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Notch signalling and the initiation of neural development in the *Drosophila* eye

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SUMMARY

Neural determination in the *Drosophila* eye occurs progressively. A diffusible signal, Dpp, causes undetermined cells first to adopt a 'pre-proneural' state in which they are primed to start differentiating. A second signal is required to trigger the activation of the transcription factor Atonal, which causes the cells to initiate overt photoreceptor neurone differentiation. Both Dpp and the second signal are dependent on Hedgehog (Hh) signalling. Previous work has shown that the Notch signalling pathway also has a proneural role in the eye (as well as a later, opposite function when it restricts the number of cells becoming photoreceptors – a process of lateral inhibition). It is not clear how the early proneural role of Notch integrates with the other signalling pathways involved. We provide evidence that Notch activation by its ligand Delta is the second Hh-dependent signal required for neural determination. Notch activity normally only triggers Atonal expression in cells that have adopted the pre-proneural state induced by Dpp. We also report that Notch drives the transition from pre-proneural to proneural by downregulating two repressors of Atonal: Hairy and Extramacrochaetae.

Key words: Notch, Delta, Atonal, Decapentaplegic, *Drosophila*, Eye, Neurogenesis

INTRODUCTION

During the development of multicellular organisms the specification of cells in a complex pattern depends largely on intercellular signalling. Among the best known intercellular signalling pathways is that triggered by the transmembrane receptor Notch (Artavanis-Tsakonas et al., 1999). Notch signalling defines an evolutionarily conserved pathway that is involved in multiple decisions during *Drosophila* development. Like other pathways, the outcome of Notch signalling is dependent on the context of the signalling event. This multiplicity of functions is well illustrated in the *Drosophila* eye, where Notch signalling has successive roles in promoting and inhibiting neural differentiation (Cagan and Ready, 1989; Baker et al., 1990; Parks et al., 1995; Baker et al., 1996; Baker and Yu, 1997; Li and Baker, 2001).

Drosophila has a compound eye comprising about 750 ommatidia that form a regular hexagonal array. At the beginning of the third larval instar, a groove known as the morphogenetic furrow begins to sweep anteriorly across the eye imaginal disc. This furrow marks the start of overt neural differentiation, although some markers are expressed a little anterior to the furrow (Ready et al., 1976). The photoreceptors develop in a stereotyped order behind the furrow, first the R8 photoreceptor, followed by the subsequent recruitment of the other photoreceptors from the surrounding undetermined cells (Tomlinson, 1985; Tomlinson and Ready, 1987; Freeman, 1997). The earliest manifestation of neural differentiation is the expression of the proneural bHLH transcription factor Atonal, upon which R8 specification depends (Jarman et al., 1994; Jarman et al., 1995). Atonal is first expressed in a weak uniform stripe of cells in front of the morphogenetic furrow; this stripe is then upregulated and refined into proneural clusters of cells expressing high levels of Atonal and from these, smaller groups of 2-3 cells, the 'R8 equivalence groups', emerge. Finally, the expression of Atonal is restricted to the single cell in each cluster, which will become the R8 photoreceptor (Jarman et al., 1994; Jarman et al., 1995; Baker et al., 1996; Dokucu et al., 1996; Baonza et al., 2001).

Notch signalling is required for successive steps of R8 determination, specifically in the regulation of Atonal (Baker et al., 1990; Parks et al., 1995; Baker et al., 1996; Dokucu et al., 1996; Baker and Yu, 1997; Baker and Yu, 1998; Li and Baker, 2001). At first, Notch promotes neural differentiation; later it inhibits it. Consequently, the phenotype of Notch loss of function varies with time. When the function of Notch is completely removed from very early stages, neural differentiation does not occur. Similar results with loss of the Notch ligand Delta (Dl), but not with the alternative ligand Serrate (Ser), indicates an early proneural function of Delta-to-Notch signalling (Baker and Yu, 1997; Ligoxygakis et al., 1998; Li and Baker, 2001). Conversely, later loss of Notch function, once Atonal expression is refined to proneural clusters, causes excess R8s to form, indicating a function for

Notch in the restriction of the number of cells that finally express Atonal (Cagan and Ready, 1989; Baker et al., 1990; Baker and Zitron, 1995; Baker et al., 1996; Baker and Yu, 1997). This process of 'lateral inhibition' also occurs in other neural tissues in the fly, and has been extensively studied and characterised (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999). By contrast, the mechanisms and genes that mediate the earlier proneural function of Notch have remained uncertain.

