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In the first paragraph of the Materials and methods section, the units of concentration of the morpholinos injected should be mg/ml.

The authors apologise to readers for this mistake.

Notch resolves mixed neural identities in the zebrafish epiphysis

Elise Cau*, Aurelie Quillien* and Patrick Blader†

Manipulation of Notch activity alters neuronal subtype identity in vertebrate neuronal lineages. Nonetheless, it remains controversial whether Notch activity diversifies cell fate by regulating the timing of neurogenesis or acts directly in neuronal subtype specification. Here, we address the role of Notch in the zebrafish epiphysis, a simple structure containing only two neural subtypes: projection neurons and photoreceptors. Reducing the activity of the Notch pathway results in an excess of projection neurons at the expense of photoreceptors, as well as an increase in cells retaining a mixed identity. However, although forced activation of the pathway inhibits the projection neuron fate, it does not promote photoreceptor identity. As birthdating experiments show that projection neurons and photoreceptors are born simultaneously, Notch acts directly during neuronal specification rather than by controlling the timing of neurogenesis. Finally, our data suggest that two distinct signals are required for photoreceptor fate specification: one for the induction of the photoreceptor fate and the other, involving Notch, for the inhibition of projection neuron traits. We propose a novel model in which Notch resolves mixed neural identities by repressing an undesired genetic program.

KEY WORDS: Notch, Neural specification, Zebrafish, Epiphysis, Photoreceptor, Projection neuron

INTRODUCTION

The formation of a functional nervous system relies on the production of an amazing diversity of neuronal subtypes. Given the high level of complexity of the vertebrate nervous system, understanding the basis of neuronal subtype diversification remains a challenging issue. Nonetheless, several mechanisms have been implicated in the specification of neuronal subtype identity. For example, secreted molecules such as sonic hedgehog (Shh) and bone morphogenetic proteins (BMPs) act as morphogens to pattern the neural tube along the dorsoventral axis. In the ventral spinal cord, graded Shh signaling defines five distinct compartments in which neuroepithelial cells express different sets of homeodomain transcription factors. The expression of these distinct sets of homeodomain transcription factors specifies the identity of the neurons born from these progenitors (Price and Briscoe, 2004). Basic helix-loop-helix transcription factors (bHLH) homologous to the *Drosophila* proneural genes also play a role in defining neuronal subtype identity (Brunet and Ghysen, 1999). For example, within the V2 domain (one of the five ventral domains defined by the graded effect of Shh), Mash1 specifies the identity of *Chx10*⁺ interneurons (Parras et al., 2002). Finally, specific transcription factors are dedicated to the specification of peculiar neurotransmitter phenotypes. For instance, the bHLH transcription factor Ptf1a is necessary for the specification of GABAergic neurons in the mouse cerebellum, spinal cord and retina, and is sufficient to drive GABAergic traits when overexpressed in the dorsal telencephalon (Fujitani et al., 2006; Glasgow et al., 2005; Hoshino, 2006). However, our understanding of the acquisition of the various identities that make up the nervous system is far from complete.

The Notch signaling pathway plays a central role in the generation of diversity within the *Drosophila* nervous system. Upon binding by ligands from the DSL family (for Delta, Serrate, Lag2), Notch is proteolyzed and its intracellular domain (Notch-intra) translocates to the nucleus, where, together with co-factors, it activates transcription (Bray, 2006). Notch is required at several stages during neural development in the fly. First its activity is crucial for the selection of a neural progenitor from a pool of competent cells, a process referred to as lateral inhibition. In this context, Notch signaling functions as a feedback loop in which the activation of target genes by Notch-intra leads to a diminished expression of proneural genes. As proneural genes control the expression of the Delta ligands, activation of Notch within a cell leads to a reduced signaling to neighboring cells. The Notch pathway thus provides a mechanism by which small differences in proneural gene expression between neighboring cells can be readily amplified, thus singling out one cell expressing a higher level of proneural genes from a pool of equivalent cells (Simpson, 1997). Once this selection has occurred, the expression of the proneural genes endows the cell with neural potential, a process referred to as neural determination.

A second role of Notch in the fly nervous system concerns cell fate diversification. For instance, in the eye, cell-cell communication via Notch allows sorting of two distinct photoreceptor subtype identities called R3 and R4. In this context, Notch signaling is initially biased by the activity of a polarizing signal acting through the Frizzled receptor that leads to stronger expression of Delta in the presumptive R3 (Fanto and Mlodzik, 1999). Similarly, mechanosensory organ precursors generate cells that communicate through Notch to specify the four distinct identities that compose the sensory organ. These identities are generated through sequential binary decisions. First, the sensory organ precursor divides to generate two intermediate progenitors (pIIb and pIIa) that communicate via Notch to establish their respective identities. These cells divide again to generate four cells, the identities of which are established through communication between sister cells via Notch. In this case, Notch signaling is biased by the asymmetric segregation

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of Notch interactors during cell division (Bardin et al., 2004). In addition, the activity of Notch in such binary decisions involves targets distinct from proneural genes (Guo et al., 1995; Okabe et al., 2001).

In vertebrates, the Notch pathway plays a clear role in the selection of a neural progenitor from a pool of competent cells through the regulation of proneural genes (Lewis, 1996). By controlling this process, Notch signaling affects the timing of cell birth and differentiation. A correlation has been observed between the timing of cell birth and the identity of the neural cell produced in many neuronal lineages (Temple, 2001). Therefore, it remains controversial whether the Notch pathway diversifies cell fate through the regulation of the timing of neurogenesis or acts directly in specifying neuronal subtype identity. For example, the Notch pathway plays a role in specifying two distinct populations of GABAergic interneurons (called KA' and KA'') at the expense of motoneuron fate in the ventral spinal cord of the zebrafish. However, although in the case of the KA'' cells the effect of Notch is primarily to control the timing of neurogenesis (Yeo and Chitnis, 2007), Notch directly controls the KA' cell fate (Shin et al., 2007). Recent work performed in the mouse retina also suggests a direct role for Notch in the specification of neuronal subtype identity. Conditional inactivation of the *Notch1* gene induces the production of an excess of photoreceptors at the expense of other cell fates. Interestingly, this effect is independent of the timing of *Notch1* inactivation, which suggests a direct activity for Notch in cell fate specification (Jadhav et al., 2006; Yaron et al., 2006). Finally, Notch plays a role in specifying two distinct subtypes of interneurons in the ventral spinal cord (Peng et al., 2007). These two neuronal types are born from *Lhx3*+ progenitors and seem to be produced simultaneously, as judged by the expression of molecular markers. However, in absence of birthdating studies, the possibility remains that Notch indirectly influences cell fate by controlling the timing of neuronal differentiation.

We have begun to analyze the mechanisms that govern cell fate diversification in the zebrafish epiphysis, or pineal gland, a small diencephalic structure involved in light detection and the regulation of circadian rhythms (Foster and Roberts, 1982; Natesan et al., 2002). This simple structure contains only two neuronal types: photoreceptors and projection neurons (Masai et al., 1997). Precursors for epiphysial neurons arise from a subdomain of the dorsal diencephalon that expresses the homeodomain transcription factor *floating head* (*flh*). *Flh* is required for the expression of two proneural genes, *achaete/scute homolog 1a* (*ascl1a*) and *neurogenin1* (*ngn1*), which are in turn redundantly required for neuronal production within the epiphysis. However, genetic perturbation of this *Flh*/proneural genes cascade affects both photoreceptors and projection neurons, indicating that *flh*, *ascl1a* and *ngn1* are not involved in the specification of neuronal subtype identity (Cau and Wilson, 2003; Masai et al., 1997). Thus, although we understand how neurons are produced in the epiphysis, the mechanism by which these neurons acquire their identity remains to be discovered.

