

An instructive function for Notch in promoting gliogenesis in the zebrafish retina

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SUMMARY

The Gal4-UAS technique has been used to misexpress a constitutively active Notch receptor variant (*notch1a-intra*) in the developing zebrafish retina. This is the first study to use this technique to misexpress genes and assess their function in neural development of the zebrafish. Expression of activated Notch1a either ubiquitously, driven by a *heat-shock70* promoter, or in a spatially regulated manner, controlled by the *deltaD* promoter, causes a block in neuronal differentiation that affects all cell types. Developing cells take on either a glial fate or remain undifferentiated. A large number of cells eventually undergo apoptosis. These phenotypic effects of activated Notch1a are expressed cell autonomously. Cells within central regions of the retina adopt a glial fate if they express activated Notch1a in a time window that extends from 27

to 48 hours postfertilization. This period corresponds mainly to the time of origin of ganglion cells in the normal retina. Activation of *notch1a* at later stages results in defects in cell type specification that remain restricted to the ciliary marginal zone, whereas neuronal types are specified normally within the central region. These observations indicate that glial differentiation is initiated by Notch1a-*intra* expressing cells, which become postmitotic in the same time window. Our results strongly suggest that Notch1a instructs a certain cell population to enter gliogenesis, and keeps the remaining cells in an undifferentiated state. Some or all of these cells will eventually succumb to apoptosis.

Key words: Notch1a, Gliogenesis, Zebrafish, Retina, Apoptosis

INTRODUCTION

The cells of the vertebrate retina differentiate into seven different major classes, one of which is glial (the Müller cells), while the other six are neuronal: the rod and cone photoreceptors, the horizontal, bipolar, amacrine and ganglion cells (Cajal, 1893; Dowling and Boycott, 1966). These seven major classes, however, comprise a large variety of different cell types, which can be classified on the basis of morphological and functional criteria. The cells of the retina develop from multipotential progenitor cells, giving rise to progeny that can differentiate into members of all the seven different cell classes (Turner and Cepko, 1987; Holt et al., 1988). However, at least some progenitor cells are biased toward the production of particular cell types. Thus, the VC1.1⁺ progenitor cells produce large numbers of amacrine and horizontal cells and, as development progresses, an increasing number of rods, but no cone cells (Alexiades and Cepko, 1997). Even though progenitor cells are very heterogeneous in their differentiation potential, and the time of cell birth is apparently not a major determinant of cell fate (Belecky Adams et al., 1996), a progressive restriction occurs, as later progenitor cells produce fewer different cell types (Alexiades and Cepko, 1997). A temporal sequence in the appearance of the different cell types has been described for several vertebrate species. Within a given region of the retina,

ganglion cells, horizontal cells and amacrine cells, as well as cones, are specified first, followed by bipolar cells, rods and Müller cells (see review by Cepko et al., 1996).

A similar sequence for the differentiation of the retinal cell types has been observed in the zebrafish. The first cells to become postmitotic are ganglion cells, which are located in a ventronasal patch at about 28 hours postfertilization (hpf) (Nawrocki, 1985; Hu and Easter, 1999). At about 37 hpf, all ganglion cells of the central region of the retina are postmitotic (Hu and Easter, 1999). Cell differentiation within the remaining layers of the retina progresses in the same direction (Raymond et al., 1995; Schmitt and Dowling, 1996; Passini et al., 1997; Hu and Easter, 1999). Thus, the first postmitotic cells of the internal nuclear layer appear at 38 hpf, and the first photoreceptor cells at about 48 hpf (Larison and BreMiller, 1985; Nawrocki, 1985; Raymond et al., 1995; Hu and Easter, 1999). At about 60 hpf, approximately 90% of the neurones in the central region of the retina have differentiated and the various layers are clearly distinguishable (Malicki, 1999). No data on the time of origin of the Müller cells are available for the zebrafish.

The molecular basis of cell type diversification within the developing retina has been the object of several studies in different animal species (reviewed by Cepko et al., 1996; Harris, 1997). It is currently assumed that the competence of retinal progenitors to respond to environmental cues is

modified in a sequential fashion. Progenitors thus become progressively restricted in their developmental potential and give rise to differentially specified progeny in response to specific signals.

One of those signals is mediated by the Notch pathway, which plays a prominent role in cell fate specification in the retina. Previous studies (Dorsky et al., 1995; Dorsky et al., 1997; Bao and Cepko, 1997; Henrique et al., 1997) used lipofection or replication-defective retroviruses to misexpress specific variants of Notch and Delta homologues in the developing retina. The results of most of these studies suggest that Notch signalling maintains cells in an undifferentiated state, thus contributing to specify the different cell types by making cells susceptible to regulatory signals at specific stages (Dorsky et al., 1995; Dorsky et al., 1997; Henrique et al., 1997). In still other studies, the histogenetic effects of misexpressing *HES1*, one of the target genes of Notch signalling, have been analysed. In such animals, retinal differentiation is blocked; conversely a null mutation in *HES1* leads to premature differentiation of neuronal types that differentiate at earlier stages (Ishibashi et al., 1994; Ishibashi et al., 1995; Tomita et al., 1996). Recently, a number of papers have been published which imply that Notch signalling promotes glial cell development in rodents (Furukawa et al., 2000; Morrison et al., 2000; Gaiano et al., 2000). Similar results have been obtained by misexpressing *Hes5*, another target of Notch signalling, in the mouse retina (Hojo et al., 2000). In one of these papers (Morrison et al., 2000), an instructive function of Notch signalling in promoting glial development is proposed on the basis of *in vitro* studies.

We have used the Gal4-UAS system for targeted gene expression (Fisher et al., 1988; Brand and Perrimon, 1993) to express a constitutively active Notch receptor (*notch1a-intra*; Takke et al., 1999) in the developing zebrafish retina. This technique was recently adapted for the zebrafish (Scheer and Campos-Ortega, 1999) and the mouse (Rowitsch et al., 1999), and here we present, for the first time, results from a functional analysis of a neural developmental process using the Gal4-UAS technique in zebrafish. Our results indicate that Notch signalling in the developing retina blocks neuronal differentiation, causing cells either to enter gliogenesis or remain undifferentiated and/or die, instead of adopting a neuronal fate. These observations strongly suggest that Notch signalling has an instructive effect on retina cells, promoting glial development.