The movement of the morphogenetic furrow and subsequent photoreceptor differentiation depends on the secreted protein Hedgehog (Hh) (Heberlein et al., 1993; Ma et al., 1993; Domínguez and Hafen, 1997). Hedgehog is expressed in the differentiated photoreceptors behind the furrow and diffuses anteriorly to trigger the initiation of Atonal expression just ahead of the furrow (Heberlein et al., 1993; Ma et al., 1993; Domínguez and Hafen, 1997; Borod and Heberlein, 1998; Domínguez, 1999). The effects of Hh on Atonal expression are partly mediated by the secreted TGFβ family member, Decapentaplegic (Dpp), which is expressed within and ahead of the morphogenetic furrow in response to Hh signalling (Blackman et al., 1991; Heberlein et al., 1993; Ma et al., 1993; Pignoni and Zipursky, 1997). Although loss of Dpp signalling does not block neural differentiation or the propagation of the furrow, the rate of furrow progression is slowed and the expression of several genes anterior to the furrow is impaired (Chanut and Heberlein, 1995; Burke and Basler, 1996; Wiersdorff et al., 1996; Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000). It has therefore been proposed that Dpp acts at long range to define a 'pre-proneural' region anterior to the furrow and that shorter-range Hh signalling subsequently converts this pre-proneural region into a proneural region (Greenwood and Struhl, 1999).

Although this pre-proneural state is not well molecularly defined, several genes other than Atonal are expressed in a domain that broadly corresponds to this region. Thus, the negative regulators of Atonal expression, Hairy and Extramacrochaetae (Emc), both show elevated expression in this zone, as does the transcriptional activator Daughterless (Brown et al., 1995). It has been proposed that the acquisition of the pre-proneural state is associated with these transcriptional changes: it is a primed state, where cells are ready to differentiate as R8s (because they express Atonal and Daughterless) but are held in check because they also express the inhibitors Hairy and Emc (Greenwood and Struhl, 1999).

We have examined the proneural function of Notch signalling in the context of other signalling pathways involved in early ommatidial differentiation. We find that Notch activation by Delta is sufficient to trigger neural differentiation only in cells that have already received an inductive signal to become pre-proneural. Our results support the model that suggests that Dpp is sufficient for the acquisition of the pre-proneural state (Greenwood and Struhl, 1999) and extend it by showing that the transition from the pre-proneural state to the proneural state (upregulation of Atonal expression) depends on Delta/Notch-induced repression of the negative regulators of Atonal, Hairy and Emc. We synthesise a model that integrates this proneural role of Notch with the function of Dpp and Hh signalling in the early stages of *Drosophila* eye development.

MATERIALS AND METHODS

Genetic strains

We have used the following null or strong loss of function alleles: $N^{54/9}$ (null), Dl^{Rev10} (strong loss of function) and med^{8} (null). All these stocks are described in FlyBase (http://fly.ebi.ac.uk:7081/). We used one reporter line, a P[lacZ] insertion in *emc* (emc^{P5C}); (Garrell and Modolell, 1990). The UAS lines used were: *UAS-Dl* (Huppert et al., 1997), *UAS-brk* (Lammel et al., 2000), *UAS-Ser* (de Celis and Bray, 1997) and *UAS-dpp* (FlyBase).

Generation of mosaics

Mitotic clones were generated by Flp-mediated mitotic recombination (Xu and Rubin, 1993). In all cases, recombination was induced in second instar larvae by a 1 hour 30 minute heat shock at 37°C. Mutant clones for Dl^{Rev10}, med^{8} and double med^{8} Dl^{Rev10} were marked by the absence of β-galactosidase, using *y w hsp70-flp;* FRT^{82} *arm-lacZ M(3)/TM6B* stock. These flies were crossed to: *y w;* FRT^{82} Dl^{Rev10}*/TM6B, y w;* $FRT^{82}med^{8}$ */TM3,* and FRT^{82} med^{8} Dl^{Rev10} */TM3.* Mutant clones for $N^{54/9}$ were marked by the absence of GFP crossing males *Ubi-GFP* FRT^{18A}; *hsp70-flp MKRS/+* by females y w $N^{54/9}$ FRT^{18A}*/FM6.*

Flip-out clones

Clones of cells expressing Dl, Ser, Dpp and Hh were generated using the UAS/GAL4 system. Females of *UAS-Dl* or *UAS-brk* or *UAS-Ser* or *UAS-dpp* or *UAS-Hh.* genotypes were crossed to males *y w hsp70-FLP1.22; Act5C<FRT yellow*$^{+}$ *FRT>GAL4 UAS-GFP/+*.