In this paper, we examine the role of the Notch pathway in specifying the two neuronal subtypes of the epiphysis. We show that a reduction or a gain of Notch activity modifies the proportion of the two cell types compared with wild type. This effect is independent of cell birthdate as projection neurons and photoreceptors are born simultaneously. We propose that Notch controls the specification of neuronal subtype identity independently of its role on the timing of neurogenesis in this simple neuronal lineage.

MATERIALS AND METHODS

Strains and developmental conditions

Embryos were reared at 28.5°C and staged according to standard protocols (Kimmel et al., 1995). Embryos homozygous for *mib^{ta52b}* (Itoh et al., 2003) or *dla^{hi781}* (Amsterdam et al., 2004) and *after-eight* (Holley et al., 2002) mutations were obtained by intercrossing heterozygous carriers. Tg(HuC:GFP), Tg(AANAT2:GFP), Tg(hs:Gal4) and Tg(UAS:Nintra) transgenic lines have been described previously (Park et al., 2000; Scheer et al., 2002; Gothilf et al., 2002) as has the sequence for *dld* MO (Holley et al., 2002). Sequence for *dldm* MO and *dld* 5'MO are 5'-aaaGagctat-GattaCtcCtcGat-3' and 5'-agaggatctgaactgtgtgaaact-3', respectively. Although *dld* and *dldm* MO were injected at 2.5 ng/μl, *dld* 5'MO was injected at 3 ng/μl.

DAPT treatments

Embryos were raised in embryo medium containing DAPT (Calbiochem) at 100 μM and DMSO (1%), as previously described (Geling et al., 2002). Control embryos were incubated in an equivalent concentration of DMSO.

Birthdating of neurons with 5-bromo-2-deoxyuridine

Embryos were incubated in embryo medium with 10 mM BrdU and 8% DMSO for 20 minutes on ice followed by 2 hours at 28.5°C. BrdU incorporation was detected by immunohistochemistry using an anti-BrdU antibody (G3G4, 1/1000, Developmental Studies Hybridoma Bank).

In situ hybridization

In situ hybridization was performed using an in situ hybridization robot (Intavis AG, protocol available upon request). The following digoxigenin-labeled antisense riboprobes were used: *flh* (Talbot et al., 1995), *lhx3* (Glasgow et al., 1997), *ascl1a* (Allende and Weinberg, 1994), *ngn1* (Blader et al., 1997), *islet1* (Appel et al., 1995), *dla*, *dld*, *dld* (Haddon et al., 1998) and exorhodopsin, red opsin and rhodopsin (Forsell et al., 2001; Mano et al., 1999).

Immunostaining

Antibody staining was performed as previously described (Masai et al., 1997) using the following primary antibodies: FRet43, 1/200 (Larison and Bremiller, 1990), anti-Islet1 (39.4D5; 1:200, Developmental Studies Hybridoma Bank), anti-GFP (1/1000, Torrey Pines Biolabs), anti HuC/D (1/400, Molecular Probes), Pax6 (1/1000) (Carriere et al., 1993) and caspase 3 (1/200; BD Pharmingen); and the following secondary antibodies (Molecular Probes): Alexa 488-conjugated goat anti-rabbit IgG (1/1000), Alexa 546-conjugated goat anti-mouse IgG (1/1000), Alexa 647-conjugated goat anti-mouse IgG (1/1000), Alexa 546-conjugated goat anti-mouse IgG1 (1/100) and Alexa 647-conjugated goat anti-mouse IgG2 (1/100).

Image acquisition and counts

Confocal acquisition was performed using a Leica (SP2) and ImageJ software was used for cell counting. For each condition a minimum of three embryos was analyzed.

RESULTS

Photoreceptors and projection neurons of the zebrafish epiphysis can be distinguished using stable molecular markers

The zebrafish epiphysis contains two neuronal subtypes, photoreceptors and projection neurons, that occupy distinct subdomains of the epiphysial vesicle (Fig. 1A). These neurons can also be distinguished using molecular markers, such as FRet43 and *lhx3*, which label photoreceptor and projection neurons, respectively (Cau and Wilson, 2003; Glasgow et al., 1997; Masai et al., 1997). We noticed that the total number of FRet43 and *lhx3*-expressing cells at 48 hours represents fewer than half of the total number of Islet1+ epiphysial neurons, suggesting either that these markers are transiently expressed or label distinct subtypes of projection neurons

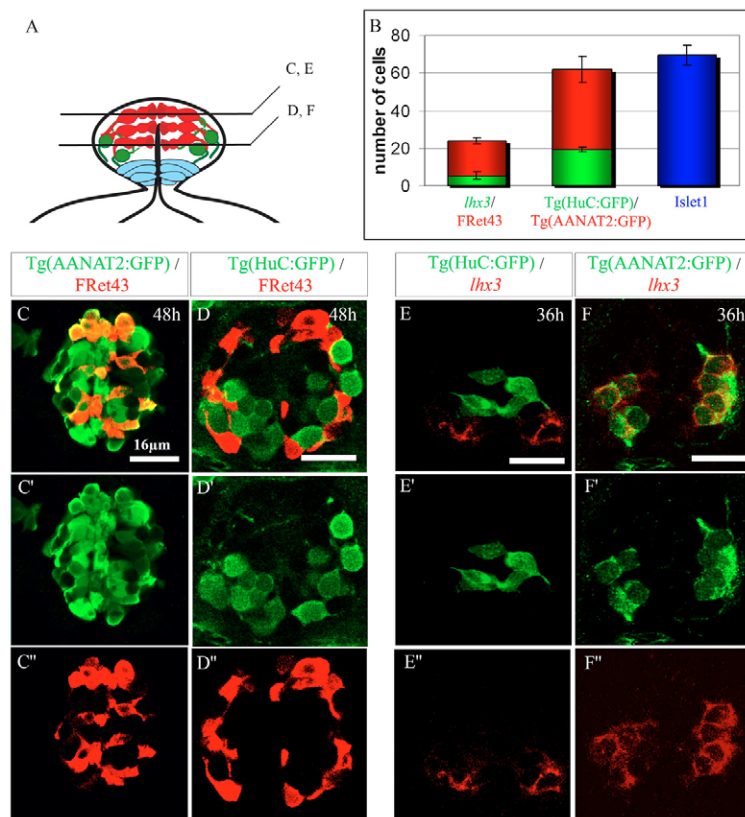


Fig. 1. Characterization of the two categories of epiphysal neurons. (A) Schematic diagram of the epiphysal vesicle in frontal section. Dorsal is upwards. The photoreceptors (in red) lie dorsally and medially compared with the more ventrolateral projection neurons (in green). Ventrally located neuroepithelial cells are in light blue. (B) Average numbers of cells positive for markers of projection neurons (green) or photoreceptors (red) and total number of Islet1+ neurons (blue). A minimum of three embryos were analyzed for each stage. Error bars represent the standard deviation. (C-F'') Confocal sections of the epiphysis from Tg(AANAT2:GFP) (C-C'',E-E'') and Tg(HuC:GFP) transgenic embryos (D-D'',F-F'') labeled at 48 hours with the photoreceptor marker FRet43 or at 36 hours with the projection neurons marker *lhx3*. Anterior is upwards. Scale bars: 16 μ m.

and photoreceptors (Fig. 1B). Thus, to allow us to quantify the total number of projection neurons and photoreceptors, we searched for other markers of these two cell populations.

