

Mutations affecting neurogenesis and brain morphology in the zebrafish, *Danio rerio*

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SUMMARY

In a screen for embryonic mutants in the zebrafish a large number of mutants were isolated with abnormal brain morphology. We describe here 26 mutants in 13 complementation groups that show abnormal development of large regions of the brain. Early neurogenesis is affected in *white tail* (*wit*). During segmentation stages, homozygous *wit* embryos display an irregularly formed neural keel, particularly in the hindbrain. Using a variety of molecular markers, a severe increase in the number of various early differentiating neurons can be demonstrated. In contrast, late differentiating neurons, radial glial cells and some non-neural cell types, such as the neural crest-derived melanoblasts, are much reduced. Somitogenesis appears delayed. In addition, very reduced numbers of melanophores are present posterior to the mid-trunk. The

wit phenotype is reminiscent of neurogenic mutants in *Drosophila*, such as *Notch* or *Delta*. In mutant *parachute* (*pac*) embryos the general organization of the hindbrain is disturbed and many rounded cells accumulate loosely in the hindbrain and midbrain ventricles. Mutants in a group of 6 genes, *snakehead* (*snk*), *natter* (*nat*), *otter* (*ott*), *fullbrain* (*ful*), *viper* (*vip*) and *white snake* (*wis*) develop collapsed brain ventricles, before showing signs of general degeneration. *atlantis* (*atl*), *big head* (*bid*), *wicked brain* (*win*), *scabland* (*sbd*) and *eisspalte* (*ele*) mutants have different malformation of the brain folds. Some of them have transient phenotypes, and mutant individuals may grow up to adults.

Key words: neurogenesis, neurogenic genes, somitogenesis, hindbrain, brain ventricles, adhesion, primary neurons, zebrafish

INTRODUCTION

Neural development in vertebrates starts during gastrulation with the induction and patterning of the neural plate. The morphogenic transformation of the neural plate into the neural tube and the differentiation of neuronal cell types in different waves of neurogenesis follow. Finally, precise connection between neurons in the various brain regions are established. The complexity of this process is overwhelming, suggesting interactive mechanisms between cells and their neighbors or cells and their environment. The genetic control of many of these processes is not well understood in vertebrate organisms. In *Drosophila*, local cell-cell interactions play an important role in coordinating developmental decisions required for the generation of different cell types (for reviews, Campos-Ortega, 1993; Ghysen et al., 1993). Neurogenic genes, such as *Notch*, *Delta* and others are involved in this process in many different tissues (Hartenstein et al., 1992). The products of these genes

interact through lateral inhibition, committing a cell to a neural fate, and neighboring cells become epidermis (Seydoux and Greenwald, 1989; Heitzler and Simpson, 1991). Loss-of-function mutations cause overproduction of neurons – the neurogenic phenotype (Lehmann et al., 1983) – due to the mis-specification between the neural and epidermal pathways (Heitzler and Simpson, 1991). Gain-of-function mutations lead to overproduction of epidermis at the expense of neural structures (Palka et al., 1990) and can be mimicked by extracellular truncations of *Notch* (Struhl et al., 1993).

Homologs of these genes have been cloned in vertebrates, such as *Notch* (Coffman et al., 1990; Weinmaster et al., 1991; Ellisen et al., 1991; Reaume et al., 1992 del Amo et al., 1992; Bierkamp and Campos-Ortega, 1993; Lardelli and Lendahl, 1993) *Delta* (Chitnis et al., 1995; Henrique et al., 1995; Bettenhausen et al., 1995) and a rat ligand for *Notch* (Lindsell et al., 1995). Misexpression of constitutively active forms of *Notch* in cultured mammalian cells suggests that it is involved

in the signaling pathways for cell fate decisions (Kopan et al., 1994; Nye et al., 1994). However, in a loss-of-function mutation in the *Notch1* locus no effect on neurogenesis could be detected (Swiatek et al., 1994; Conlon et al., 1995). In *Xenopus*, the ectopic expression of *X-Delta-1* inhibits the genesis of primary neurons, and the interference of endogenous *X-Delta-1* activity causes overproduction of primary neurons (Chitnis et al., 1995). Although the progress in understanding neurogenesis in vertebrates is very encouraging, it is still not clear how and to what extent these genes are involved in cell-cell interaction and cell fate specification.

In addition to cellular interactions by lateral inhibition, the development of neurons further depends on interactions with molecules in their environment. Studies of individual cell types have revealed a tremendous diversity in the molecules that regulate the development of cells in the nervous system. These include cell adhesion molecules and the extracellular matrix (for reviews, Hynes and Lander, 1992; Müller and Kypta, 1995; Venstrom and Reichardt, 1993). These molecules have been shown to influence a variety of processes such as neuronal migration, survival, axonal growth and guidance, synapse formation and glial differentiation.

The morphogenesis of the neural tube in fish differs slightly from that of other vertebrates. There are two forms of neurulation in vertebrates. Primary neurulation, which occurs mainly in amphibia, birds and mammals (Karfunkel, 1974), proceeds by invagination of the neural plate to form a neural groove, which subsequently closes to form a neural tube. In contrast, secondary neurulation, which occurs in teleosts (Schmitz et al., 1993) and in the caudal region of the trunk in many other vertebrates (Hughes and Freeman, 1974; Nakao and Ishizawa, 1984; Schoenwolf and DeLongo, 1980), proceeds by the formation of a dense nerve rod, which then forms a lumen by cavitation. Although the morphogenetic movements accompanying formation of the neural keel in the zebrafish are different from that in other vertebrates, the zebrafish utilizes a mechanism similar to primary neurulation by infolding of the neural plate (Papan and Campos-Ortega, 1994). Mitoses occur preferentially on the ventricular side (Schmitz et al., 1993), and the siblings frequently form bilateral cell clusters across the midline (Kimmel et al., 1994; Papan and Campos-Ortega, 1994). Following its formation, the anterior end of the embryonic neural tube in every vertebrate, from fish to human, partitions into three primary brain vesicles, forebrain, midbrain and hindbrain. Further differentiation involves thickening of the wall in some locations and evagination in others. By 24 hours, several prominently sculptured regions can be seen in the zebrafish brain, including telencephalon, diencephalon, dorsal midbrain (tectum), ventral midbrain (tegmentum), cerebellum and seven hindbrain rhombomeres (Kimmel, 1993). The embryonic neurocoel is larger in the brain than in the spinal cord. Within the brain vesicles it forms expansions called ventricles.

In a large scale screen for mutants affecting the development of the zebrafish embryo, we have isolated about 100 mutants with visible brain phenotypes. In complementation tests between mutants with similar phenotypes, we have identified 16 genes that are involved in general brain development and morphogenesis (Table 1). In this paper we describe the phenotypes of 13 genes involved in different aspects of neural development, such as cell fate decision, cell-cell adhesion, and formation of the brain ventricles. *parachute* mutant embryos

have loose cells in their brain ventricles and a displacement of some neuronal cell types in the hindbrain. Mutations in a group of six genes, *snakehead*, *natter*, *otter*, *fullbrain*, *viper* and *white snake*, cause early failure of ventricle formation of the brain, severe retardation and early death. Mutants in five genes, *atlantis*, *big head*, *wicked brain*, *scabland* and *eisspalte* display extra or abnormal folds in the brain. We have concentrated our analysis on *white tail*, the only gene identified in our screen that displays a neurogenic phenotype.

MATERIALS AND METHODS

Maintenance and mutagenesis of fish

Fishes were maintained and mutagenized as described by Brand et al. (1995); Haffter et al. (1996) and Mullins et al. (1994). Embryos were collected by natural spawning, raised at 28.5°C, and staged as described by Westerfield (1994) and Kimmel et al. (1995). Sometimes the embryos were treated with 0.2 mM 1-phenyl-2-thiourea (PTU), which inhibits tyrosinase (Blagoeva and Stoichev, 1979), to maintain their optical transparency (Westerfield, 1994).