Clones of ectopic co-expression of two genes were generated crossing females: *UAS-dpp* or *UAS-Hh* or *UAS-brk to males y w hsp70-FLP1.22*; *Act5C<FRT yellow*$^{+}$ *FRT> GAL4 UAS-GFP/+*; *UASDl/TM6b*.

To analyse the expression pattern of *emc* in clones of ectopic expression of *Dl* females emc^{P5C} were crossed to males *y w hsp70-FLP1.22; Act5C<FRT yellow*$^{+}$ *FRT>GAL4 UAS-GFP/+; UAS-Dl/TM6b*

Clones were induced 24-48 or 48-72 hours after egg laying by 12 minute heat shocks at 37ºC. The flip-out of the *<FRT yellow*$^{+}$ *FRT>* cassette results in the expression of the transcriptional activator GAL4 gene under the control of the *Act5C* promoter (Ito et al., 1997) and consequently the activation of the genes under the regulation of the UAS sequences. These clones were detected by expression of GFP, and were analysed in third instar larvae.

Immunohistochemistry

Eye imaginal discs from third instar larvae were stained as described (Gaul et al., 1992). The following antibodies were used: rabbit and mouse anti-β-galactosidase (Cappel); rabbit anti-Atonal (1:100) (Jarman et al., 1993); mouse and rat anti-Elav (used at 1:50 and 1:100 respectively) (O'Neill et al., 1994) and mouse anti-Hairy (Paddock et al., 1993). Anti-Elav was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Alexa 488- and 594- (Molecular Probes), and Cy5- (Jackson ImmunoResearch) conjugated secondary antibodies were used at dilutions of 1:200.

RESULTS

Notch signalling induces neural differentiation in cells near the morphogenetic furrow

We confirmed the observation of Baker and Yu (Baker and Yu, 1997) that loss of Notch signalling leads to a loss of neural differentiation. As expected, cells within clones of a null allele of *Notch* fail to upregulate Atonal expression from its initial low, uniform level (Fig. 1A,B). This implies that Notch