The transgenic line Tg(AANAT2:GFP), in which regulatory elements of the zebrafish serotonin-*N*-acetyltransferase-2 control the expression of a GFP reporter, has been described to label photoreceptors (Gothilf et al., 2002). To confirm this, we performed double-labeling experiments with photoreceptor markers, such as FRet43 and a complex opsin probe (containing exorhodopsin, rhodopsin and red opsin), or projection neuron markers, such as *lhx3* and Pax6 (Masai et al., 1997). As expected, although we observed co-labeling of FRet43 or opsin with GFP in Tg(AANAT2:GFP) embryos, the expression of *lhx3* and Pax6 was largely excluded from cells expressing the AANAT2 transgene (Fig. 1C-C'',E-E''); see Fig. S1A,A',C,C' in the supplementary material). Interestingly, however, we observed a few GFP+ cells that express *lhx3* or Pax6 in some Tg(AANAT2:GFP) embryos (Fig. 7C; see Fig. S1C,C' in the supplementary material). This either reflects the occasional activation of *lhx3* and Pax6 in photoreceptors or the presence of cells with a transient mixed identity.

The RNA-binding protein HuC is a marker of newly born neurons and a transgenic line containing regulatory elements upstream of the *huC* gene driving GFP, Tg(HuC:GFP), has been reported to reproduce this pattern (Park et al., 2000). To our surprise, however, we observed that GFP from the Tg(HuC:GFP) transgenic line only labels a subset of epiphysal neurons. The lateral position of these GFP-expressing cells suggests that they are projection neurons (Fig. 1D-D''). Consistent with this, we observed that the vast majority of *lhx3*+ and Pax6+ cells co-express the *HuC:GFP* transgene (Fig. 1F-F''); see Fig. S1B,B' in the supplementary material), whereas no co-expression was observed with opsin or FRet43 (Fig. 7C and data not shown). We thus consider Tg(HuC:GFP) to be a marker of projection neurons.

Finally, to confirm that the GFP expression from the transgenes is stably detected in epiphysal neurons, we quantified Tg(HuC:GFP)+ and Tg(AANAT2:GFP)+ cells at 48 hours of development. We observed an average of 42.4 ± 7 Tg(AANAT2:GFP)+ cells and an average 19.2 ± 1.06 Tg(HuC:GFP)+ cells per embryo (Fig. 1B). At the same stage, the epiphysis contains 69.5 ± 5.32 Islet1+ neurons. Thus, unlike FRet43 and *lhx3*, these transgenes stably label the entirety of the two neuronal subtypes in the epiphysis.

***deltaB* and *deltaD* are expressed specifically in projection neurons**

Notch signaling has been implicated in cell fate choice in invertebrates, but its role is more controversial in vertebrate neural lineage specification (Bardin et al., 2004; Fanto and Mlodzik, 1999; Jadhav et al., 2006; Shin et al., 2007; Yaron et al., 2006; Yeo and Chitnis, 2007). A role for Notch in binary decisions is often associated with an asymmetric expression of Notch ligands (Fanto and Mlodzik, 1999). Interestingly, two ligands *deltaB* and *deltaD* show preferentially lateral expression in the zebrafish epiphysis (Cau and Wilson, 2003) (Fig. 2B,C). By contrast, *deltaA* shows widespread expression in the epiphysal territory (Fig. 2A). We hypothesized that the lateral expression of *deltaB* and *deltaD* corresponds to projection neurons and performed double-labeling experiments to confirm this idea. At 24 hours, cells double-labeled with a *deltaA* probe and GFP were observed in both Tg(HuC:GFP) and Tg(AANAT2:GFP) embryos (Fig. 2D,D',G,G'). Thus, *deltaA* is expressed in both photoreceptors and projection neurons. By contrast, *deltaB* and *deltaD* co-expressed GFP in Tg(HuC:GFP) (Fig. 2E,E',F,F') but not in Tg(AANAT2:GFP) (Fig. 2H,H',I,I'). These results show an enrichment of the expression of Delta genes in projection neurons with *deltaB* and *deltaD* being expressed selectively in projection neurons.

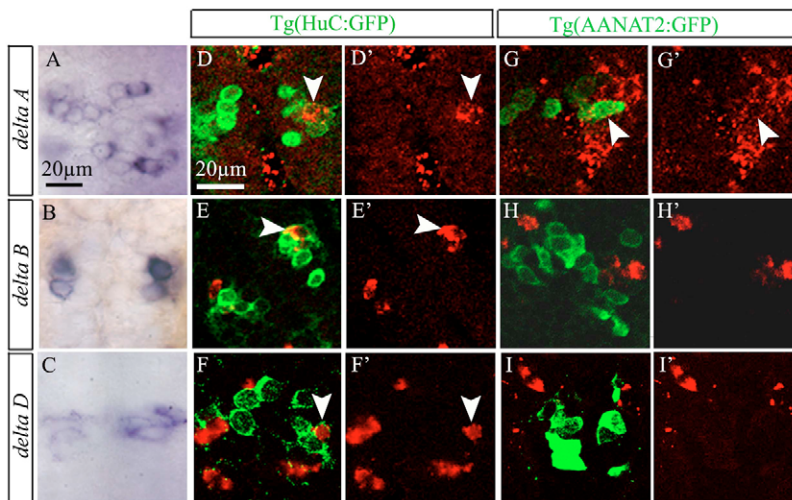


Fig. 2. *deltaB* and *deltaD* are expressed specifically in projection neurons. (A-C) Dorsal view of epiphysis, showing expression of *deltaA*, *deltaB* and *deltaD* in wild-type embryos at 18 hours. (D-F') Confocal section of the epiphysis, showing expression of *deltaA*, *deltaB* and *deltaD* (red) in Tg(HuC:GFP) embryos at 24 hours. (G-I') Confocal section of the epiphysis, showing expression of *deltaA*, *deltaB* and *deltaD* (red) in Tg(AANAT2:GFP) embryos at 24 hours. Anterior is upwards. Scale bars: 20 μ m. White arrowheads indicate double-labeled cells.

Reducing Notch signaling increases neurogenesis in the epiphysis

The asymmetry in the expression of *deltaB* and *deltaD* led us to test a role for Notch signaling in the zebrafish epiphysis using embryos mutant for *mindbomb* (*mib*); *mib* encodes a ring ubiquitin ligase that modifies Delta, thereby potentiating its activity as a ligand for Notch (Itoh et al., 2003; Le Borgne and Schweisguth, 2003). As other ring-ubiquitin ligases of the Mib or Neuralized (Neur) families could partially compensate for mutations in *mib* (Lai et al., 2005; Le Borgne et al., 2005; Zhang et al., 2007a; Zhang et al., 2007b), we also used the γ -secretase inhibitor DAPT, as a more general way of inhibiting the Notch pathway (Geling et al., 2002).

As the Notch pathway is known to affect general neuronal production, we first checked the number of epiphysial neurons using an antibody against Islet1. At all stages analyzed, we observed an increase in the number of Islet1+ neurons in a *mib* mutant background compared with wild type. For example, at 48 hours, *mib* mutant embryos contain an average of 80 Islet1+ cells compared with 66 cells in wild-type embryos (Fig. 3A). When DAPT was administered from 9 hours, which corresponds to the beginning of epiphysial specification, we also observed an increase in the number of Islet1+ cells. This increase was significantly stronger than that observed in *mib* mutants (Fig. 3A). In addition, *mib* mutant embryos treated with DAPT showed the same number of Islet1+ cells as wild-type DAPT-treated embryos, indicating the existence of remnant Notch activity in *mib* mutants.