MATERIALS AND METHODS

A fish line carrying one of the *UAS:myc-notch-intra* insertions described in Scheer and Campos-Ortega (1999) was used as effector. For the construction of the activator plasmids, the *HindIII* fragment of *Gal4* from pGATB (Brand and Perrimon, 1993) was fused to the SV40 small intron and polyadenylation site, and either a 6 kb fragment from the *deltaD* promoter (S. H. and J. A. C.-O., unpublished), or a 1.5 kb fragment from the *hsp70* promoter (Halloran et al., 2000), was cloned upstream of the translational start site of *Gal4*. Microinjection of DNA was as described previously (Scheer and Campos-Ortega, 1999).

Hybridization of digoxigenin-labelled *Gal4* RNA probes to embryo whole mounts was as described previously (Bierkamp and Campos-Ortega, 1993). For this purpose the *HindIII Gal4* fragment from

pGATB was cloned into the *HindIII* site in pBluescript (KS), and digoxigenin-labelled *Gal4* RNA probes were prepared using an RNA labelling kit (Boehringer Mannheim). Embryos and larvae of different genotypes were embedded in Araldite, 4-5 μm transverse sections were made with an ultramicrotome (LKB) and stained with Methylene Blue.

Immunohistochemistry

Antibody staining was as described by Westerfield (Westerfield, 1993) with some modifications. The lenses of embryos and larvae older than 48 hpf were removed with sharp needles and the animals incubated for 30 minutes with 0.01% collagenase/PBT before bleaching in 1% H_2O_2 /PBT overnight. The EliteABC kit (Vectastain) was used to enhance the sensitivity of the staining. All incubation steps were performed at 4°C overnight in 5% DMSO/5% horse serum/PBT. For myc antibody staining, the embryos were treated with a hybridoma supernatant containing a monoclonal anti-myc antibody (1:2). The 16A11, zn-8, FRet43, FRet11 and zrf-1 antibodies (kindly given to us by the Monoclonal Antibody Facility, University of Oregon) were diluted 1:50 for incubation. The antibody against phosphorylated histone H3 was from Upstate Biotechnology. The secondary antibody was in all cases a HRP-linked goat anti-mouse antiserum (1:500; Dianova). The stained specimens were embedded in Araldite and 4-5 μm transverse sections were made with an ultramicrotome (LKB).

Cell transplantations

Donor embryos, derived from crosses between either *deltaD:Gal4/+* or *hsp70:Gal4/+* and *UAS:myc-notch1a-intra/+* fish, were injected at the 1- to 2-cell stage with a mixture of Texas Red and biotin dextran (1:9; total concentration of 5% w/v in 0.2 M KCl) and allowed to develop until the 1000-cell/30% epiboly stage. Groups of 20-30 cells were then removed from the donors and isochronically transplanted into wild-type hosts. The genotype of individual donor embryos was established by PCR after the transplantation, and the host embryos were investigated at 24 hpf for the presence of Texas Red-labelled cells in the retina, fixed at 3-6 dpf, treated with streptavidin-HRP and stained with DAB. The animals were embedded in Araldite and transverse thick sections were made by hand with a razor blade.

RESULTS

As in other fish and in amphibians (Harris, 1997), the central region of the zebrafish retina is clearly distinct from the peripheral, ciliary marginal zone, where the stem cells are located and from which the retina grows during the lifetime of the animal (Harris and Perron, 1998). Cells within these two zones behave differently in response to Notch signalling at a given time point. Unless otherwise indicated, our observations refer exclusively to the central region.

Throughout this study we used as the effector gene a stably inserted *UAS:notch1a-intra* construct (in the figures abbreviated *UAS:nic*) that had been tagged with a tandem array of six copies of the Myc epitope (described by Takke et al., 1999; Scheer and Campos-Ortega, 1999). Analogous versions of this construct were tested in several animal species and invariably found to encode constitutively active Notch receptors (Coffman et al., 1993; Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993; Austin et al., 1995; Dorsky et al., 1995; Takke et al., 1999). Transgenic insertions of two different *Gal4* activator constructs were used to activate expression of *notch1a-intra*. The first activator, called *deltaD:Gal4*, is based on a genomic 6 kb fragment from the promoter region of the zebrafish *deltaD* gene (Dornseifer et al.,

1997; Haddon et al., 1998), which contains all regulatory elements of this gene (S. H. and J. A. C.-O., unpublished). Staining with an anti-myc antibody of the progeny of crosses of *deltaD:Gal4* fish with fish carrying a *UAS:myc-notch1a-intra* insertion shows that the topological distribution of reporter gene expression is essentially identical to that of the transcripts of the endogenous gene (not shown). Most cells in the central regions of the developing retina at 27 hpf, and all cells in the same region at 40 and 72 hpf, express the myc epitope (Fig. 1A,B). Therefore, *deltaD:Gal4* activated *notch1a-intra* is expressed in mitotic as well as in postmitotic cells.

The second activator construct, *hsp70:Gal4*, carries a zebrafish *heat-shock70* promoter (Halloran et al., 2000), which activates transcription of *UAS:myc-notch1a-intra* ubiquitously immediately after heat shock (for 30 minutes at 40°C, not shown). The translation product can be detected with the anti-myc antibody as early as 1 hour after heat shock in all cells of the body (not shown). To assess the persistence of the Notch1a-intra protein, 27 hpf embryos were heat shocked for 30 minutes at 40°C and stained with the anti-myc antibody 24 and 48 hours later. Staining was very strong 24 hours after heat-shock in several organs, such as the retina (Fig. 1C), the neural tube, the notochord and, at much lower intensity, the somites (not shown). To test whether the epitope detected by the anti-myc antibody corresponds to the chimeric myc-notch1a-intra protein or to degradation products, a western blot was prepared with proteins from *hsp70:Gal4;UAS:myc-notch1a-intra* embryos 24 hours after a 30 minutes heat-shock (40°C) at 24 hpf, which was then probed with the anti-myc antibody. The result shows a band of about 91 kDa, the size expected for the chimeric protein (Fig. 1D). The retina still contains detectable amounts of the myc epitope 48 hours after heat shock. However, no attempt was made to detect the chimeric protein by western blotting in these animals.