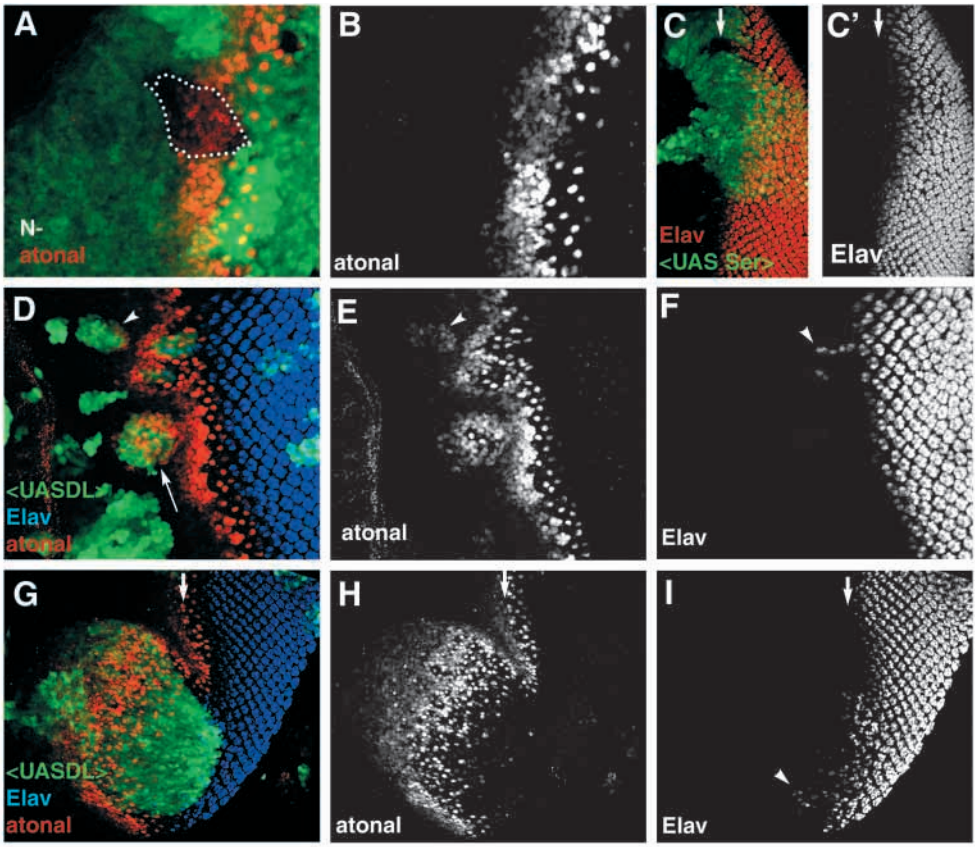

Fig. 1. Activation of Notch signalling is sufficient to induce the accumulation of Atonal at high levels. (A,B) In $N^{54/9}$ mutant clones (which lack green GFP staining), Atonal (red) expression is maintained at low levels and is not upregulated as it is in neighbouring wild-type tissue. The broken line indicates the border of the clone. (C,C′) Clones of ectopic expression of *Ser* (green) do not induce ectopic neural differentiation. Photoreceptor differentiation is visualised by Elav expression (red); white arrows indicate the approximate position of the morphogenetic furrow. (D-I) Clones of ectopic expression of *Dl* (green). The expression of Atonal and Elav are shown in red and blue, respectively, in D,G. (D-F) Clones of *Dl*-expressing cells within 12-15 cell diameters of the furrow induce the expression of Atonal at high levels autonomously as well as in the cells surrounding the clone (e.g. white arrow in D). In clones that are partially within the competent zone (arrowhead in D,E) Atonal is only activated in the cells nearest to the morphogenetic furrow. Occasionally, we observe Elav-positive cells anterior to normal Elav expression (arrowhead in F). (G-I) Large clones of *Dl*-expressing cells that cross the morphogenetic furrow causes its anterior displacement. The expression of Atonal behind the morphogenetic furrow is disorganised, and the number of Atonal-expressing cells isolated seems increased. We also observe Elav-expressing cells in advance of its endogenous expression (arrowhead in I), and this expression is disorganised. White arrows indicate the approximate position of the morphogenetic furrow. Here, and in all figures, anterior is towards the left.

signalling is required for the initiation of neural development but not for the first low level expression of Atonal. To examine in detail the role of Notch signalling in promoting neural differentiation, we have made clones of cells expressing the Notch ligand Delta and examined their ability to induce neural differentiation. In the wing disc, similar ectopic expression of Delta in clones induces the activation of Notch signalling within the clone as well as non-autonomously in cells surrounding it (Baonza et al., 2000; Baonza and Garcia-Bellido, 2000)

Clones were generated using the Gal4/UAS system combined with the Flip-out technique (see Materials and Methods) and third instar larval eye discs were labelled with different markers to assess neural development. We find that the phenotype of Delta-expressing clones depends on their position with respect to the morphogenetic furrow. Clones in the anterior part of the disc have no effect unless they are within 12-15 cell diameters of the furrow. Within this zone close to the furrow, Delta induces the ectopic expression of Atonal, both autonomously within the clone and non-autonomously, in cells surrounding the clone (n=25) (Fig. 1D-F). We observe that in some of these clones (n=6) there are also cells ectopically expressing the neural antigen Elav (Figs 1D-F, 2A). This indicates that once Atonal expression is activated, the full neural program is initiated. Thus, the primary proneural function of Notch signalling is the activation of Atonal.

Consistent with the neural-promoting properties of Delta, clones that span the furrow from posterior to anterior cause the anterior displacement of Atonal and Elav expression (n=45). This displacement implies that the furrow has accelerated as it moved through the clone (Fig. 1G-I). In the region of these clones that lies posterior to the furrow, we observe that the domain of Atonal expression is expanded and that the Atonal-expressing cells are disorganised and more numerous. In this region we also observe repression of neural differentiation, visualised with the expression of Elav (Fig. 1G-I). This later phenotype reflects the function of Notch signalling pathway in preventing neural differentiation posteriorly to the morphogenetic furrow (Sun and Artavanis-Tsakonas, 1996).