Whole-mount in situ hybridization and antibody protocols

Whole-mount in situ hybridization and antibody stainings were performed as described previously (Hammerschmidt and Nüsslein-Volhard, 1993). For antibody stainings, anti-Islet-1 (1:500; Korzh et al., 1993), anti-GABA (1:500; Sigma), anti-myf-5 (1:200; Santa Cruz), anti-myogenin (1:200; Santa Cruz) and anti-GFAP (1:1000; DAKO) polyclonal antibodies and zrf-1, zn-5 (1:1000; Trevarrow et al., 1990), 3A10 (1:500; Furley et al., 1990), MF20 (1:100; Bader et al., 1982), 4D9 (1:500; Patel et al., 1989), anti-acetylated tubulin (1:1000; Sigma; Piperno and Fuller, 1985) monoclonal antibodies were used. For in situ hybridization, digoxigenin-labeled RNA probes were synthesized from *krox-20* (Oxtoby and Jowett, 1993), *Zash-1a*, *Zash-1b* (Allende and Weinberg, 1994), *pax[zf-b]* (Krauss et al., 1991), *TRP-2* (Kelsh et al., personal communication) and *Notch* (Bierkamp and Campos-Ortega, 1993) cDNA clones. Photographs were taken on an Axiophot photomicroscope (Zeiss).

RESULTS

In this paper we describe mutants in 13 genes that are involved in early neurogenesis, development of the hindbrain and formation of the brain ventricles (Table 1). We will first describe the phenotype of *white tail* in some detail, followed by a brief description of *parachute*, and an overview of the phenotypes of two groups of genes that affect early ventricle formation, and brain folding, respectively.

Neural hyperplasia in *white tail*

The gene *white tail* is represented by a single recessive allele. No other mutants with a similar phenotype have been isolated in our screen. During segmentation stages, homozygous *wt* embryos show an irregularly formed neural keel, particularly in the area of the hindbrain (Fig. 1A,B). Additionally, the somite boundaries in the posterior segments are indistinct (Fig. 1C,D) and the notochord is often slightly undulated. In the pharyngula period (24-48 hours), no rhombomeric boundaries can be seen (Fig. 1E,F). Melanophore pigmentation is absent in the posterior trunk and tail (Fig. 1G,H), and no migratory neural crest cells can be seen in the lateral pathway of the posterior trunk (Raible et al., 1992; data not shown).

Table 1. Overview of mutants

Gene name	Alleles*	CNS phenotypes	Other phenotypes	Reference
I. Early neural phenotype				
<i>white tail (wit)</i>	<i>ta52b</i>	Indentation in mid- and hindbrain	Pigmentation, somitogenesis	a,b
<i>parachute (pac)</i>	<i>tm101b,tj250a, tg299b</i>	Loose cells in mid- and hindbrain		
	<i>tg1a(s),tc248c(v)</i>	Cell clumps in hindbrain		
II. Collapsed ventricles				
<i>snakehead (snk)</i>	<i>tu236,tj223,to273a, tm52,tw3b,tl30 m115,m273,m523</i>	Brain ventricles collapsed	Ear, jaw, heart	c,d
<i>natter (nat)</i>	<i>tl43c,ta219b</i>	Brain ventricles collapsed	Heart	e
<i>otter (ott)</i>	<i>ta76b, m628,m673</i>	Brain ventricles collapsed	Ear, jaw, heart	c,d
<i>fullbrain (ful)</i>	<i>tq279, m133,m157</i>	Brain ventricles collapsed	Ear, pigmentation, heart	b,c,d
<i>viper (vip)</i>	<i>ta52e</i>	Brain ventricles collapsed	Heart	e
<i>white snake (wis)</i>	<i>tr241, m427</i>	Brain ventricles collapsed	Pigmentation, motility, heart	d
III. Abnormal brain folds				
<i>atlantis (atl)</i>	<i>tc234b(v),tg1b(v), tm48(v),ty86a(v)</i>	Extra neural bridges in hindbrain		
<i>big head (bid)</i>	<i>to15c(v)</i>	Enlarged midbrain		
<i>wicked brain (win)</i>	<i>tk254a</i>	Bumpy brain	Neural degeneration	
<i>scabland (sbd)</i>	<i>tm337c</i>	Bumpy brain	Jaw	
<i>eisspalte (ele)</i>	<i>ty77e</i>	Dent after midbrain-hindbrain boundary		
IV. Other mutants				
<i>bashful (bal)</i>	<i>tp82,tm220a,tr259, tf235,tv36,tt206, tp86,tm267a,tj209, to265,tc245a,tq210, tb244f,tc248f,tr203</i>	Bumpy brain	Notochord differentiation, axonal pathfinding	f,g
<i>grumpy (gup)</i>	<i>ti228b,tj229a,tx221, tl17b,tm61,tp42, tg210</i>	Bumpy brain	Notochord differentiation, axonal pathfinding	f,g
<i>sleepy (sly)</i>	<i>te223,te333,tj215b, ti272a,to216a,tm89, tp16,ts33a,ti263a</i>	Bumpy brain	Notochord differentiation, axonal pathfinding	f,g

*All are lethal except marked. s, semiviable; v, viable.

References: a, van Eeden et al., 1996a; b, Kelsh et al., 1996; c, Whitfield et al., 1996; d, Schier et al., 1996; e, Chen et al., 1996; f, Odenthal et al., 1996; 1996b; g, Karlstrom et al., 1996.

Despite the complex phenotype, the development of mutant embryos does not seem to be generally retarded. The general structure and the shape of the posterior trunk and tail look rather unaffected (Fig. 1C,D); muscle cell differentiation seems to be normal (data not shown); the major subdivisions of the brain are present, and the otic vesicle length (Kimmel et al., 1995) seems to be normal (data not shown).

Due to the early CNS phenotype, we assayed the expression of a number of neuronal markers in *wit* homozygous mutant embryos. The monoclonal antibody (mAb) 3A10 recognizes a neurofilament-specific epitope (Furley et al., 1990) that is prominent in the Mauthner cells (Hatta, 1992 and Fig. 2A), a set of primary hindbrain interneurons which have been studied in many vertebrates (for review, Faber and Korn, 1978). In *wit* mutant embryos, 3-4 Mauthner cells, instead of a single cell, can be detected per hemi-rhombomere 4. Additionally, other identified hindbrain interneurons labeled by 3A10 are increased in number as well (Fig. 2B). The medial longitudinal fascicle is wider than that in wild type, suggesting an increase in axons and therefore in the neurons that give rise to these axons (compare Fig. 2A and 2B).

Does this increase in neuronal number apply only for specific neurons such as the Mauthner cell or generally for many kinds of neurons? To address this question, we used

other specific and well characterized neuronal markers. The Islet-1 antibody detects LIM homeobox-containing proteins that are present in early differentiating neurons (Korzh et al., 1993). As early as in the 8-somite stage we find a striking increase in the number of Islet-1-positive cells in *wit* embryos (Fig. 2C-F). At this stage in the zebrafish embryo the trigeminal ganglia are labeled (Fig. 2C). In addition, there are two laterally distributed lines of Rohon-Beard sensory cells, and two medially distributed lines of primary motoneurons (Fig. 2E). In *wit* embryos, the trigeminal ganglia get larger (Fig. 2D), smaller motoneurons along the anteroposterior axis increase in number, and the bigger Rohon-Beard cells get more numerous not only along the anteroposterior axis but also along the mediolateral axis (Fig. 2F). By the 20-somite stage, not only CNS cells, such as the Rohon-Beard sensory cells, motoneurons and epiphyseal neurons (Fig. 2G,H), but also Islet-1-positive cells in the PNS, such as trigeminal ganglia (as mentioned before), and the presumptive anterior and posterior lateral line ganglia, are increased in number (Fig. 2G,H). Acetylated tubulin mAb labeling reveals similar results: axons of the anterior commissure (AC) and the postoptic commissure (POC) form thicker bundles and neuronal somata in the spinal cord (such as Rohon-Beard cells) are increased in number (data not shown).