Expression of activated Notch1a causes loss of neuronal markers

Individual progeny of crosses between either *deltaD:Gal4* or *hsp70:Gal4* fish with *UAS:myc-notch1a-intra* fish were genotyped by PCR. Mutant progeny of the former cross are in fairly good general physiological conditions and live for a few days after hatching (until 6-7 dpf; days postfertilization), whereas those of the latter cross are more strongly affected and die earlier (at 4-5 dpf). Striking pattern defects were found in the retinas of *deltaD:Gal4;UAS:myc-notch1a-intra* individuals (Fig. 2B), and even stronger defects were seen in those of *hsp70:Gal4;UAS:myc-notch1a-intra* larvae following heat shock at 27 hpf (30 minutes at 40°C) and fixation at 3 dpf (Fig. 3B,C). Sections of eyes from these animals stained with methylene blue demonstrate that the normal layered organisation of the retina is hardly distinguishable. Neither the characteristic palisade-like arrangement of outer segments of the photoreceptor cells nor plexiform layers can be distinguished, although isolated islands of neuropile are visible among clusters of cell bodies.

The use of specific antibodies allows one to further dissect the patterning defects. The 16A11 antibody recognises an antigen expressed by amacrine and ganglion cells (Link et al., 2000), whereas FRet43 (Larison and BreMiller, 1990) and FRet11 (Schmitt and Dowling, 1996) stain double cones

and rods, respectively. The number of cells expressing the corresponding antigens is strongly reduced in *deltaD:Gal4;UAS:myc-notch1a-intra* (Fig. 2C,D,G-J), and such cells are absent altogether in *hsp70:Gal4;UAS:myc-notch1a-intra* 3 dpf retinas that had been heat-shocked with 40°C for 30 minutes at 27 hpf (Fig. 3E and not shown). Similar observations were made using the zn-8 antibody, which stains the axons of the ganglion cells (Trevarrow et al., 1990). Although a rudimentary optic nerve is occasionally present, the number of axons in these nerves is always strongly reduced (Fig. 2E,F).

Activation of Notch1a leads to excessive and premature gliogenesis

zrf-1 recognises radial glia cells (Trevarrow et al., 1990). In the wild-type retina, the earliest signs of immunoreactivity to *zrf-1* are found at approximately 5 dpf, in fibres that traverse the plexiform and nuclear layers (Fig. 4A-C), corresponding to the Müller cells. However, following *deltaD:Gal4*-mediated activation of *notch1a-intra*, *zrf-1*-positive cells can already be distinguished at 2 dpf, that is to say several days prior to the time at which cells expressing this marker appear in the wild type (Fig. 4D). The number of *zrf-1*-positive cells increases, and at 6 dpf apparently all cells of the inner layers of the retina express the antigen strongly and have differentiated fibres traversing the retina (Fig. 4E-F).

Heat-shock-mediated activation of *notch1a-intra* at early developmental stages, e.g. tailbud or early somite stages (10-12 hpf), results in anophthalmic animals (not shown). Heat-shock pulses at later stages allow eye development and lead to gliosis, the strength and topological location of which clearly depends on the stage at which *notch1a-intra* is expressed.

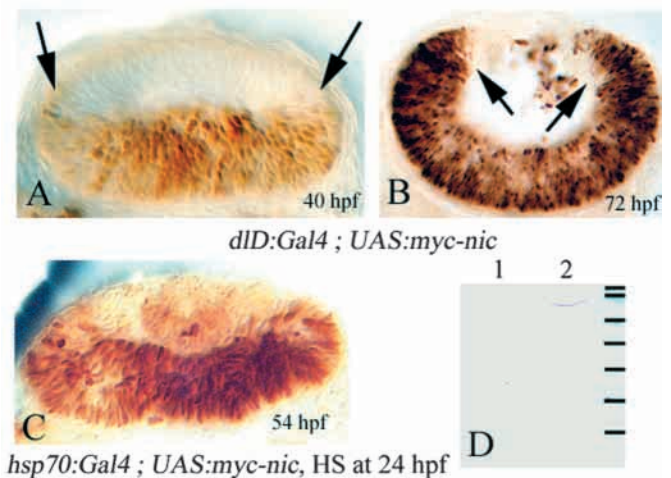


Fig. 1. (A,B) Anti-myc antibody stainings of *deltaD:Gal4;UAS:myc-notch1a-intra* (*nic*) embryos at 40 hpf (A) and 72 hpf (B). Notice that the number of cells expressing the myc epitope increases with age, although all central retina cells contain the epitope, and that the ciliary marginal zone is devoid of myc expression (arrows in A and B). (C) An anti-myc antibody staining of a *hsp70:Gal4;UAS:myc-notch1a-intra* animal, which had been heat-shocked at 24 hpf and fixed at 54 hpf. (D) A western blot with protein extract from 48 hpf embryos, which had been heat-shocked at 24 hpf and probed with anti-myc antibody. Lane 1, wild-type; lane 2, *hsp70:Gal4;UAS:myc-notch1a-intra* embryos. The right lane shows the SeeBlue™ Plus2 marker (Novex). Dorsal is to the left in all pictures.