Expression of the prepattern transcription factor *floating head* (*flh*) is detected in the epiphysial anlage from 9 hours of development and its activity is required for epiphysial neurogenesis as it has been shown that few Islet1+ neurons are formed in *flh* mutant embryos (Masai et al., 1997). To determine whether the increased neuronal production observed in *mib* mutant and DAPT-treated embryos resulted from an increase in the size of the presumptive epiphysial territory, we assayed *flh* expression in *mib* mutant and DAPT-treated embryos; *flh* expression was analyzed at the stage where the first post-mitotic neurons can be detected (Masai et al., 1997). No change was detected in the size of the presumptive epiphysial territory in embryos with reduced Notch signaling (Fig. 3B,C; data not shown). The increase in the number of Islet1+ cells detected in embryos with compromised Notch signaling might, alternatively, result from increased neurogenesis within the epiphysial anlage. To explore this possibility, we assayed the expression of the proneural genes

ascl1a and *ngn1*, which are redundantly required for the production of epiphysial neurons downstream of Flh (Cau and Wilson, 2003). In contrast to *flh* expression, both mutation of *mib* and DAPT treatment affect the expression of *ascl1a* and *ngn1* (Fig. 3D-G; data not shown). Indeed, in wild-type and mock-treated embryos, we observed 10-15 *ascl1a*+ cells compared with 20-25 cells in embryos with reduced Notch signaling at 16 hours of

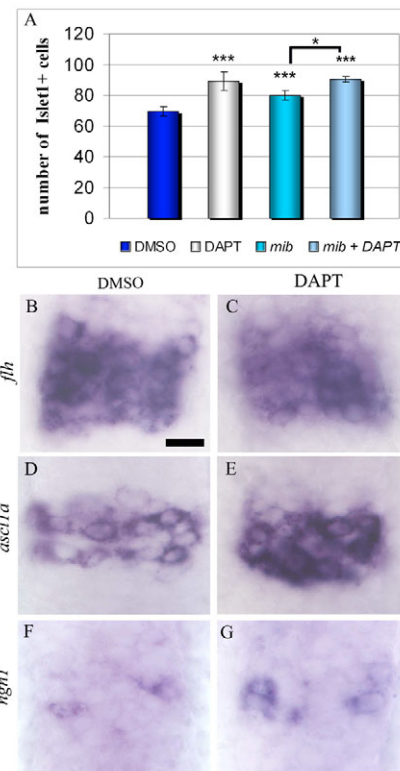


Fig. 3. Increased production of neurons in *mib* and DAPT-treated embryos. (A) Average numbers of Islet1+ cells in the epiphysis of mock-treated embryos, *mib* mutant and DAPT-treated embryos at 48 hours. (B-G) Dorsal view of epiphysis, showing expression of *flh*, *ascl1a* and *ngn1* in mock or DAPT-treated embryos at 16 hours. Error bars represent the standard deviation. * $P < 0.05$; *** $P < 0.0005$ using a *t*-test. Scale bar: 10 μ m. Anterior is upwards.

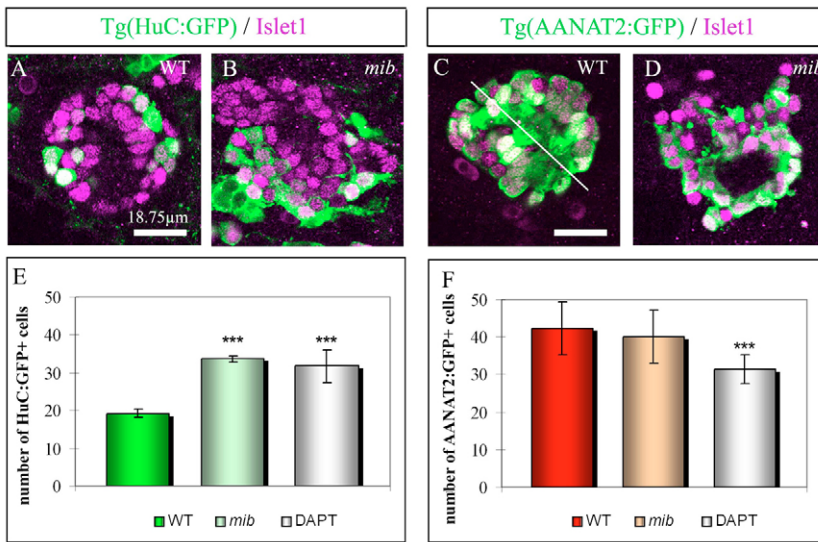


Fig. 4. Modification of neuronal subtype identity in *mib* and DAPT-treated embryos. (A,B) Expression of GFP (green) and Islet1 (purple) in wild-type (WT) and *mib*;Tg(HuC:GFP) transgenic embryos at 48 hours. As Tg(HuC:GFP) labels other structures close to the epiphysis and as the epithalamus of *mib* embryos is highly disorganized, Islet1 serves to identify epiphysal neurons. (C,D) Expression of GFP (green) in Tg(AANAT2:GFP) transgenic embryos shown as confocal sections with Islet1 (purple). In wild-type embryos, Tg(AANAT2:GFP)+ photoreceptors are arranged in two mirror-imaged rows with the outer segments of the cells located at the midline (white line), this stereotyped organization is lost in *mib* embryos. Scale bars: 18.75 μ m. (E,F) Average numbers of GFP+ cells (green) in Tg(HuC:GFP) (E) or in Tg(AANAT2:GFP) embryos (F) in the epiphysis of wild-type, *mib* or DAPT-treated embryos at 48 hours. Anterior is upwards. Error bars represent the standard deviation * $P < 0.05$; *** $P < 0.0005$ using a *t*-test.

development (Fig. 3D,E; data not shown). Similarly, we observed more *ngn1*+ cells in embryos with reduced Notch signaling than in wild-type embryos at 16 hours; 5-10 cells in Notch compromised versus fewer than five cells in wild-type embryos (Fig. 3F,G; data not shown). Thus, the Notch pathway is required to inhibit neuronal production in the epiphysal anlage. This effect most probably reflects a role for Notch in maintaining epiphysal cells in a progenitor state through the downregulation of the expression of *ascl1a* and *ngn1*, in a manner similar to that described in other areas of the nervous system.

Increases projection neuron numbers in embryos with reduced Notch signaling

We next looked at the identity of the neurons produced in *mib* mutant and DAPT-treated embryos. We observed a strong increase in the number of Tg(HuC:GFP)+ cells in *mib* mutants compared with wild type (Fig. 4A,B,E). In addition, quantification of cells expressing *lhx3*+ indicate that at any given stage *mib* mutant embryos contain twice as many *lhx3*+ cells as wild-type embryos, suggesting a continuous production of supernumerary projection neurons in a *mib* mutant background (data not shown). By contrast,