In addition to the identified early differentiating neurons mentioned above, several other interneurons increase in *wit* as well. The antibody against the neurotransmitter gamma aminobutyric acid (GABA) labels several different types of interneurons in the brain and spinal cord (Bernhardt et al., 1992; Macdonald et al., 1994). The increase of GABA labeling can be shown at the 20-somite stage (data not shown). In the pharyngula period, not only is the GABA labeling in the hindbrain increased but also the segmented organization of the hindbrain appears disrupted (data not shown). *pax-b*, also called *pax2* (Krauss et al., 1991; Püschel et al., 1992; Mikkola et al., 1992) expression in the hindbrain and spinal cord appears increased in *wit* embryos while the expression domain at the midbrain hindbrain boundary appears normal in size (compare Fig. 2I,J).

Zash-1a and *Zash-1b* are two *achaete-scute* homologs, which encode transcription factors containing basic helix-loop-helix domains (Allende and Weinberg, 1994). At about the 15-somite stage, the expression of *Zash-1a* and *Zash-1b* mRNA in *wit* is increased in many regions compared to normal embryos (Fig. 3A-D). In contrast, by the pharyngula period, their expression is decreased, especially the segmented expression pattern in the hindbrain (compare Fig. 3E,F and 3G,H and see discussion).

The increase in cell number is not restricted to neuroectodermal regions, but is also observed in at least two mesodermal tissues. The polster, which is a part of the prechordal mesoderm (Kimmel et al., 1990; Hatta and Kimmel, 1993) is larger in *wit* embryos as seen by Islet-1 labeling (Fig. 2D; Inoue et al., 1994). *pax-b* shows an increased area of the expression domain in the pronephric duct (Fig. 2I,J).

In summary, by using several markers, we can show that many early differentiating neurons, including sensory neurons, interneurons and primary motoneurons, as well as some mesodermal cell types, are strikingly increased in number.

Late differentiating neurons, glial cells and neural crest-derived cells are reduced in *wit*

The preceding data suggest that *wit* mutants develop an increased number of early differentiating neurons. How do these additional cells arise? One possibility is that they increase at the expense of other cell types within the neuroepithelium. We investigated other neural cell types using markers for late differentiating neurons, glia and neural crest derivatives.

During the later pharyngula period, zn-5 mAb labeling can be seen in commissural axons and dorsal interneurons in the hindbrain (Trevarrow et al., 1990, Fig. 4A), as well as in ventral axons of the secondary motoneurons in the spinal cord (Pike et al., 1992). These late differentiating neurons do not appear before 24 hours postfertilization (hpf) and their somata are smaller than those of primary neurons (Kimmel and Westerfield, 1990). In *wit* homozygous embryos, zn-5 mAb labeling is reduced in the hindbrain at 36 hpf (Fig. 4A,B). By 48 hpf, there is no detectable zn-5-positive labeling of secondary motoneuron axons in the spinal cord in *wit* homozygous embryos (data not shown), which suggests that the secondary motoneurons are strongly reduced in number compared to normal embryos. In contrast, the zn-5 labeling of the optic nerve and ganglion cell layer in the head is increased (data not shown), which shows that the reduction in zn-5 labeling is not due to general retardation.

We also examined the expression of four markers that recognize radial glial cells in the zebrafish (Trevarrow et al., 1990; Marcus and Easter, 1995). The *zrf-1* labeling is not only highly reduced in the retina (Fig. 4C,D) and the hindbrain (Fig. 4E,F) but also in the diencephalon, tectum and spinal cord (data not shown). This reduction can be detected as early as the 17-somite stage. From 24 hpf to 48 hpf, the radial glial labelings, prominent in the hindbrain in wild type, cannot be detected in *wit* homozygous embryos (Fig. 4E,F and data not shown). It appears that not all glial cell types are affected, as the expression of glial fibrillary acidic protein (GFAP), which is expressed in several glial cell types (Eng and Shiurba, 1988), looks normal by 24 hpf (data not shown). In contrast, the other three *zrf* antibodies did not show obvious reduction in labeling like *zrf-1*; however, the segmented labeling in the hindbrain of wild-type embryos is not seen in *wit* embryos (data not shown). *zrf-2*, *zrf-3* and *zrf-4*, in addition to radial glial cells, may also

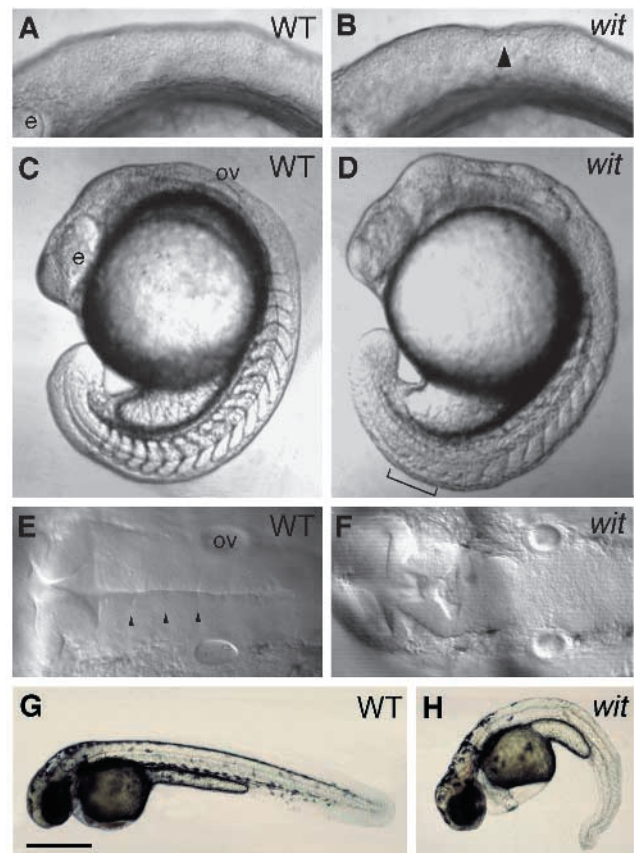


Fig. 1. Phenotype of live *wit^{ta52b}* embryos. If not specified, all the following figures are rostral to the left, dorsal to the top. Lateral views of wild-type (A) and mutant (B) embryos at 9-somite stage. The indentation in the hindbrain (arrowhead) is indicated. Lateral views of wild-type (C) and mutant (D) embryos at 17-somite stage. The indistinct somite boundaries (bracket) are indicated. Dorsal views of wild-type (E) and mutant (F) embryos at 26 hours postfertilization (hpf). Normal rhombomeric boundaries (arrowheads) are not seen in mutant embryos. Lateral views of wild-type (G) and mutant (H) embryos at 36 hpf. Note that there are no visible pigmented melanophores in the posterior trunk of mutant embryo in H. e, eye; ov, otic vesicle. Bar, 200 μ m (A,B,E,F); 335 μ m (C,D); 640 μ m (G,H).

label some other neuronal cell types (Westerfield, 1994 and data not shown).

Neural crest cells give rise to a variety of different cell types, including neurons, melanophores, endocrine cells and various connective tissue types (for review, Le Douarin et al., 1993). As described above, in *wit* mutants neither pigmented melanophores, nor neural crest cells migrating on the lateral migration pathway are visible in the posterior trunk and tail. The zebrafish *TRP-2* homolog labels melanoblasts prior to melanin synthesis (R. Kelsh, unpublished observation). While *TRP-2* labeling in the head and anterior trunk of *wit* mutants is indistinguishable from that of wild-type siblings, in the posterior trunk/tail there are essentially no *TRP-2* labeled cells in mutant embryos (compare Fig. 4G,H). Together, these results suggest that at least one neural crest derivative (melanophore) is not formed in the posterior trunk and tail.