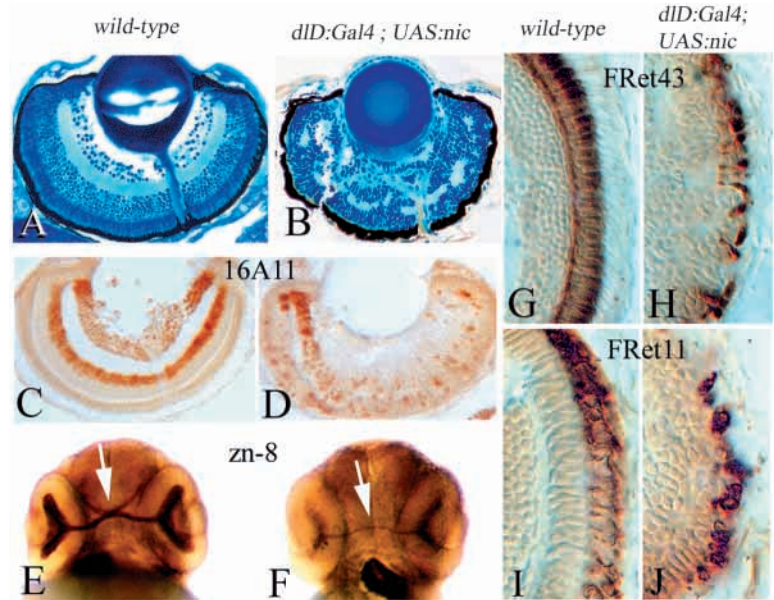


Fig. 2. Neuronal development is impaired following expression of activated notch1a. A and B show Methylene Blue-stained sections through the retina of 5 dpf wild-type (A) and *deltaD:Gal4; UAS:myc-notch1a-intra* (*nic*) (B) larvae. Note the perturbation of the normal layered organisation in the mutant. (C–J) Neuronal development is strongly suppressed in *deltaD:Gal4; UAS:myc-notch1a-intra* animals (D,F,H,J), as compared to wild-type (C,E,G,I). (C,D) Sections from retinas stained with the 16A11 antibody. (E,F) Whole-mounts stained with zn-8 (48 hpf embryos, white arrows point to the optic chiasm). (G,H) Sections stained with FRet43 (5 dpf), (I,J) Sections stained with FRet11 (5 dpf). Notice that the number of cells expressing the corresponding marker is strongly reduced in the mutants. These cells are situated in outer layers of the retina.

Following a 30 minute 40°C pulse at 27 hpf, the eyes develop but 16A11-, FRet11- and FRet43-positive cells are absent at 3 dpf (Fig. 3D–E), whereas the number of *zrf-1*-positive cells is high (Fig. 4G). It is remarkable that, although all cells of the retina expressed *myc-notch1a-intra*, as judged by the *myc* antibody, immunoreactivity to *zrf-1* was present only within central regions of the retina, with the highest activity concentrated in the innermost layers. However, *zrf-1*-positive

cells were less well developed, with shorter fibres, than following activation of *notch1a-intra* with *deltaD:Gal4* (compare Fig. 4F with 4G). Heat-shock treatments later, at 35 hpf, resulted in fewer *zrf-1*-positive cells at 3 dpf, but their distribution was similar to that in the previous case (Fig. 4H).

Patterning in the retina at 3 dpf is still strongly affected by heat shocks at 48 hpf (Fig. 3C), but ganglion cells, amacrine cells and double cones expressed their characteristic markers,

Fig. 3. Neuronal development is impaired following expression of activated Notch1a. Temporal evolution of retinal defects. (A–C) Methylene Blue-stained sections of wild-type (A) and *hsp70:Gal4; UAS:myc-notch1a-intra* (*nic*) (B,C) 3 dpf larvae that had been heat-shocked at 27 (B) and 48 hpf (C). Nuclei of differentiated ganglion cells (white arrows) and a rudimentary optic nerve (black arrow) can be seen in C. (D) wild-type and (E,F) *hsp70:Gal4; UAS:myc-notch1a-intra* 3 dpf larvae that had been heat-shocked at 27 hpf (E) and 48 hpf (F), and stained with the 16A11 and FRet43 antibodies. Whereas activation of *notch1a-intra* expression at 27 hpf completely blocks neuronal differentiation (E), patterning and immunoreactivity of neuronal markers is less affected after heat shocks at 48 hpf. The arrows in F point to cells expressing the neuronal markers 16A11 and FRet43. (G) Wild-type and (H) *hsp70:Gal4; UAS:myc-notch1a-intra* 3 dpf animals that had been heat-shocked at 48 hpf, and stained with FRet11. Presumptive rods are organised in palisades (arrow in H), but do not express the FRet11 antigen. (I) A 6 dpf *hsp70:Gal4; UAS:myc-notch1a-intra* larva that had been heat-shocked at 3 dpf and stained with Methylene Blue. Defects are restricted to the ciliary marginal zone (arrow).

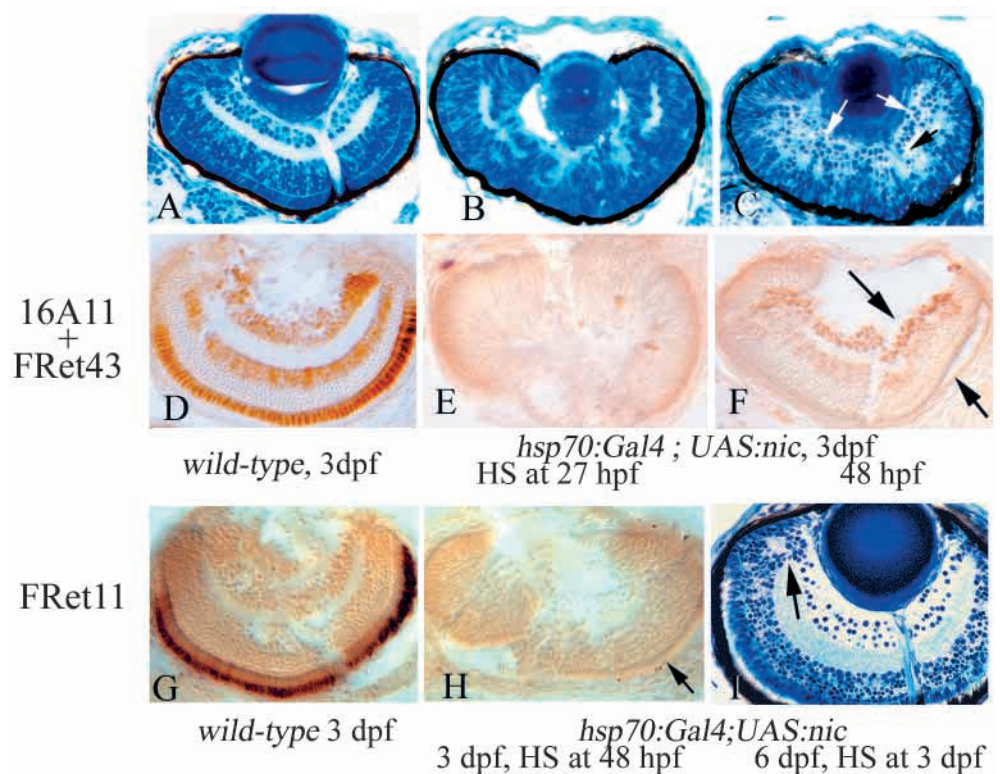
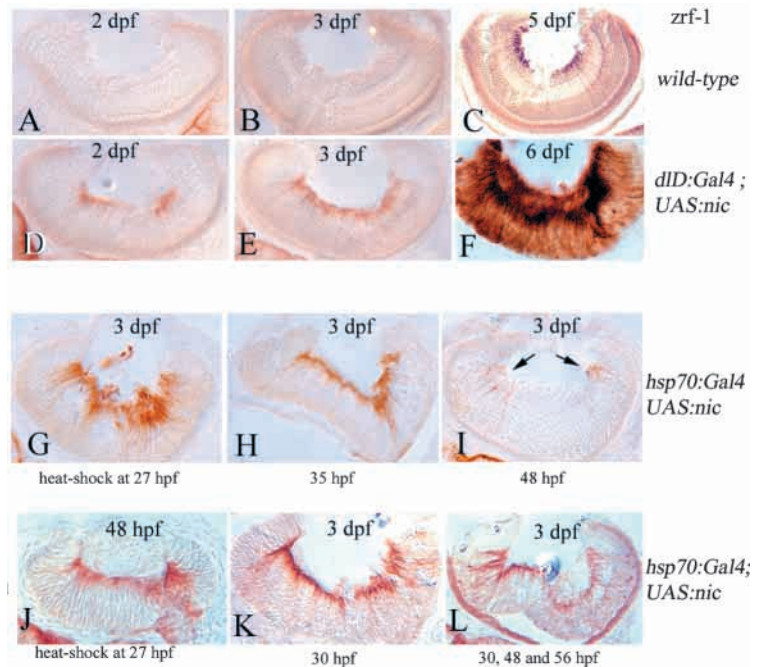