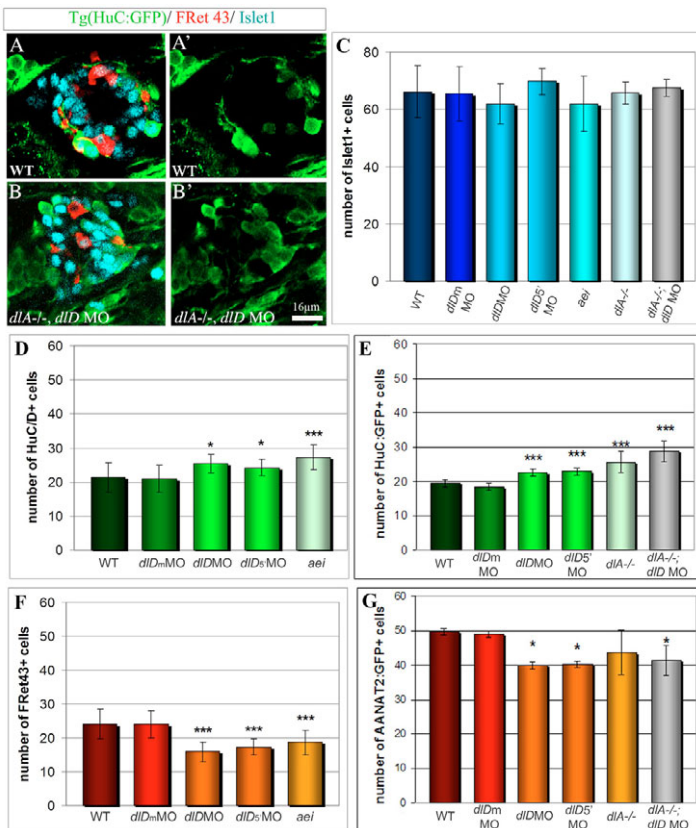


Fig. 5. Impaired photoreceptor/projection neurons ratio in embryos deficient for Delta genes. (A-B') Confocal sections from wild-type (A-A') and *deltaA*^{-/-}, *deltaD*-morphant epiphysis (B-B') showing GFP from Tg(HuC:GFP) (green), FRet43 (red) and Islet1 (purple) at 48 hours of development. (C-G) Average number of Islet1+ (C), HuC/D+ (D), Tg(HuC:GFP)+ (E), FRet43+ (F) and Tg(AANAT2:GFP)+ cells (G) in 48 hours embryos depleted for the function of *deltaA* and/or *deltaD*. Anterior is upwards. Scale bar: 16 μ m. Error bars represent the standard deviation *** $P < 0.0005$ using a *t*-test.

a similar increase in the numbers of Tg(AANAT2:GFP)+ cells was not detected in *mib* mutant embryos (Fig. 4C,D,F). Furthermore, counting FRet43+ cells shows that photoreceptors are produced with the normal timecourse in a *mib* mutant background (data not shown). Nonetheless, the relative number of photoreceptors decreases from 68.8% in wild-type embryos to 54.3% in *mib* mutants as a function of the total number of specified neurons.

Embryos treated with DAPT from 9 hours, show an increase in the number of Tg(HuC:GFP)+ cells compared with wild type (Fig. 4E). However, in contrast to *mib* mutant embryos, early treatment with DAPT decreases the absolute number of Tg(AANAT2:GFP)+ cells (Fig. 4G). This difference might be due to the relative penetrance of the two conditions, *mib* being generally weaker than DAPT treatment. Alternatively, DAPT treatment might lead to an increase in apoptosis of one or both neural cell type. To look at this further, we used an antibody against activated caspase 3. Indeed, although there is a slight increase in the number of caspase+ cells in *mib* mutant versus wild-type epiphyses, there is a strong increase in embryos treated with DAPT from 9 hours (see Fig. S2 in the supplementary material). To address whether a specific cell type is more likely to undergo apoptosis after early DAPT treatment, we performed anti-caspase staining in combination with staining for markers of neuronal identity. As caspase+ cells include both unspecified neurons and neurons of either projection neuron or photoreceptor identity, we conclude that there is no specificity to the apoptosis induced by early DAPT treatment; a similar observation was made in *mib* mutants embryos (see Fig. S2 in the supplementary

material). The loss of cells in DAPT-treated embryos most probably explains the apparent decrease in the absolute number of photoreceptors observed after early DAPT treatment: loss of projection neurons by apoptosis is masked by the significant increase in this cell type in the absence of Notch signaling. Nonetheless, our results suggest that Notch both controls neuronal number and represses the projection neuron fate.

Reducing *deltaA* and *deltaD* function specifically affect neuronal identity

Next, we tested the functions of the Delta genes in the epiphysis. For this, we used a retroviral insertion mutant in the *deltaA* gene (Amsterdam et al., 2004). For *deltaD*, we used a previously reported morpholino (*dld* MO) (Holley et al., 2002), as well as a second morpholino directed further upstream in the 5' UTR of the gene (*dld* 5' MO). Embryos with reduced *deltaA* and/or *deltaD* activity exhibit an increase in the number of Tg(HuC:GFP)+ cells (Fig. 5A,A',B,B',E). In parallel, we observed a decrease in the number of Tg(AANAT2:GFP)+ cells upon reduction of *deltaD* or *deltaA* and *deltaD* functions (Fig. 5G). Thus, as for *mib* mutants or early DAPT-treated embryos, the relative number of photoreceptors falls from 72.4% to 62.9% and 63.5% in the absence of DeltaA and DeltaD function, respectively. Interestingly, however, reducing *deltaA* and/or *deltaD* activity had no effect on the total number of Islet1+ neurons compared with wild type (Fig. 5C). Although we observed similar effects in both conditions of *deltaD* knock down, no phenotype was obtained upon injection of a control morpholino in which the sequence of the *dld* MO harbors five mismatches

