous degeneration of the neuroepithelium in these mutants. Firstly, developmental arrest of neuroepithelial cells could result from a block in cell division. Secondly, neuroepithelial precursor cells might divide normally but fail to differentiate. Thirdly, neuroepithelial cells are more prone than other tissues to suffer from altered cellular metabolism.

In our screen we have isolated mutants that develop general cell degeneration by 12 hours, much earlier than class IV NED mutants start degeneration (Kane et al., 1996). In these mutants, all cell types except those that have already undergone their final mitosis (e.g. primary neurons and notochord cells), display non-apoptotic cell death. The accumulation of abnormal chromosomal mitotic figures in these mutant embryos suggest that in these mutants the cell cycle might be affected (Kane et al., 1996). Genes involved in the cell cycle are generally required for cell survival. As muscle and epidermal cells continue to divide in class IV mutants, these genes are most likely not required for cell survival. However, we cannot eliminate the possibility that neuroepithelial cells are simply more sensitive to defects in cellular metabolism or respiration. The brain is an organ of high energy consumption and is exclusively dependent on the aerobic metabolism of glucose (for example, see Kandel and Schwartz, 1993). Defects in the metabolic pathways or anti-oxidative cascades could therefore predominantly cause neural degeneration before significantly causing damages to other tissues. The large number of the NED mutants found in our screen, may reflect a requirement of those genes, especially for neuronal survival.

It has been postulated that neurotrophic factors like nerve growth factor (NGF) support neuronal cell survival by regulating metabolic activities essential for their survival. Lack of neurotrophins known to date does not explain the phenotype of the class IV mutants, since none of the gene knock-out-mice in NGF, other neurotrophins or their receptors, cause such a massive neuroepithelial cell death as is observed in class IV NED mutants, but display a deletion of only a subset of neurons (for example, see Crowley et al., 1994). To date, there is no report of a gene whose absence leads to massive neuroepithelial cell death in the nervous system in the mouse. The essential difference between genes involved in cellular metabolism and factors indirectly regulating neuroepithelial metabolism is that the former acts cell-autonomously and the latter non-cell-autonomously. Cell transplantation experiments could help to investigate the cell-autonomous action of the mutated genes. Mutations that function in a non-cell-autonomous manner might show the presence of a neurotrophic factor that is generally required for neuroepithelial cell survival.

Concluding remarks

An intrinsic cell death program is linked to a variety of dys-

functions of genes. Some of the causes are related to specific processes of development. In the light of our study on a small sample of NED mutants, cell death is revealed to be, in 3 cases, the only early visible phenotype of a patterning defect. Therefore, the use of vital cell death stain methods detecting the pattern of apoptosis could help to distinguish NED mutants. Based on the frequency of early patterning defects detected in our 40 mutants, we would predict that a significant fraction of NED mutations are due to CNS patterning defects. In the light of this perspective, a close examination and classification of NED mutants employing vital cell death staining would be fruitful, since the screening of class I NED mutants by changes in gene expression would locate patterning mutants within the background of other NED mutations. A careful characterization of the NED mutants already isolated could also give new perspectives on the significance of cellular death, as well as crucial information on the regulation of programmed cell death during embryonic development.

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