Somitogenesis is delayed, but myogenesis is not affected in *wit*

In live *wit* homozygous embryos, somite boundaries in the posterior trunk and tail are less distinct than in normal embryos (Fig. 1D). In the zebrafish, somites appear in an anterior to posterior progression at a constant rate of one somite every half hour (Hanneman and Westerfield, 1989). Although homozygous *wit* embryos can hardly be distinguished from wild type before the 8-somite stage, we can show that there is a delay in somitogenesis before this stage. We counted the somite number, kept individual

embryos separate and determined the phenotype after 24 hpf. The delay is detected from the onset of somitogenesis (data not shown). At the 2-somite stage, the recognizable somite number

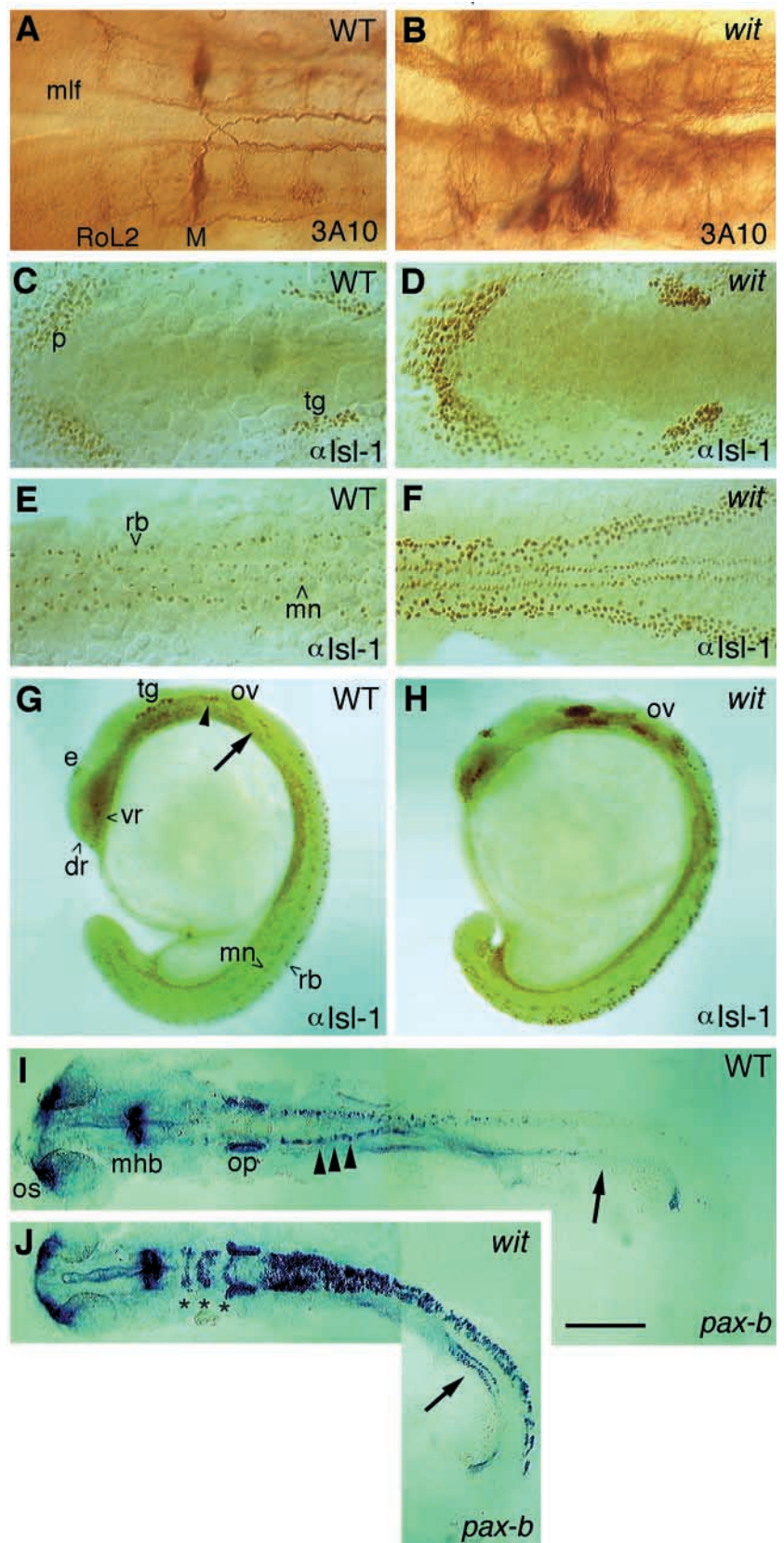


Fig. 2. Neuronal hyperplasia in *wit^{a52b}*. All are dorsal views and rostral to the left, except for G and H, which are lateral views and rostral to the top. Hindbrain reticulospinal neurons in wild-type (A) and mutant (B) embryos labeled with mAb 3A10 at 36 hpf. Anterior parts of wild-type (C) and mutant (D) embryos and of trunk parts of wild-type (E) and mutant (F) embryos labeled with Islet-1 antibody at the 8-somite stage. Wildtype (G) and mutant (H) embryos labeled with Islet-1 antibody at 20-somite stage. The presumptive anterior lateral line ganglion (arrowhead) and presumptive posterior lateral line ganglion (arrow) are indicated. *pax-b* expression in wild-type (I) and mutant (J) embryos at 24 hpf. The CoSA neurons (arrowheads) and pronephric duct (arrows) are indicated (Mikkola et al., 1992). In mutant embryos, not only is the *pax-b* expression in spinal interneurons and pronephric duct increased, and there is also an increased expression in presumptive r2, r3 and r4 (asterisks). dr, dorsorostral cluster; e, epiphysis; M, Mauthner cell in r4; mhb, midbrain-hindbrain boundary; mlf, medial longitudinal fascicle; mn, primary motoneurons; op, otic placode; os, optic stalk; ov, otic vesicle; p, polster; rb, Rohon-Beard cells; RoL2, RoL2 neurons in r2; tg, trigeminal ganglia; vr, ventrorostral cluster. Bar, 100 μ m (A,B); 160 μ m (C-F); 255 μ m (G,H); 400 μ m (I,J).

Table 2. Delay of somitogenesis in *wit*^{ta52b} demonstrated by molecular makers

Stage	myf-5		myogenin	
	+/+ & +/-*	-/-	+/+ & +/-	-/-
11s	13.9±0.3(30)†	8.8±0.4(11)	11.3±0.5(21)	7.9±0.4(7)
18s	19.7±0.8(48)	12.4±0.7(13)	16.8±1.1(28)	12.1±0.7(13)

Values are means±s.d.
 *Genotype of embryos.
 †Segmented and labeled somite number. The number in parentheses is counted embryos.

in *wit* is about 90% of that in wild type demonstrated by statistics from a reasonable number of embryos, but it decreases further to around 60% by the 17-somite stage (compare Fig. 1C,D).

The delay in somite formation also can be shown by molecular markers. By the 11-somite stage, the difference of labeled and segmented somite number between wild types and *wit* homozygous embryos is about 3 somites, as assayed by expression of Myogenin (Table 2), whereas the difference is about 5 somites in Myf-5 labelings (Table 2 and Fig. 5A,B). By the 18-somite stage, the wild-type embryos have about 5 and 7 more labeled and segmented somites than *wit* embryos as determined by Myogenin and Myf-5 labeling, respectively (Table 2). These results support the observations in live embryos. The Myf-5 pattern reveals that, in homozygous *wit* embryos, in addition to the delay in somitogenesis, the somites are less sharply segmented and the caudal somite remains are unsegmented (Fig. 5A,B). MF20, which is a marker for myosin heavy chain (Bader et al., 1982 and Kopan et al., 1994), is expressed at normal levels except for the unsegmentation of somites in the posterior (Fig. 5C,D), and somitic myogenesis appears to be normal. Furthermore, the organization of myofibrils is normal in *wit* homozygous embryos (data not shown).