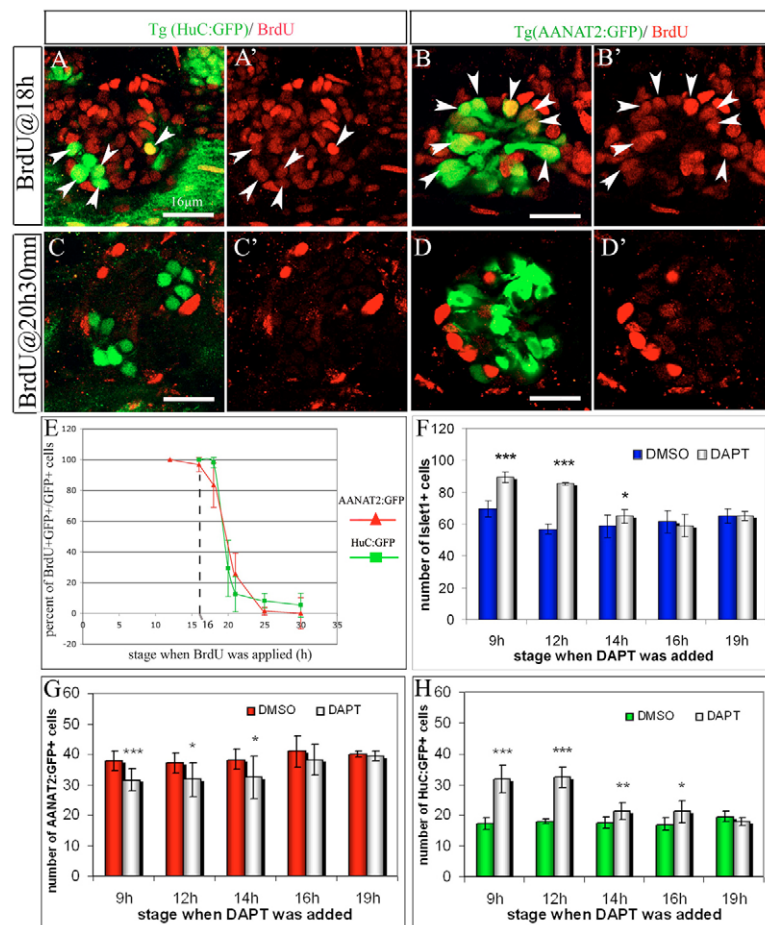


Fig. 6. The photoreceptors and the projection neurons are born simultaneously and Notch activity is required in cycling progenitors. (A-D') Co-labeling of BrdU (red) with either Tg(HuC:GFP) (A,C) or Tg(AANAT2:GFP) (B,D) in 48-hour-old embryos that have been subjected to a 2-hour BrdU-pulse starting at 18 hours (A-B') or 20.5 hours (C-D'). White arrowheads indicate double-labeled cells over total number of GFP+ cells after a 2-hour pulse of BrdU. Embryos transgenic for Tg(HuC:GFP) or Tg(AANAT2:GFP) were subjected to a pulse of BrdU starting from various stages. The percent of BrdU+ cells was evaluated at 48 hours. **(E-H)** Average numbers of Islet1+ neurons, Tg(AANAT2:GFP)+ and Tg(HuC:GFP)+ cells in the epiphysis of DMSO and DAPT-treated embryos. The x-axis indicates the stage at which the treatment starts. Anterior is upwards. Scale bars: 16 μ m. Error bars represent s.d. * P <0.05; ** P <0.001; *** P <0.0005 using a t-test.

(*dldm* MO; Fig. 5C-G). We also confirmed the specificity of the *deltaD* morpholinos used in this study by analyzing the number of Islet1+, HuC/D+ and FRet43+ cells in the *deltaD* mutant *after eight* (*aei*). In all three cases, results with *aei* mutant embryos were comparable with those generated by morpholino injection (Fig. 5C,D,F). Thus, reducing *deltaA* and *deltaD* function affects neuronal subtype identity. Furthermore, it does so without modifying total neuronal numbers. These results suggest that the Notch effects on neuronal number and on neuronal identity reflect two distinct activities.

Photoreceptors and projection neurons are born during the same time window

In several neural lineages, different neural subtypes are produced at different times, suggesting that specification of these cells could result from a change in the competence of their progenitors over time (Temple, 2001). To understand whether the effect of reducing Notch signaling on the specification of projection neurons and photoreceptors reflects a role in the time of birth of these two populations, we performed birthdating experiments using bromodeoxyuridine (BrdU) pulses in Tg(AANAT2:GFP) or Tg(HuC:GFP) transgenic embryos. When BrdU was applied before or at 18 hours, most transgene-expressing cells were also BrdU+, indicating that the majority of epiphyseal progenitors are still dividing at these stages (Fig. 6A,A',B,B',E). By contrast, after a pulse at 20.5 hours, most GFP+ cells were BrdU negative (Fig. 6C,C',D,D',E). The similarity between the incorporation curves obtained for projection neurons and photoreceptors indicate that the birthdate is the same for the two cell types. These results rule out sequential production of neuronal types as a possible mechanism by which Notch regulates neuronal specification in this system.

Notch controls neuronal numbers and specification in dividing progenitors

Reduction of Notch activity alters both the total number of epiphyseal neurons, as well as the identity of these neurons. We next searched for the stages at which Notch activity was required for these two activities by treating embryos with DAPT from various stages of development. Although an increase in the total number of Islet1+ neurons was observed when DAPT was administered from stages up to 14 hours, treatment starting at or after 16 hours did not affect neuronal number (Fig. 6F). Similarly, we observed a decrease in the number of Tg(AANAT2:GFP)+ cells when DAPT was administered from up to but not after 14 hours (Fig. 6G). Surprisingly, however, we observed a statistically significant increase in the number of Tg(HuC:GFP)+ cells when DAPT treatment was started at stages up to 16 hours (Fig. 6H). These results suggest that cell fate can still be modified at a stage when inhibition of Notch activity no longer affects the total number of neurons. We conclude that the effect of Notch on neuronal numbers and on neuronal identity reflects distinct sequential activities. Furthermore, as 98.03±3.4% of the future Tg(HuC:GFP)+ and 83.4±14.4% of the future Tg(AANAT2:GFP)+ cells have not exited their last S phase at 18 hours (Fig. 6E), our results indicate that Notch activity is required in cycling progenitors for both the control of neuronal number and the specification of neuronal identity.

Notch signaling is required to resolve mixed identity

We noted that treatment with DAPT at 16 hours increases the number of Tg(HuC:GFP)+ cells without a concomitant diminution of the number of Tg(AANAT2:GFP)+ cells or an increase in the

total number of neurons. One possible explanation is that in the absence of a functional Notch pathway, some cells are unable to choose between a photoreceptor or a projection neuron identity and therefore retain markers for both identities. We performed double staining with an antibody against HuC/D in a Tg(AANAT2:GFP) background to assess this possibility; the HuC/D antibody recapitulates the expression of Tg(HuC:GFP) except for a few ventrally located cells that are HuC/D+ but Tg(HuC:GFP)- and that we interpret to be newly born projection neurons (data not shown). In wild-type or mock-treated embryos, we observed a low occurrence of HuC/D+/Tg(AANAT2:GFP)+ cells (4.1% of specified epiphyseal neurons). A similar frequency of

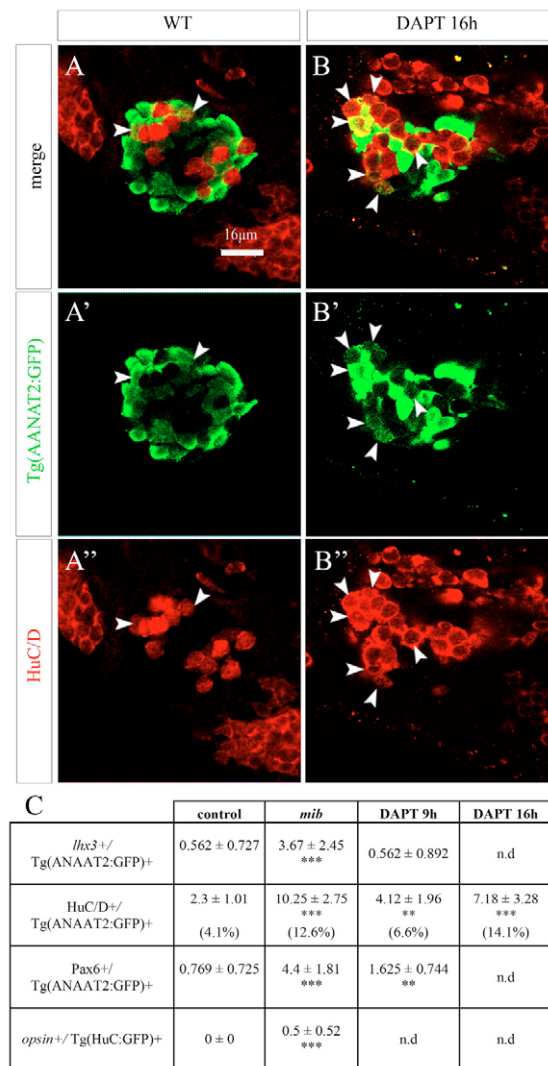


Fig. 7. Role for Notch in the resolution of a mixed identity.