We have also produced similar clones expressing the alternative Notch ligand, Serrate, and find that, unlike Delta-expressing cells, they cause no neural induction ahead of the furrow. Conversely, when posterior to the furrow, *Ser*-expressing clones behave like those expressing Delta and prevent neural differentiation (Fig. 1C,C′) (Sun and Artavanis-Tsakonas, 1996). This implies that anterior to the furrow, the two Notch ligands are not equivalent in their ability to activate the receptor. We have not explored the reason for this, but note that the Notch glycosyltransferase Fringe, which makes Notch resistant to Serrate, is strongly expressed anterior to the furrow (Cho and Choi, 1998). The inability of Serrate to induce

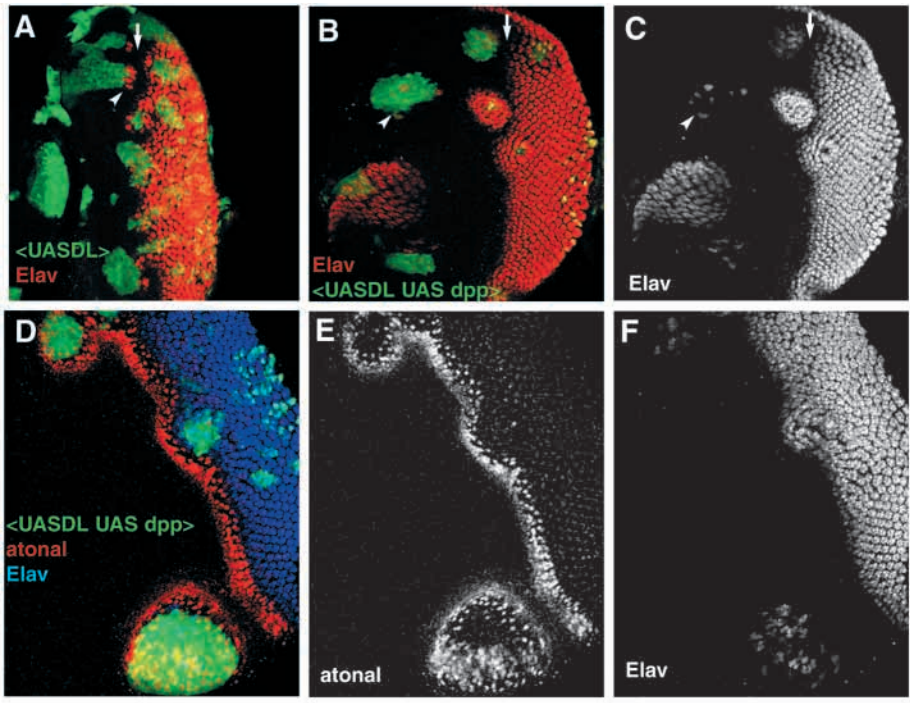

Fig. 2. Co-expression of *dpp* and *Dl* can induce neural differentiation in all regions ahead of the morphogenetic furrow. The white arrows indicate the approximate position of the morphogenetic furrow. (A) Induction of neural differentiation (visualised by Elav expression in red) in clones ectopically expressing *Dl* alone (green) is limited to a band of cells near of the morphogenetic furrow (arrowhead). (B-F) Clones ectopically expressing *Dl* and *dpp* (green). (B,C) Clones co-expressing *dpp* and *Dl* can trigger neural differentiation in all regions anterior to the morphogenetic furrow, even in clones far from the morphogenetic furrow (arrowhead); compare with A. (D-F) The first effect caused by these double overexpressing clones is the activation of Atonal (red). Again, this induction occurs in all the cells surrounding the clone even when they are distant from the competent region. In most cases, Atonal expression is observed several cells away from the border of the clone; within the clone, only isolated cells express Atonal. We explain this result as the consequence of the Atonal expression inducing an ectopic morphogenetic furrow (see text).

proneural Notch signalling is consistent with previous reports, which showed that loss of Serrate caused no effects on eye development (Baker and Yu, 1997; Ligoxygakis et al., 1998).

These results imply that there is a zone of about 12-15 cell diameters ahead of the morphogenetic furrow, where the activation of Notch signalling by Delta, but not by Serrate, is sufficient to trigger neural fate.

Co-expression of Delta and Dpp is sufficient to trigger neural differentiation

The simplest explanation of the results described above is that some signal or signals emanating from the cells posterior to, or within, the morphogenetic furrow are necessary for the specification of a neural competence zone ahead of the furrow. Within this zone, cells can respond to Delta-induced Notch activation by upregulating Atonal expression. A candidate for such a signal is the secreted protein Dpp. Dpp is expressed within the furrow in response to Hh signalling and has been proposed to define a 'pre-proneural' state in a zone anterior to the furrow (Blackman et al., 1991; Heberlein et al., 1993; Ma et al., 1993; Pignoni and Zipursky, 1997; Greenwood and Struhl, 1999). In order to analyse whether the function of Dpp is sufficient to generate the condition necessary for the neural activation by Notch signalling, we have induced clones that simultaneously express ectopic *dpp* and *Delta* (see Materials and Methods).