In summary, we have shown that in *wit* mutant embryos the number of early differentiating neurons is increased. In contrast, late differentiating neurons, some glial cells and neural crest derivatives are decreased. We find an additional effect on somite formation. A similar spectrum of phenotypes, including the effect on the mesoderm, has been observed for neurogenic genes in *Drosophila*, such as *Notch* and *Delta*. Therefore we examined the expression of the zebrafish *Notch* homolog (Bierkamp and Campos-Ortega, 1993). While in the normal embryo, during somitogenesis, high levels of *Notch* transcripts can be detected in the presomitic mesoderm and developing somitomeres (Fig. 5E; Bierkamp and Campos-Ortega, 1993), this expression is reduced in *wit* homozygous embryos (Fig. 5F).

In *parachute*, the brain is disorganized

In our screen, we found 5 alleles of different strengths of *parachute* (Table 1). Mutant embryos can be identified around the 10-somite stage by a slightly uneven neural keel or a small bulge in the hindbrain area (Fig.

6A,B). Later, in the pharyngula period, the brain ventricles are filled with rounded-up cells and the hindbrain region is thinned while displaying an uneven surface (Fig. 6D). In the hindbrain ventricles of the weak alleles, some cell clumps are located which have a filopodia-like structure (Fig. 6E). During the hatching period abnormal growth in the hindbrain is often seen, which is associated with a bump that is devoid of pigmentation (data not shown).

Hindbrain segments consist of one segment center region and one segment border region, which contain reticulospinal neurons and commissural neurons, respectively. Centers are divided from the borders by curtain-like rows of glial fibers (Trevarrow et al., 1990). In *zrf-1* mAb (Trevarrow et al., 1990) labeled normal embryos, these segmented structures are visible (Fig. 7A), while they appear disorganized in *pac* homozygous embryos (Fig. 7B). The disorganization also holds true for neurons as indicated by labeling with antibodies against acetylated tubulin (Fig. 7C,D).

Using the 3A10 mAb, we found that the position of the Mauthner cells is altered in *pac* mutant embryos (Fig. 8B-D,

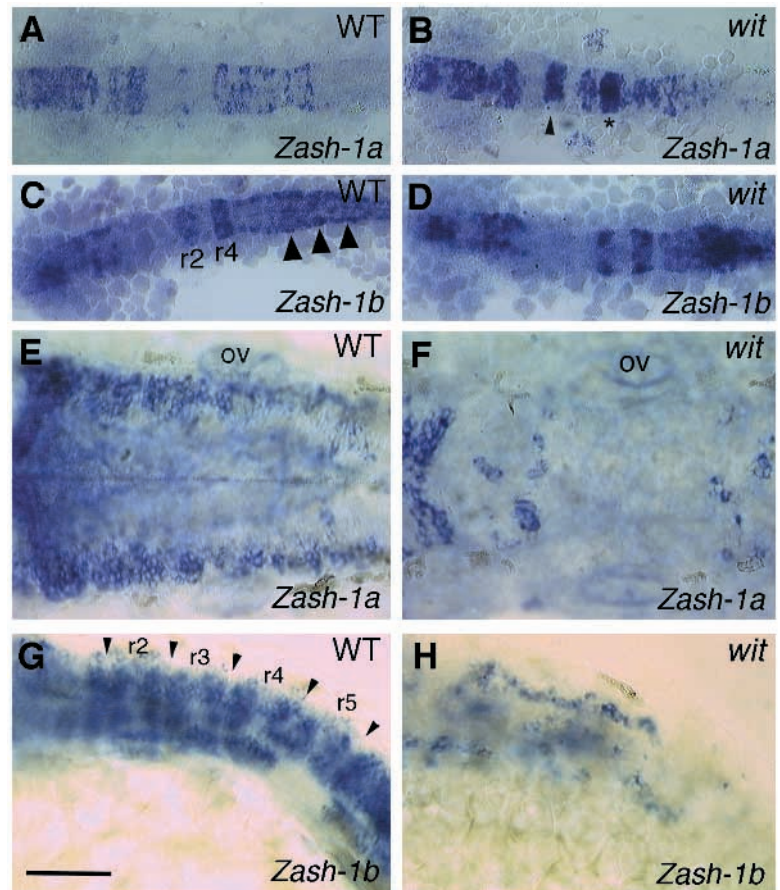


Fig. 3. The expression pattern of two zebrafish *achaete-scute* homolog genes in *white tail*. *Zash-1a* expression in wild-type (A) and mutant (B) embryos at the 17-somite stage. The presumptive mhb (arrowhead) and presumptive r3 (asterisk) are indicated. *Zash-1b* expression in wild-type (C) and mutant (D) embryos at 15-somite stage. The expression in the spinal cord (triangles) is indicated. Wildtype (E) and mutant (F) embryos at 26 hpf labeled with *Zash-1a* RNA probe. Wildtype (G) and mutant (H) embryos at 26 hpf labeled with *Zash-1b* RNA probe. ov, otic vesicle; r(n), rhombomere (n). Bar, 320 µm (A-D); 100 µm (E-H).

for detail see legend). 3A10 mAb not only labels Mauthner cells in rhombomere 4 but also some other reticulospinal interneurons in adjacent rhombomeres (Fig. 8A and Hatta, 1992). Somata positions of all 3A10-positive neurons excluding the Mauthner cells are relatively unaffected, and the pathfinding of their axons is normal or slightly affected (Fig. 8). Although a frequent displacement of Mauthner cells was observed, a less stained and more medial neuron in r4 is still present (compare Fig. 8A-C), suggesting the Mauthner cell moved from its normal position in *pac* embryos. At least in weak alleles, the *pac* phenotype gets milder with age, and some individuals may survive to adulthood.

The 'snakehead' group of genes: collapsed ventricles and retardation of development

In our screen we isolated a number of mutants displaying very early effects on brain morphology. A group of genes, the 'snakehead' group, *snakehead* (Fig. 9B), *natter*, *otter*, *fullbrain*, *viper* and *white snake* have very similar phenotypes. In the pharyngula period, the brain region is very much reduced in size, and the brain ventricles appear collapsed. Mutant embryos also have heart failures, enlarged heart cavity and display signs of general retardation. They die in the early larval period. Although the general phenotype of genes in this group is very similar, there are locus-specific differences, listed in Table 1. We have examined the expression patterns of *krox-20* (Oxtoby and Jowett, 1993) and *Engrailed* (Patel et al., 1989) in *snk*, *nat* and *ott*, but no detectable abnormalities can be found (data not shown).

atlantis and other genes affecting vesiculation of the brain

Mutations in a group of three genes, *atlantis*, *big head* and *wicked brain*, all cause abnormalities in brain size or folding during the pharyngula period. In *atlantis* additional folds are often seen (Fig. 9D), while in *wicked brain* the folding is irregular, and *big head* embryos have larger ventricles. Many mutant embryos develop a swimbladder and may grow up to normal adults which do not display an obvious phenotype. Each gene has its own characteristic features as described in Table 1. The expression patterns of *krox-20* (Oxtoby and Jowett, 1993) and *Engrailed* (Patel et al., 1989) is normal in *atl* (data not shown).