Fig. 4. Excessive and premature differentiation of glial cells following expression of activated notch1a. (A-C) Wild-type and (D-F) *deltaD:Gal4; UAS:myc-notch1a-intra (nic)* animals at 2 dpf (A,D), 3 dpf (B,E) and 5-6 dpf (C,F), all stained with the zrf-1 antibody. Note that, in the mutants, reactivity to the antibody is already detectable in central regions of the retina at 2 dpf, and increases considerably until 6 dpf, whereas immunoreactivity first appears in the wild type around 4-5 dpf. (G-I) 3-dpf *hsp70:Gal4; UAS:myc-notch1a-intra (nic)* retinas, heat-shocked at 27 hpf (G), 35 hpf (H), and 48 hpf (I). Note that the most severe gliosis results from expression of activated Notch1a at earlier stages (G), that gliosis is less intense after activation of Notch1a at 35 hpf (H), and that pulses at 48 hpf bring about excessive glial development only outside the central retina (arrows in I). (J) A *hsp70:Gal4; UAS:myc-notch1a-intra* 48 hpf embryo that had been heat-shocked at 27 hpf. Note that the severity of gliosis is comparable to that in G, despite the age difference. (K,L) 3 dpf *hsp70:Gal4; UAS:myc-notch1a-intra* larvae after a single heat shock (K) at 30 hpf, or repeated heat shocks at 30, 48 and 56 hpf (L) to show that activated Notch1a is expressed at the same time as the glial phenotype. The severity of glial hyperplasia is comparable in both cases despite the use of repeated heat shocks in L.



although very weakly (Fig. 3F). Presumptive rods did not express the FRet11 antigen in these animals, but were arranged in characteristic palisades (Fig. 3G-H). The defects in gliogenesis following heat-shock at 48 hpf are virtually absent from central regions of these retinas, as judged from the number of zrf-1 cells (Fig. 4I). However, obvious defects are visible internal to the ciliary marginal zone, where newly originated and differentiating cells are located (Fig. 4I). Even later heat-shock pulses, at 3 dpf, result in pattern defects exclusively within the marginal region of the 6 dpf larva, whereas the central regions are apparently normal (Fig. 3I).

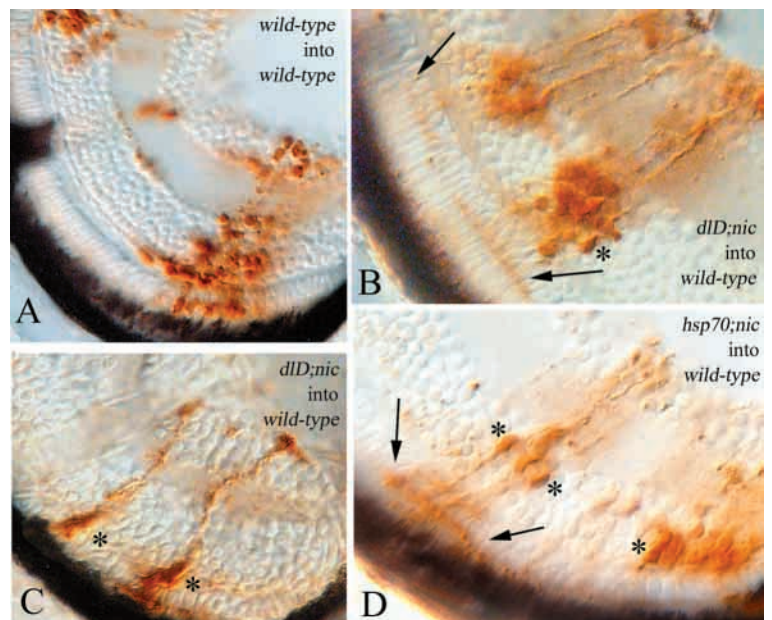
Therefore, premature and excessive glial development can be elicited in central regions of the retina only between 27 and 48 hpf, i.e. during the time at which mainly ganglion cells become postmitotic in untreated embryos (Hu and Easter, 1999). Activation of Notch1a at 48 hpf leads to pattern defects without accompanying gliosis. Activation at still later stages does not affect the central retina.