(A-B'') Confocal sections of Tg(AANAT2:GFP) embryos at 48 hours, stained with a HuC/D antibody. Embryos were either mock treated (A-A'') or DAPT treated (B-B'') from 16 hours. Anterior is upwards. White arrowheads indicate double-labeled cells. (C) Average numbers of cells double-labeled for various projection neuron/photoreceptor marker combinations. In the case of HuC/D/Tg(AANAT2:GFP) + cells, the percent of double-labeled cells was calculated over the total number of cells expressing either marker. Numbers indicate average±s.d. ** $P < 0.001$; *** $P < 0.0005$ using a t -test. nd, not determined.

huC+/*Tg*(AANAT2:GFP)+ cells was observed in embryos double labeled for *huC* transcripts (data not shown). Interestingly, double-labeled cells were more numerous in *mib* mutants and embryos treated with DAPT at 16 hours (12.6 and 14.1%, respectively; Fig. 7A-C). We also observed an increase in the number of cells double-labeled for *Tg*(AANAT2:GFP) and the projection neurons markers *lhx3* or *Pax6* upon reduction of Notch signaling (Fig. 7C). Finally, we used a complex opsin probe to assess co-labeling with the *Tg*(HuC:GFP) transgene. Although in a wild-type context these two markers are exclusive, we observed rare co-labeled cells in *mib* mutants (Fig. 7C, 50% of *mib* mutant embryos show one double-labeled cell). These results suggest that epiphyssial neurons pass through a state where they express markers for both subtype identities and that Notch is required for the resolution of such mixed identity.

Constitutive activation of Notch represses projection neuron identity

Embryos with impaired Notch activity show a decrease in the number of photoreceptors relative to the total number of epiphyssial neurons. This observation raises the possibility that the Notch pathway plays an instructive role in specifying the photoreceptor fate. To address this, we used a previously described *hs:Gal4/UAS-Nintra* system (Scheer et al., 2002).

As shown in other studies, a graded response to Notch signaling is achieved depending on the temperature of heat-shock activation (Shin et al., 2007). Indeed, whereas embryos subjected to a strong heat shock (0.5 hours at 40°C) at 9 hours show very few epiphyssial neurons, upon milder activation (1 hour at 38°C) a wild-type number of *Islet1*+ neurons in the epiphysis was observed (Fig. 8C and data not shown). Nonetheless, mild activation of Notch signaling produced a strong decrease in the number of cells labeled with projection neurons markers (Fig. 8A,B,D). Consistent with results from late treatment with DAPT, the reduction of the numbers of *Tg*(HuC:GFP)+ cells was not observed when heat shock was induced at 24 hours, a stage where the majority of epiphyssial progenitors have passed their last S phase (Fig. 8D). Unexpectedly, the number of photoreceptors remains unchanged regardless of the stage at which Notch signaling is activated (Fig. 8A,B,E; data not shown). Thus, although neurons are produced

normally under mild Notch activation, projection neurons fail to be specified. Furthermore, preventing the specification of projection neurons is not sufficient to induce the transformation of unspecified cells into photoreceptors. We conclude that Notch signaling represses the projection neuron fate but is not instructive for photoreceptor identity, which presumably requires other inducing signals.

DISCUSSION

Composed of only two neuronal subtypes, the zebrafish epiphysis provides a simple system in which to address how neuronal identities are specified. Here, we have analyzed the role of Notch signaling in this model. Our results suggest that the Notch pathway controls both the total number of neurons formed, as well as the balance between their identities. Furthermore, our results show that the effect of Notch in the specification of epiphyssial neurons is strikingly different from the 'binary switch model' that has previously been described in either vertebrates or invertebrates. Below, we discuss our results and propose a model for how Notch signaling functions in this simple system.

Notch regulates cell number and identity in a short time window in cycling epiphyssial progenitors

Our results indicate that Notch signaling plays two distinct roles in the epiphysis: it regulates the number of neurons produced and the balance between projection neuron and photoreceptor identity. Such a dual role for Notch signaling has been already described in other areas of the vertebrate nervous system (see Shin et al., 2007). Interestingly, our DAPT time course and birthdating studies suggests that these two decisions occur in dividing epiphyssial progenitors. Furthermore, the very short delay observed between the two Notch-driven decisions raises the issue of the how epiphyssial progenitors adapt to such rapid changes in the level of Notch activation. One attractive hypothesis is that determination and specification of neuronal subtype identity employ different Notch signaling components. Indeed, we have shown that reduction of *deltaA* and *deltaD* functions alters the balance between the projection neuron and photoreceptor fates without affecting the total number of neurons. Alternatively, the control of neuronal numbers and identity

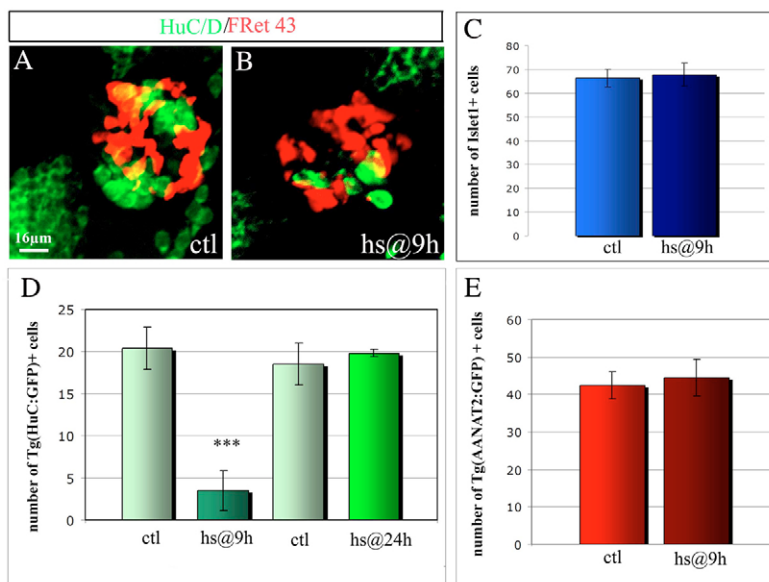


Fig. 8. Repression of projection neuron identity upon constitutive activation of Notch. (A,B) Confocal projections of a control (A) and a *Tg*(*hs:Gal4*); *Tg*(*UAS-Nintra*) double transgenic (B) embryo 48 hours after a heat shock performed at 9 hours (B). Cells are labeled with *HuC/D* and with *FRet43*. (C-E) Average numbers of *Islet1*+ cells (C), *Tg*(*HuC:GFP*)+ (D) and *Tg*(*AANAT2:GFP*)+ cells (E) at 48 hours in control and *Tg*(*hs:Gal4*); *Tg*(*UAS-Nintra*) double transgenic embryos heat shocked at 9 or 24 hours. As the constitutive expression of Notch intra impairs the formation (or the migration) of the parapineal organ, which originates from the epiphysis (Concha et al., 2003), we counted the total numbers of *Islet1*+ in epiphysis and parapineal in the control embryos. Scale bar: 16 μ m. Error bars represent s.d. * P <0.05; *** P <0.0005 using a *t*-test.

could involve the same Notch ligands with the effects on neuronal number and identity reflecting differences in the sensitivity of the two processes to the absolute levels of ligand present. In this case, determination and specification of neuronal subtype identity might employ different intracellular components downstream of Notch.