Other brain mutants

Mutants in two further genes (*scabland*, *eisspalte*) display disorganized brains during the pharyngula period. Both have additional phenotypes and show retarded development (Table 1). General brain morphology is also abnormal in three genes, *bashful*, *grumpy*, and *sleepy*, affecting notochord differentiation (Odenthal et al., 1996). Mutations of all three genes also cause abnormal axonal pathfinding (Karlstrom et al., 1996). A number of other genes affecting specific regions of the brain, which have been identified in our screen, are described in accompanying papers (Brand et al., 1996; Heisenberg et al., 1996).

DISCUSSION

In our screen, we isolated about 100 mutants that show a major brain phenotype. Most of the mutants were found at the second day of development when the folding of the brain vesicles is still ongoing. Several of the mutants mentioned in this paper display defects which can already be detected at much earlier stages of development. The *snakehead* group phenotype, as well as *sbd* and *ele* show general signs of retardation. Because of their very early effects on the brain morphology, we kept

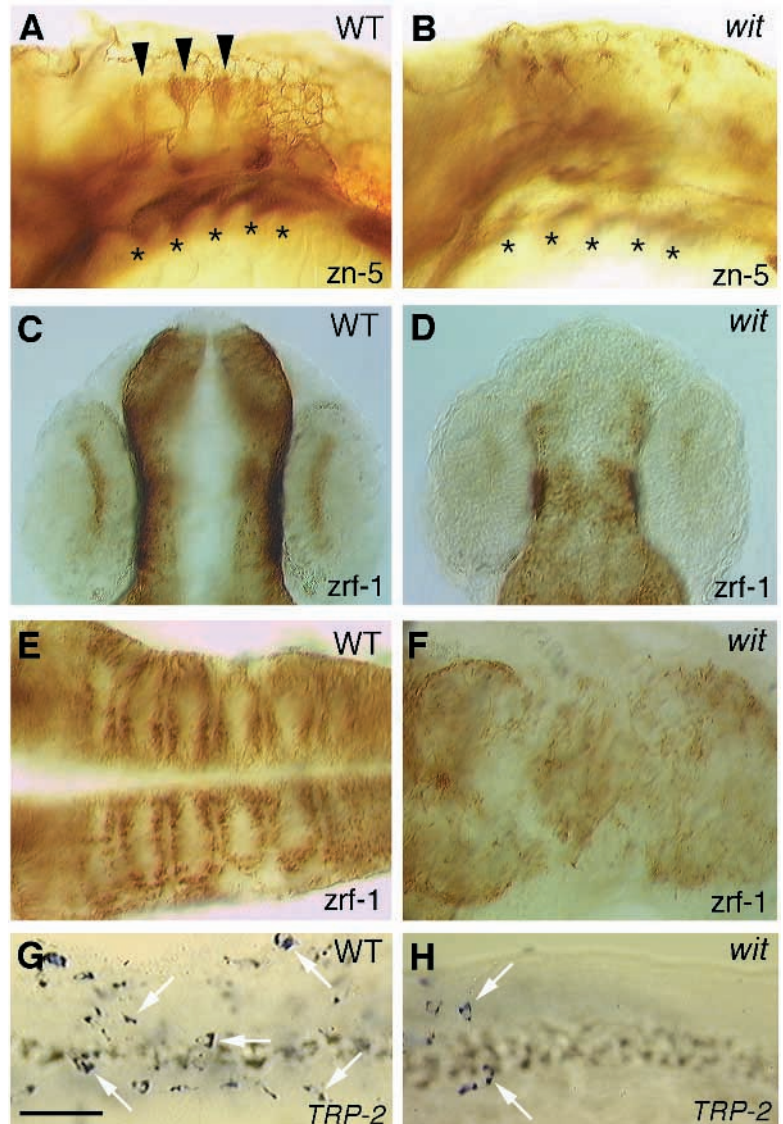
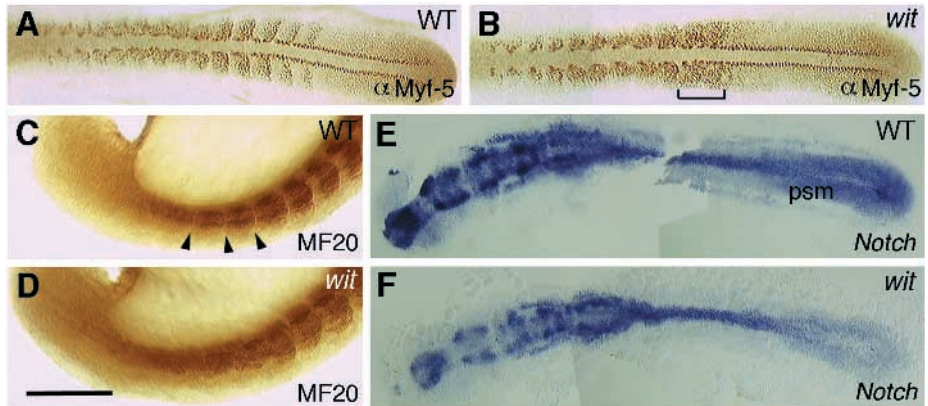


Fig. 4. Some cell types are decreased in *wit^{ta52b}*. C and D are rostral to the top. A, B are lateral views; C-H are dorsal views. Wildtype (A) and mutant (B) embryos labeled with zn-5 mAb at 36 hpf. The dorsal hindbrain neurons (arrowheads; Trevarrow et al., 1990) are decreased in mutant embryos. The pharyngeal endoderm (asterisks; Schilling and Kimmel, 1994) is not affected. The pharyngeal endoderm in B is out of focus. Wildtype (C,E) and mutant (D,F) embryos labeled with zrf-1 mAb. C,D are at 24 hpf; E,F are at 38 hpf. Radial glial fibers in the retina (Westerfield, 1994) and hindbrain (Trevarrow et al., 1990) are decreased. *TRP-2* expression pattern of wild-type (G) and mutant (H) embryos in the anterior trunk at 24 hpf. The labeled melanophores (arrows) are decreased in H. Bar, 125 μ m (A-D); 100 μ m (E-H).

Fig. 5. Somitogenesis is delayed, but somitic myogenesis is normal in *wit*^{ta52b}. A,B,E,F dorsal views. C,D lateral views. Myf-5 antibody labeling in wild-type (A) and mutant (B) embryos at the 11-somite stage. MF20 labeling in wild-type (C) and mutant (D) embryos at the 17-somite stage, rostral to the top. The somite boundaries (arrowhead) are indicated. *Notch* expression in wild-type (E) and mutant (F) embryos at the 12-somite stage. psm, presomitic mesoderm. Bar, 400 μ m (A,B); 240 μ m (C,D); 475 μ m (E,F).



the *snakehead* group as well as *sbd* and *ele*, in contrast to those displaying retardation signs later, because we suspected specific patterning defects to be the primary cause of the phenotypes. This expectation was not substantiated, by analysis with some molecular markers. Further analysis will reveal the cause of the ventricle collapse in mutant embryos of *snakehead* group. The phenotypes of the *atlantis* group displaying additional and/or abnormal brain folds, which are not associated with retardation or degeneration, are apparently also not caused by misspecification of specific regions of the brain. Surprisingly, in the case of *atl* and *bid*, mutant individuals can recover and grow up to apparently normal and fertile adults. It is tempting to speculate that in these mutants functions are impaired that can be taken over by other genes if necessary.

The gene *parachute*, for which five alleles have been isolated, has a unique phenotype with cells apparently detaching from the surface of the outfolding neural tube. This is reminiscent of a small group of genes that were classified as causing skin defects (van Eeden et al., 1996a), as well as the dominant phenotype of epiboly mutants (Kane et al., 1996). In *pac* mutant embryos, several neurons were found to be displaced in an irregular fashion. Our tentative interpretation is that *pac* is required in processes of cell contact and adhesion within the central nervous system.