Simultaneous expression of activated Notch1a and glial markers

The premature expression of glial markers suggests that gliogenesis is initiated shortly after activation of

notch1a-intra transcription by Gal4. However, it is also conceivable that Notch1a-intra exerts a permissive function in this process and that specification of a given cell as a Müller glia cell occurs only when Notch1a-intra is no longer present. This does not seem to be the case, as *hsp70:Gal4; UAS:myc-notch1a-intra* embryos (heat-shocked at 27 hpf and fixed 24 hours later) express the myc and zrf-1 epitopes within central regions of their retinas (Figs 1C, 5D). For technical reasons (both antibodies had been raised in mouse) double staining could not be done on the same specimens. However, it seems highly probable that both markers are simultaneously expressed by the same cells, as the anti-myc antibody stains all cells within the central region of the developing retina, thus including the zrf-1-positive cells, and the Myc-Notch1a-intra protein persists for at least 24 hours (see above). Nevertheless,

Fig. 5. Glial development is induced cell autonomously. (A) An example of transplantation of wild-type cells into a wild-type host. Note that labelled perykarya are distributed throughout all layers of the retina, and that the cells can differentiate into any of the normal cell types of the retina. (B,C) *deltaD:Gal4; UAS:myc-notch1a-intra (nic)* and (D) *hsp70:Gal4; UAS:myc-notch1a-intra* cells, which had grown in wild-type hosts. Note that all labelled cells differentiate characteristics of Müller cells, i.e. perykarya within the outer nuclear layer (asterisks) and extending from the basal membrane but not extending beyond the inner segments of the photoreceptors (arrows in B and D). These characteristics can be clearly seen in the two cells in D, which grew isolated from other mutant cells.



to ensure that the cells do indeed express Notch1a-intra and glial epitopes at the same time, repeated heat-shocks were applied. *hsp70:Gal4;UAS:myc-notch1a-intra* embryos were heat-shocked at 30, 48 and 56 hpf, and fixed and processed with the zrf-1 antibody at 3 dpf (Fig. 4L). The glial phenotype of these retinas is as severe as that of embryos of the same age that had received a single heat shock at 30 hpf (compare Fig. 4K with 4L). All these data strongly suggest that the presence of Notch1a-intra is causally related to the deficit in neuronal differentiation and the gliosis.

***notch1a-intra* expression leads to gliogenesis cell autonomously**

Cell transplantations following the technique described by Link et al. (Link et al., 2000) were used to test whether the gliogenesis defects are expressed cell autonomously. Following isochronic transplantation of either *deltaD:Gal4;UAS:myc-notch1a-intra* or *hsp70:Gal4;UAS:myc-notch1a-intra* cells into wild-type hosts, the mosaic distribution of labelled cells in the retina of the wild-type hosts was similar in all cases, irrespective of the genotype of the donors (Fig. 5). That is to say, patches of labelled cells alternated with unlabelled cells; in some cases, single donor cells were completely surrounded by host cells. However, the phenotype of the transplanted cells clearly depended on the genotype of the donor. Cells from either wild-type donors or from those carrying only one of the two transgenes differentiated into any of the different cell types of the retina, as judged from the morphology of the labelled cells (Fig. 5A). Thus, labelled cells when surrounded by unlabelled cells were distributed in columns extending from the photoreceptor to the ganglion cell layer, with perikarya located in all layers. In contrast to this, cells transplanted from embryos carrying both transgenes either adopted a glial phenotype or remained undifferentiated, that is, with a round appearance and devoid of cell processes (Fig. 5B-D). When differentiated, the labelled cells were also grouped in columns, but the processes never extended beyond the level of the inner segments of the photoreceptors. Within small cell groups, the perikarya of labelled cells were located exclusively in the outer nuclear layer; in large, compact groups of *deltaD:Gal4; UAS:myc-notch1a-intra* cells, perikarya were occasionally found in other layers of the retina. When progeny of transplanted cells grew as isolated single cells or patches, their glial morphology could be observed unambiguously (Fig. 5C). In addition, biotin could also be detected in cells without processes and in dead or dying cells distributed throughout the retina (not shown).

Activation of *notch1a-intra* strongly reduces the number of mitoses

To test the hypothesis that Notch signalling affects the proliferation of the cells in the developing retina (Ishibashi et

al., 1995; Henrique et al, 1997; Furukawa et al., 2000), we estimated the number of mitoses in 2- and 3-dpf embryos, which were either wild type, *deltaD:Gal4; UAS:myc-notch1a-intra* or *hsp70:Gal4; UAS:myc-notch1a-intra*, that had been heat-shocked for 30 minutes at 30 hpf. Following staining with the mitosis marker phosphohistone H3 the numbers of labelled cells observed were consistently lower in the mutants than in the wild-type, and the effect was severe after activation of *notch1a-intra* expression with *hsp70:Gal4* (Table 1 and Fig. 6A-C). Mitotic activity was apparently normal within marginal zones, but strongly reduced within central regions.

Activated Notch 1a causes retinal cells to enter apoptosis

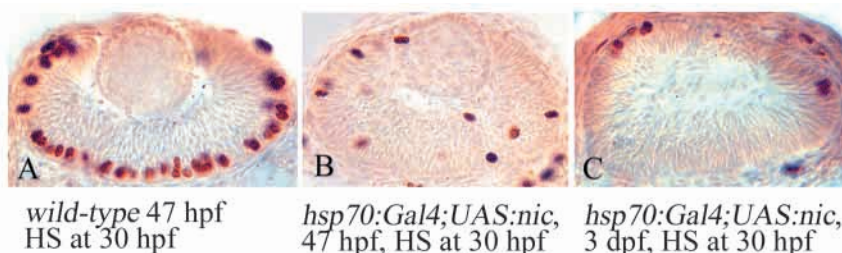
As mentioned above, a relatively large number of retina cells of *notch1a-intra*-expressing embryos and larvae do not develop either a glial or a neuronal phenotype. This is particularly striking when *notch1a-intra* is activated by early heat-shocks, as neuronal markers are completely absent. We assume that these cells remain undifferentiated as they do not exhibit any process nor do they express neural markers (neuronal or glial). To assess the role that cell death may play in the fate of these non-glial and non-neuronal cells, we performed TUNEL staining in wild-type and transgenic fish (*hsp70:Gal4;UAS:myc-notch1a-intra* and *deltaD:Gal4;UAS:myc-notch1a-intra*) of different ages, following the protocols described by Abdelilah et al. (Abdelilah et al., 1996) and Daly and Sandell (Daly and Sandell, 2000).