Notch resolves a mixed photoreceptor/projection neuron identity

A role for Notch in binary cell fate decisions has already been reported in vertebrates (Shin et al., 2007; Peng et al., 2007). Here, we present evidence that cells of a mixed photoreceptor/projection neuron identity can be observed in the wild-type epiphysis, albeit with a low frequency. Furthermore, cells expressing markers of both neural subtypes are more numerous in the epiphysis of embryos with reduced Notch activity. These results suggest that epiphysial progenitors pass through a transient phase of double identity and that Notch is required to resolve this. As we observe an increase in the number of cells expressing markers of projection neurons in Notch compromised embryos, it appears that the increase in the number of cells with mixed identity reflects that Notch is required to repress projection neuron identity in these cells. However, although reduction of Notch activity promotes the formation of projection neurons, the constitutive activation of the pathway inhibits the projection neuron fate but does not induce the transformation of these neurons into photoreceptors. Although we cannot rule out that activation of the photoreceptor fate requires a different threshold or mode of Notch activity than the Tg(hs:Gal4); Tg(UAS:Nintra) system provides, our data suggest that the Notch pathway does not play an instructive role in specifying the photoreceptor fate. In this regard, the situation found in the epiphysis is strikingly different from the previously described cases of Notch triggering a 'binary fate decision' both in *Drosophila* and vertebrates (Fanto and Mlodzik, 1999; Guo et al., 1995; Shin et al., 2007). For example, in the ventral spinal cord, loss of Notch activity induces the production of an excess of motoneurons at the expense of KA' interneurons and a reciprocal excess of KA' interneurons at the expense of motoneurons is induced upon constitutive activation of the pathway (Shin et al., 2007).

As activating Notch is not sufficient to activate the photoreceptor fate in the epiphysis, we postulate the existence of a photoreceptor inducing signal. We propose that mixed identity cells have received the postulated photoreceptor-inducing signal but have not yet downregulated the projection neuron program via Notch signaling. Three possibilities can be envisaged for what happens to cells with a mixed identity when Notch activity is impaired: they die, they retain markers of both identities or they finally adopt one of the two fates in a stochastic manner. We have shown that reduction of Notch activity induces cell death in both Tg(HuC:GFP) and Tg(AANAT2:GFP)+ cells. Interestingly, a negative correlation is observed between the presence of dying cells and the presence of cells with a mixed identity upon DAPT treatment. Indeed, we observe a relatively high frequency of mixed identity cells and no significant increase in apoptosis upon late DAPT treatment, while the opposite is observed upon early DAPT treatment. However, it is not possible to ascertain whether dying cells correspond to mixed identity cells and thus to establish a causal link between the failure to resolve such identity and apoptosis. By contrast, as we observe an excess of projection neurons at the expense of photoreceptors in embryos expressing reduced levels of Delta ligands, we would predict that at least some cells with mixed identity downregulate the photoreceptor program and adopt a projection neuron fate.

Towards a model of epiphysial cell type specification

Our results show that Notch controls both neuronal numbers and neuronal subtype identity in the zebrafish epiphysis and a model summarizing how this might be achieved is presented in Fig. 9. First, Notch effects on neuronal number and fate appear to occur in dividing precursors. However, impairing Notch activity at 16 hours modifies cell fate without modifying neuronal number. Thus, the choice between neuronal subtype identities is made slightly later than the decision to differentiate. Therefore, the first role of

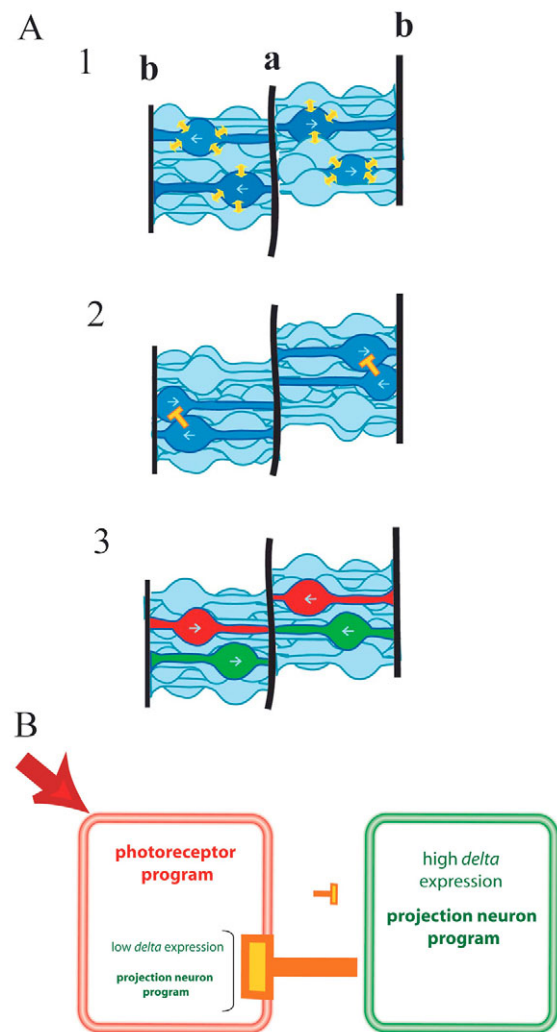


Fig. 9. A model for neuronal subtype specification in the zebrafish epiphysis.

(A) Schematic representation of the neuroepithelium. (1) Neural progenitors (dark blue) are selected from a pool of neuroepithelial cells (light blue) through Notch signaling (yellow arrow). Selected cells migrate to the basal side of the epithelium (blue arrow indicates the direction of movement) where they encounter new neighbors. (2) Selected neural progenitors again communicate via Notch to establish their respective identities. (3) Specified neural progenitors finish their last cell cycle. Progenitors for photoreceptors are in red, progenitors for projection neurons are in green. Apical and basal are labeled a and b, respectively. (B) Neuronal progenitors communicate via Notch (orange), thereby inhibiting both the projection neuron program and the expression of Delta genes. In parallel, a photoreceptor-inducing signal (red arrow) activates the photoreceptor program.

Notch results in the selection of one neural progenitor from a pool of equipotent cells. As the choice of a subtype identity is slightly delayed, we suggest that cells having chosen to differentiate change neighbors between the two Notch-dependent decisions. We speculate that such a change occurs as a result of interkinetic nuclear movements that neural progenitors undergo within the neuroepithelium (Frade, 2002; Sauer, 1935). Cells first decide whether they will stop dividing after they have completed their last cycle (Fig. 9A1). Then, they migrate basally where they encounter other neural progenitors which have already been selected to differentiate (Fig. 9A2). Communication between these cells would allow them to choose a fate before the completion of their last S-phase with Notch signaling inhibiting the projection neuron fate in cells having received the photoreceptor inducing signal (Fig. 9A3).

Our model implies a role for the Notch pathway in establishing cell fate through communication between cells expressing high levels of Delta (the progenitors for projection neurons) and cells expressing lower levels of Delta (the progenitors for photoreceptors). Indeed, two Notch ligands, *deltaB* and *deltaD*, are specifically expressed in projection neurons. Interestingly, the restriction of *deltaB* expression to projection neurons requires a functional Notch pathway as in *mib* mutants, we observed the expression of *deltaB* in photoreceptors (E.C., A.Q. and P.B., unpublished). This suggests that cell-cell communication via Notch is required to restrict the expression of certain Notch ligands to projection neurons (see Fig. 9B), in a manner similar to that described in the fly proneural clusters (Simpson, 1997).

Conclusion

Although the effect of Notch signaling on the spatio-temporal control of neurogenesis has been extensively studied, comparatively little is known about the role of Notch on the specification of neuronal subtype identity in vertebrates. Our results highlight a novel role for Notch. Indeed, acquisition of the photoreceptor fate in the epiphysis involves two distinct events: the induction of a photoreceptor program and the inhibition of projection neurons traits. However, although Notch is required to resolve fate choice by inhibiting the undesired genetic program, in contrast to other models in which Notch has been studied, it is not sufficient for the induction of the appropriate program. Further studies will show whether induction of other neuronal subtype identities similarly involves two distinct signals one for the induction of the appropriate fate and the other for the inhibition of inappropriate traits.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/14/2391/DC1>

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