In this paper, we describe the mutant phenotype of one gene, *white tail*, in more detail. For *wit*, only one allele was isolated in the screen, displaying a unique phenotype reminiscent of the neurogenic phenotype of several *Drosophila* mutants. As only one *wit* allele is known so far, it is not clear whether all aspects

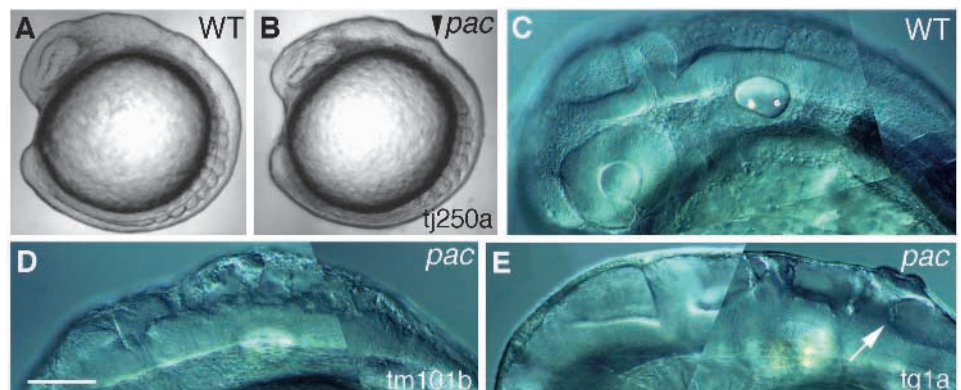
of the phenotype are caused by a single mutation, or by a deletion eliminating several genes. In addition, the strength of the allele is not known. Therefore the following interpretation of the phenotype should be regarded as tentative. Aside from the putative Notch/Delta signaling, the asymmetric protein localization mechanism is also possible (for review, Jan and Jan, 1994; Doe and Spana, 1995).

The increase of neuronal cells could be at the expense of some other cell types in *wit*

The *wit* gene is required very early in the embryo. At the two somite stage, mutant embryos can already be recognized by a delay in somite formation. Shortly thereafter, the most prominent phenotypic feature of the *wit* mutation is the striking increase of several neuronal cell types as revealed by molecular markers. This neuronal hyperplasia led us to investigate whether *wit* might be a gene with functions similar to the neurogenic genes in *Drosophila*. In these *Drosophila* mutants, neuronal hyperplasia is at the expense of other ectodermal cell types (Campos-Ortega, 1993; Hartenstein et al., 1992).

In neural development of the zebrafish, both hindbrain reticulospinal interneurons and spinal motoneurons appear during two distinct waves of neurogenesis (Kimmel and Westerfield, 1990). Early differentiating neurons are increased, while the late differentiating neurons are decreased in *wit* homozygous embryos. This could be due to the lack of late differentiating neural progenitors, which have been misspecified to early differentiating neural progenitors during the early wave of neurogenesis and/or can not acquire neuronal identity during the

Fig. 6. Phenotype of live *pac* embryo. C-E treated with 0.2 mM PTU. Lateral views of wild-type (A) and *pac*^{tj250a} (B) embryos at around the 9-somite stage. The small bulge (arrowhead) in the hindbrain is indicated. Lateral views of wild type (C) at 28 hpf, *pac*^{tm101b} (D) at 28 hpf and *pac*^{tg1a} (E) at 36 hpf. The filopodia-like structure (arrow) is indicated. Bar, 320 μ m (A,B); 200 μ m (C-F).



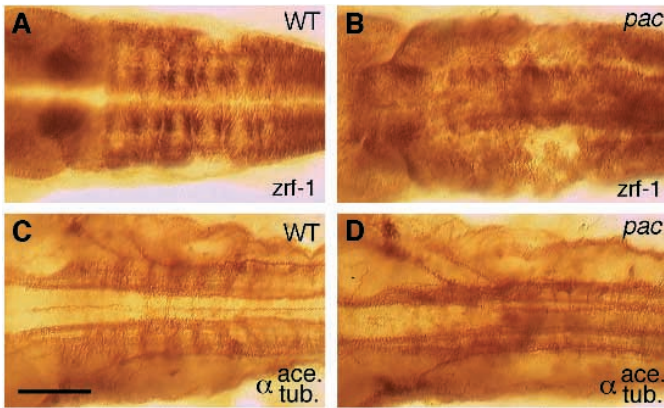


Fig. 7. Disorganization of the hindbrain in *pac*. Dorsal views of wild type (A) and *pac^{ij250a}* (B) labeled with *zrf-1* mAb. Dorsal views of wild type (C) and *pac^{ij250a}* (D) labeled with mAb against acetylated tubulin. Bar, 125 μ m.

late wave of neurogenesis. It has been proposed that *Zash-1a* and *Zash-1b* have a proneural function and are also involved in patterning of the CNS (Allende and Weinberg, 1994). We find that an increase of *Zash* expression around 15-somite stage (Fig. 3B,D) correlates with the additional early differentiating neurons found in *wit*. By 24 hpf, in contrast, we observe a strong decrease of *Zash* (Fig. 3F,H). One interpretation of this finding is that the cells that normally express *Zash* later are late differentiating neurons, and that the cells in *wit* embryos are misspecified to give rise to early differentiating neurons, expressing *Zash* during the early wave of neurogenesis.

Glial cells and neuronal cells have common progenitor cells in insects (Udolph et al., 1993), the early neural plate (Hartenstein, 1989; Soula et al., 1993), neural crest (Stemple and Anderson, 1992) and in some parts of CNS, such as the retina (Turner and Cepko, 1987), tectum (Galileo et al., 1990) and spinal cord (Leber et al., 1990). Using *zrf* mAbs and an antibody against GFAP, we show that a subpopulation of glial cells is reduced (Fig. 4D,4F and data not shown). These results suggest that the increase of neuronal cells is at the expense of glial cells, although only subsets of glial cells are reduced. Further experiments are required to investigate at which level of glial cell specification the *wit* gene is required.

The *wit* gene gets its name from a conspicuous lack of melanophores in the posterior trunk and tail. During neurulation in vertebrate embryos, the ectoderm becomes subdivided into three embryonic tissues, the neural tube, neural crest cells, and the epidermis. It has been demonstrated that if epidermis and neural plate are put adjacent to each other, neural crest cells are generated at the junction of the two tissues in amphibians (Moury and Jacobson, 1989) and in the chick (Selleck and Bronner-Fraser, 1995). However, it has been shown that in *Drosophila* neurogenic mutants, the neuronal hyperplasia is at the expense of epidermal cells (Seydoux and Greenwald, 1989; Heitzler and Simpson, 1991). Further analysis of epidermal and other neural crest derivatives is required in *wit*. As other neural crest cells, which normally migrate along the lateral pathway, could not be observed in the posterior of *wit* embryos, we propose that the neural hyperplasia observed in *wit* embryos is in part at the expense of neural crest derivatives. The restriction of the pigment phenotype to the posterior of the

embryo may be due to the incomplete lack of function of the only *wit* allele.