In the wild type, a few apoptotic cells are present in the retina from about 24 hpf on; the frequency remains similar at 48 and 60 hpf, 3 dpf and 3.5 dpf, and has decreased at 5 dpf (Fig. 7A,C). The frequency of cell death in the two mutant genotypes is several times higher than in the wild-type at all stages (Fig. 7B,D). The frequency is highest in 48 hpf embryos of both genotypes, at least relatively, as the total number of cells increases in the older retinas. Unfortunately, individual variation makes it very difficult to give precise absolute values for the frequency of cell death in the mutants.

DISCUSSION

Both types of activator transgenes used here, *deltaD:Gal4* and *hsp70:Gal4*, can drive strong Gal4 expression in the retina, and both cause similar defects by activating *notch1a-intra*, i.e. a strong reduction in numbers, or even complete disappearance, of cells expressing neuronal markers, and strong glial hyperplasia, which is initiated at very early stages and eventually leads to severe morphogenetic defects. *hsp70:Gal4* activates ubiquitous expression. However, as expression is inducible by temperature, it can be restricted to a given time

Fig. 6. Expression of activated Notch1a reduces the mitotic activity in the retina. (A-C) H3 antibody staining. A 47 hpf wild-type, and B,C are *hsp70:Gal4; UAS:myc-notch1a-intra* (*nic*), at 47 hpf and 3 dpf, respectively, all three heat-shocked at 30 hpf. Mitotic nuclei are located in external layers and are much more abundant in the wild-type than in the mutant retinas (see Table 1).



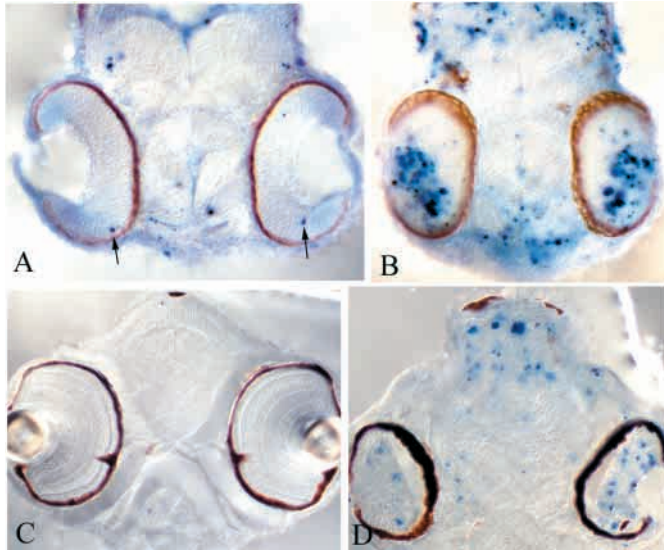


Fig. 7. The frequency of apoptosis is considerably increased following expression of activated Notch1a. TUNEL staining of wild-type (A,C) and *hsp70:Gal4; UAS:myc-notch1a-intra* (B,D) animals at 2 dpf (A,B) and 5 dpf (C,D). Dead cells are blue (arrows in A).

point. *deltaD-Gal4* activates *notch1a-intra* in cells that normally express *deltaD* (Dornseifer et al., 1997; Haddon et al., 1998). It seems likely that all cells of the central regions of the retina express *deltaD* during the critical developmental stages that we are considering, i.e. between 24 and 72 hpf, as in the *deltaD:Gal4; UAS:myc-notch1a-intra* retinas all central cells contain detectable amounts of the myc epitope. Accordingly, Notch1a-intra protein is present in mitotic as well as in postmitotic cells irrespective of whether it was activated by *deltaD:Gal4* or *hsp70:Gal4*.

Activation of Notch1a blocks neuronal development

Previous work indicated that expression of activated Notch variants blocks neuronal development in various parts of the developing CNS (Coffman et al., 1993; Henrique et al., 1995; Chitnis et al., 1995; Dorsky et al., 1995; Tomita et al., 1996; Henrique et al., 1997; Appel and Eisen, 1998; Takke et al., 1999; Furukawa et al., 2000; Morrison et al., 2000; Gaiano et al., 2000). We confirm and extend these observations. Expression of *notch1a-intra* at the critical stage of 27–48 hpf leads in 3 dpf larvae to a strong reduction, in the case of activation by *deltaD:Gal4*, or to complete absence, when activated by *hsp70:Gal4*, of cells expressing markers of ganglion cells, amacrine cells, rods and cones. The difference

in intensity of the effects caused by *deltaD:Gal4* and *hsp70:Gal4* is most probably related to the amount of Gal4 protein expressed from each activator and, consequently, the amount of Notch1a-intra protein expressed from the effector. This is supported by the observation that different insertions of the same activator induce different amounts of *Gal4* mRNA, and that the phenotypic defects in crosses with effector fish do indeed correlate with the amount of *Gal4* mRNA (not shown).

The temporal evolution of the effects indicates that neuronal development is blocked very early. As assessed in the retina of the 3 dpf larva, a single 30-minute heat shock at 27 hpf abolishes all neuronal development. Following a single heat shock at 48 hpf there is a relatively large number of 16A11- and FRet43-positive cells, although these cells express the antigens weakly, and general patterning is less affected. If the heat shock is given even later, at 3 dpf, the defects are restricted to the peripheral regions of the retina, and layering and differentiation of the retinal cell types are essentially normal. That is to say, specification of cells that had become postmitotic prior to *notch1a-intra* expression is not perturbed. However, all developing neuronal types that we have been able to assess are sensitive to activated notch1a.