Clones of cells that express *dpp* alone only induce neural differentiation along the margin of the eye discs; internal clones have no effect on neural induction (data not shown) (Pignoni and Zipursky, 1997). By contrast, clones that co-express *Dl* and *dpp* trigger neural differentiation everywhere ahead of the furrow (Fig. 2B-F). In all the clones studied (n=25), we observed ectopic expression of Atonal and Elav. The induction of neural differentiation occurs in all the cells surrounding the clone and not, as in *Delta*-expressing clones, only in the cells within the competence zone (Fig. 2, compare 2A with 2B). In most of the clones analysed (20), we found that Atonal expression was associated with an ectopic morphogenetic furrow induced by the clones. Thus, it is possible to observe clones with ectopic Atonal expression several cells away from the border of the clone and with Atonal expression restricted to isolated cells within the clone, reproducing the pattern of Atonal expression of the endogenous furrow (Fig. 2D-F). Our interpretation of this result is that once Atonal is activated within and in the cells surrounding the clone, the normal cascade of ommatidial development is triggered, inducing an ectopic furrow that begins to move away from the clone.

These observations lead us to conclude that the expression of *dpp* is sufficient to enable all cells anterior to the furrow to activate neural differentiation in response to Notch. We postulate that during normal development, Dpp primes the cells to become competent to differentiate neurally in response to Notch signalling, at a range of 12-15 cells anterior to the furrow.

Dpp signalling is necessary to promote the proneural function of Notch signalling at long range

Loss of Dpp signalling during eye development causes furrow progression to slow down but not to stop: partial redundancy allows Hh signalling to induce neural differentiation in cells in which the Dpp signalling is blocked (Burke and Basler, 1996; Wiersdorff et al., 1996; Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000). Furthermore, clones of ectopic expression of Hh always induce neural differentiation and an ectopic furrow (Heberlein et al., 1995; Domínguez, 1999) (data not shown), even beyond the zone of Dpp-influenced cells, indicating that Hh is sufficient to trigger neural differentiation. The current model is that Dpp is important for furrow progression to occur efficiently and at a normal rate, but that it