All the cell types we found reduced are neural cells born late in neurogenesis. We therefore propose that the increase in early differentiating neurons is at least in part at the expense of late differentiating neurons and/or cells with later birthdays in the nervous system. We have not investigated the fate of other ectodermal cell types yet nor do we know whether the neurogenic region in the *wit* embryo is generally larger than in wild type. From the normal size of several brain regions in *wit* embryos, (such as the midbrain-hindbrain boundary, the otic placode and the trigeminal ganglion, see Fig. 2), it rather appears that the density of early differentiating neurons is

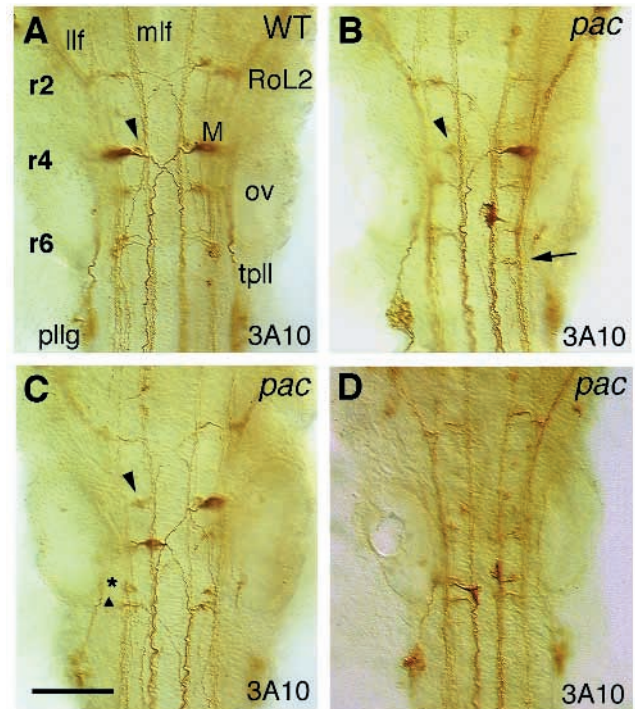


Fig. 8. Variable disorganization of hindbrain reticulospinal interneurons in *pac^{ij250a}* at 36 hpf shown by 3A10 mAb staining; dorsal views with rostral to the top. In wild type (A), the most prominently labeled Mauthner cells are located in r4, which project axons contralaterally to the mlf. RoL2 exists in r2, which projects axons contralaterally to the mlf, then to the llf. In A, B and C, MiM1 (or MiV1) (arrowheads; Mendelson, 1986a, b) is indicated. According to their position and axonal projection, the two neurons in r6 could be MiD3i (asterisk), which has an ipsilateral axon and MiD3c (triangle), which has a contralateral axon, as shown in C. B, C and D are representatively labeled embryos. In most of the cases, as shown in B, one of the Mauthner cells in rhombomere 4 is displaced to a more posterior rhombomere (r5, r6 or in between) and locates in the same side of the normal one ($n=5/10$). In B, a presumably displaced MiD3c (arrow) is indicated. In a few cases, as shown in C, the displaced Mauthner cell locates in the opposite side ($n=1/10$). Sometimes, as shown in D, both of the Mauthner cells are displaced to a more posterior rhombomere, and their axons project to the ipsilateral instead of the contralateral side ($n=3/10$). llf, lateral longitudinal fascicle; M, Mauthner cell; mlf, medial longitudinal fascicle; ov, otic vesicle; pllg, posterior lateral line ganglion; RoL2, RoL2 neurons in r2; tpll, tract of posterior lateral ganglion. Bar, 125 μ m.

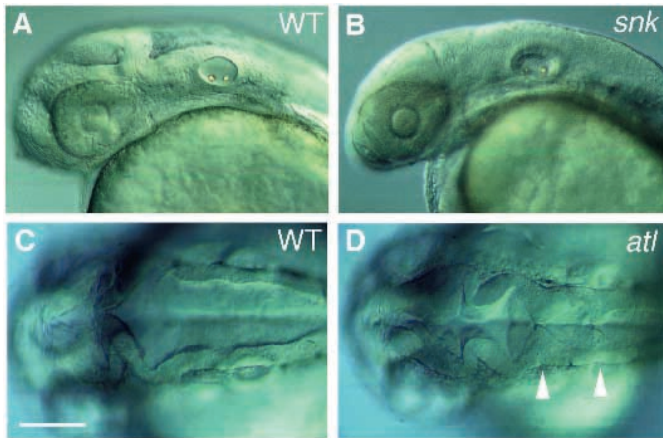


Fig. 9. Phenotypes of live *snk^{tw3b}* and *atlc^{234b}*. Lateral views of wild type (A) and *snk^{tw3b}* (B) at 36 hpf, treated with 0.2 mM PTU. Dorsal views of wild-type (C) and *atlc^{234b}* (D) embryos at 24 hpf. The extra neural folds (arrowheads) are indicated. Bar, 250 μ m (A,B); 200 μ m (C,D).

increased within a neurogenic region which is defined independently from the *wit* gene.

Somitogenesis is affected in *wit*

We find that the expression pattern of several myogenic markers is delayed and that the somite boundaries are less distinct in *wit*. Myogenesis and muscle formation, however, are normal. This suggests that somitic myogenesis is not directly linked to somitogenesis (Borman and Yorke, 1994a,b). We have isolated a number of mutations affecting somitogenesis (van Eeden et al., 1996b), and in all of them, myogenesis is also unaffected. As only one allele of *wit* has been isolated so far, it is possible that the somite phenotype is not related to the neurogenic phenotype of the *wit* gene. However, it has been demonstrated that myogenesis is normal but somitogenesis is affected in the *Notch1* knock-out mutant in mice (Conlon et al., 1995). Consistent with this, we have found that expression of zebrafish *Notch* (Bierkamp and Campos-Ortega, 1993) is affected in homozygous embryos (compare Fig. 5E,F and data not shown). We are currently determining whether *Notch* and *wit* are genetically linked.

Is *wit* a neurogenic mutant?

In many instances, the neurogenic genes are necessary for correct implementation of cell fate decisions (for review, Artavanis-Tsakonas et al., 1995). In addition, there are a number of instances where neurogenic genes are necessary for many transitions to, and maintenance of, the epithelial state (for review, Hartenstein et al., 1992). The two apparently contrasting functions may be two sides of the same coin. During neurogenesis in vertebrates, probably through lateral inhibition as in *Drosophila* (Heitzler and Simpson, 1991), neural progenitors can delaminate or partially delaminate from the ectodermal epithelium, whereas during somitogenesis, loose mesenchymal cells of presomitic mesoderm reorganize into a tightly packed epithelial ball. Both regulation of neurogenesis and somite formation may rely on acquiring/maintaining epithelial stability. Recent data have indicated the important correlation between mesenchymal/epithelial development and

neurogenic genes (Tepass and Hartenstein, 1995; Conlon et al., 1995; Mitsiadis et al., 1995).

The function of neurogenic genes in vertebrates has been analyzed in several cases. Ecotopic expression of *X-Delta-1* inhibits production of primary neurons, and interference with endogenous *X-Delta-1* activity results in an increase in primary neurons (Chitnis et al., 1995), which are reminiscent of the gain-of-function and loss-of-function mutants in neurogenic genes, respectively (for review, Campos-Ortega, 1993; Fortini and Artavanis-Tsakonas, 1993). Although the disruption of the *Notch1* did not lead to the expected neural phenotype, the mutant mice showed a problem in coordinating somitogenesis (Conlon et al., 1995). The mouse mutant corresponding to *Suppressor of Hairless* [*Su(H)*], which is involved in Notch signaling (Fortini and Artavanis-Tsakonas, 1994), also displayed somite defects (Oka et al., 1995). The *X-Delta-1*'s role in somitogenesis is still unknown, but due to its expression in presomitic mesoderm (Henrique et al., 1995) and coexpression with *X-Notch-1* in similar domains (Chitnis et al., 1995), it is likely that *X-Delta-1* is also involved in somitogenesis, playing a role similar to *Notch1*. The failure to observe a neural phenotype in *Notch1* mice could be due to the early embryonic lethality, which has been demonstrated in the study of *N-myc* (Moens et al., 1993). Although we only found one allele for *wit* in this screen, it is possible that *wit^{a52b}* is a leaky allele. We are looking for more alleles, hoping to make this point clear. Taking these results of ours and the results of others on the alterations of cell fate in the neural tube and the defects in somite segmentation, we propose that *wit* is the first neurogenic mutant in the zebrafish.

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Note added in proof

After the acceptance of this article, we found that *wit^{a52b}* does not complement or interact with *mib^{m132}* (Schier et al., 1996).

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