Notch signalling has an instructive role in retina development

The developmental fate of the cells that express activated Notch and do not adopt a neuronal fate is a controversial question. Following studies in zebrafish, *Xenopus*, chicken and mouse, some authors proposed that cells expressing activated Notch remain in an undifferentiated state (Coffman et al., 1990; Henrique et al., 1995; Chitnis et al., 1995; Dorsky et al., 1995; Tomita et al., 1996; Henrique et al., 1997; Appel et al., 1998; see a discussion in Harris, 1997). Other authors, however, reported that activation of Notch receptors within the developing CNS of mammals leads to glial development (Furukawa et al., 2000; Morrison et al., 2000; Gaiano et al., 2000).

We find that Gal4-mediated activation of *notch1a-intra* causes a large proportion of retinal cells to enter glial development in the zebrafish; this is a cell-autonomous effect, as a glial phenotype develops even when individual *notch1a-intra*-expressing cells are surrounded by wild-type cells. However, the remaining retina cells seem indeed to remain undifferentiated, as they do not express either glial or neuronal phenotypes. Repeated heat shocks and the relatively long persistence of the notch1a-intra protein ensure that activated Notch1a is still present in the cells developing a glial phenotype (see also Furukawa et al., 2000; Gaiano et al., 2000).

Therefore, our results suggest that activated Notch1a has at

Table 1. Effect of activated notch1a expression on the number of mitoses

	Genotype				
	<i>hsp70:Gal4</i> × <i>UAS:myc-</i> <i>notch1a-intra</i>	<i>hsp70:Gal4</i> × <i>UAS:myc-</i> <i>notch1a-intra</i>	<i>deltaD:Gal4</i> × <i>UAS:myc-</i> <i>notch1a-intra</i>	<i>deltaD:Gal4</i> × <i>UAS:myc-</i> <i>notch1a-intra</i>	<i>hsp70:Gal4</i> × <i>UAS:myc-</i> <i>notch1a-intra</i>
Age at heat-shock	30 hpf	30 hpf	no HS	no HS	30 hpf
Age at fixation	47 hpf	47 hpf	3 dpf	3 dpf	3 dpf
Phenotype	normal	abnormal	normal	abnormal	abnormal
Number of H3 cells/eye	538±46	74±4	368±60	172±40	68±17

least two different functions in the developing retina of the zebrafish. The most obvious function is the suppression of neuronal development associated with a diversion of a large excess of cells into glial development. Glial development is initiated as early as 48 hpf, as shown when *notch1a-intra* is activated by *deltaD:Gal4*. The temporal evolution of the gliogenic effect suggests that retinal cells of the central region are competent to react to Notch signalling by developing as glial cells only between 27 and 48 hpf, whereas activation at later stages allows cell-type specification in this region. As this time window corresponds chiefly to that of the origin of ganglion cells (Hu and Easter, 1999), we propose that these cells are the main contributors to the glial hyperplasia. The number of glial cells is higher after activation of Notch1a-intra by *deltaD-Gal4* than by heat-shock. This difference suggests that the gliogenic effects may cumulate over a period of time longer than that uncovered by the heat-shocks and corresponding to the origin of ganglion cells.

The second apparent function of activated Notch1a is to keep other retinal cells undifferentiated, at least temporarily. However, activated Notch1a also causes many cells to enter apoptosis, beginning around 48 hpf and continuing for as long as the animal survives. It also remains unclear whether apoptosis is indirect consequence of the lack of neuronal development, that is, of inductive influences normally derived from the neurones, or rather elicited by Notch1a-intra directly. The possibility that the apparently undifferentiated cells correspond to those undergoing apoptosis is an attractive hypothesis. However, as we have been unable to quantify the undifferentiated and apoptotic cells, we cannot exclude the possibility that they represent distinct populations of cells.

Relationship between Notch activation and the cell cycle

A relationship between the Delta/Notch signalling pathway and the cell cycle has been proposed by several authors (see Ohnuma et al., 1999). However, the basis for this interrelationship is largely unknown. The observations described here show a high degree of similarity to the effects of misexpressing p27^{Xic1}, a member of the Cip/Kip family of Cdk (cyclin-dependent kinase) inhibitors, in *Xenopus* (Ohnuma et al., 1999). Misexpression of p27^{Xic1} leads to premature differentiation of retinal glia cells and blocks the differentiation of at least one neuronal cell type, the bipolar cells. As a Cdk inhibitor, p27^{Xic1} causes the cell cycle to arrest, thus impeding the division of the retinal cells. However, in order for the cells to enter gliogenesis, interruption of the cell cycle as such is not sufficient; expression of p27^{Xic1} itself is required. This gliogenic function of the Cdk inhibitor is enhanced by the simultaneous expression of activated Notch. Thus, Ohnuma et al. speculate that the function of p27^{Xic1} in promoting glial development in the retina is intimately related to the Delta/Notch signal pathway (Ohnuma et al., 1999). Thus, in an indirect manner, our results support this possibility.

Our results do not support the contention, as proposed by others in mouse and chicken (Ishibashi et al., 1995; Henrique et al., 1997; Furukawa et al., 2000), that activation of Notch leads to increased cell proliferation. On the contrary, the present results indicate, first, that mitotic activity is strongly reduced, indeed almost totally suppressed, as shown by the immunoreactivity to the H3 antibody, in *notch1a-intra*-

expressing retinas. The reduction in the number of mitoses may be either a direct consequence of activated notch1a, or reflect the increased apoptotic activity – or both.

On the function of Delta/Notch signalling and cellular diversity in the developing retina

The results reported above indicate that, as a consequence of Notch1a activation, retinal cells either acquire a glial fate or remain undifferentiated and/or enter apoptosis. The premature expression of glial markers by cells that simultaneously express activated Notch1a strongly suggests an instructive rather than a permissive role for Notch signalling. In this context it is noteworthy that in *Xenopus* and rats only differentiating Müller glia cells continue to express the Notch receptor, whereas other differentiating cells of the retina apparently do not express Notch (Ohnuma et al., 1999; Furukawa et al., 2000). Under the conditions of our experiment, many other cells, besides the normally developing Müller cells, would continue to express activated Notch1a and thus be forced to initiate glial development. An instructive function for Notch signalling in promoting glial development has recently been proposed on the basis of in vitro studies using mammalian cells (Morrison et al., 2000). Our results represent the first in vivo evidence that Notch signalling may perform such an instructive function in retinal development in the zebrafish.

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