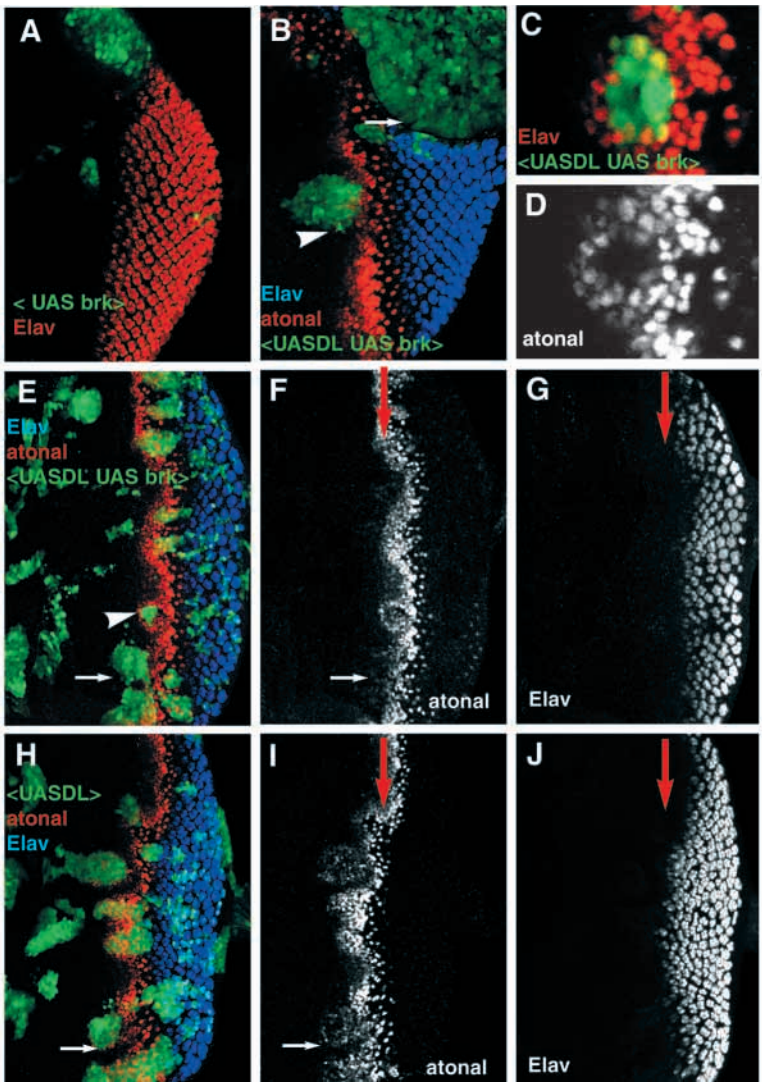

Fig. 3. The proneural function of *N* is reduced when *dpp* signalling pathway is blocked. (A) Clones of *brk*-expressing cells along the eye discs margins prevent the initialisation of the MF. (B-G) Clones co-expressing *brk* and *Dl*. (H-J) Clone of *Dl*-expressing cells. In all panels, green marks the clones, red indicates Atonal and blue indicates Elav. (B) Clones of *brk*- and *Dl*-expressing cells along the posterior eye margin show the same phenotype as clones of *brk*-expressing cells – preventing furrow initialisation, which leads to complete loss of neural differentiation (white arrow); this is a characteristic phenotype of the loss of function of the Dpp-signalling pathway. Clones ahead of the morphogenetic furrow only induce activation of Atonal in a thin band of cells immediately anterior to the morphogenetic furrow. Compare the clone indicated in B (arrowhead) with clones of *Delta*-expressing cells in H and Fig. 1; the distance from the furrow at which Atonal expression can occur is substantially reduced in the presence of *brinker*. (E-G) Several clones of *Dl*- and *brk*-expressing cells. Note the relatively poor ability of these cells to induce Atonal expression compared with clones expressing *Dl* alone (compare clones labelled with white arrows in E and F with clones of similar size and localisation labelled with white arrows in H,I). Surprisingly, despite the activation of Atonal expression, none of the double mutant clones analysed (n=30) express the neural marker Elav anteriorly to the endogenous Elav expression. Although the clones expressing *Delta* and *brinker* activate Atonal expression autonomously, there is a preferential activation of Atonal expression in neighbouring wild-type cells (when they lie near of the morphogenetic furrow – e.g. arrowhead in E); a higher magnification of this clone is shown in C,D. Red arrows indicate the approximate position of the morphogenetic furrow.

is not essential for neural differentiation to occur (Greenwood and Struhl, 1999). We have shown that Dpp signalling has an important role in promoting the proneural function of Notch signalling by generating the 'pre-proneural' state ahead of the furrow. This does not, however, rule out the possibility that Hh signalling could also produce a similar effect. If the function of Dpp signalling can be rescued by Hh signalling, then we would expect that the effects of ectopic activation of Notch signalling would be identical in a background were Dpp signalling is blocked (because in this case, Hh would replace Dpp function).

We have therefore analysed the effect of the ectopic expression of *Delta* when Dpp signalling is blocked, by inducing clones that co-express *Delta* and the negative Dpp signal regulator *brinker* (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). We validated the use of *brinker* expression as a way of inhibiting Dpp function in the eye by examining the phenotype of clones of *brinker*-expressing cells. We found that *brinker*-expressing clones indeed mimic *mad*$^{-}$ clones in their ability to prevent the initiation of the morphogenetic furrow when they occur at the posterior margin of the disc (Fig. 3A,B).

Double clones of *brinker*- and *Delta*-expressing cells only activate Atonal expression when they lie within four to five cells of the morphogenetic furrow (Fig. 3B-G). In addition, the position of the endogenous morphogenetic furrow is only slightly altered compared with control clones expressing *Dl* alone (Fig. 3; compare 3E-G with 3H-J). Thus, the proneural action of ectopic Notch signalling anterior to the morphogenetic furrow is substantially reduced in cells in which Dpp signalling is inhibited. These results suggest that despite some partially rescuing short-range signal near the furrow (which we presume to be Hh), Dpp signalling is required for the longer range ability of cells to initiate neural differentiation in response to Notch activation.

Simultaneous loss of Notch and Dpp signalling do not prevent the initial expression of Atonal

The fact that the ectopic expression of Dpp does not reproduce the effects cause by the overexpression of Hh, indicates that additional Hh-dependent signals are needed to promote neural differentiation. Our results suggest that Notch signalling could be one of these. According to this model blocking Notch and Dpp signalling would be sufficient to prevent neural differentiation as

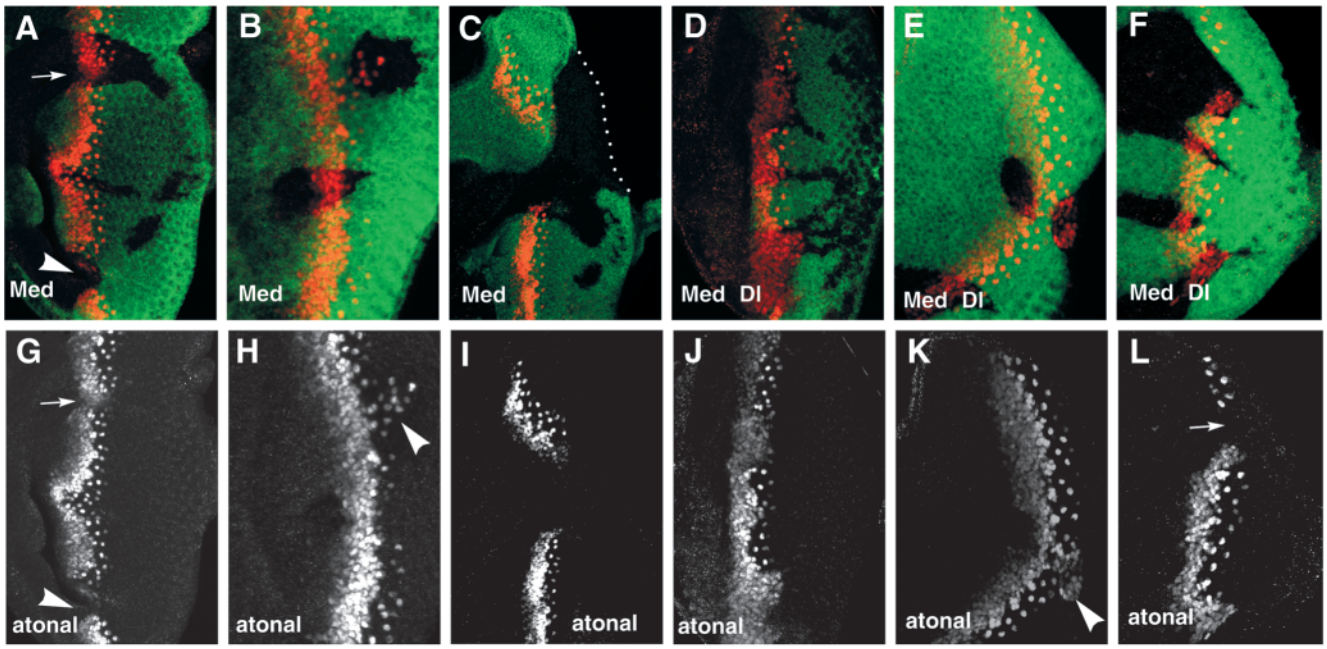

Fig. 4. The simultaneous loss of Dpp and N signalling does not prevent the initiation of Atonal expression. All clones are marked by the absence of β-galactosidase (green), Atonal is stained in red. (A-C,G-I) *medea* mutant clones. (D-F,J-L) *med Dl* double mutant clones. (A,G) In *med* clones Atonal expression is reduced (white arrow) or occasionally absent (arrowhead). (B,H) Some *med* clones posterior to the morphogenetic furrow ectopically express Atonal (white arrowhead). (C,I) *med* clones along the posterior eye margin prevent the initialisation of morphogenetic furrow. (D,J) Double mutant clones of med^{8} Dl^{rev10} express Atonal at low levels. (E,K) Occasionally posterior med^{8} Dl^{rev10} double clones ectopically express Atonal in a cluster of cells (arrowhead in K); note the difference between this and clones of *med* alone, where the ectopic expression is in isolated cells (compare K with H). (F,L) In some med^{8} Dl^{rev10} double clones there are regions where Atonal expression is totally lost (white arrow in L), a phenotype also observed in *med* clones (white arrowheads in A and G).

it would block both Hh-induced intermediate signals. To analyse this possibility, we induced double mutant clones of the strong *Delta* allele Dl^{rev10} and the *medea* allele med^{8}. Medea is the *Drosophila* homologue of the mammalian MAD-related protein Smad4, and is required for transduction of the Dpp signal (Das et al., 1998; Hudson et al., 1998). We first confirmed that clones of med^{8} along the posterior eye margin cause similar phenotypes to mad^{-} clones, preventing the initiation of the morphogenetic furrow (Das et al., 1998; Hudson et al., 1998; Fig. 4C,I). Internal clones of med^{8} can reduce the expression of Atonal, especially the initial uniform expression (Fig. 4A,G). Occasionally (1/17), the expression of Atonal is totally removed in part of the clone (Fig. 4A,G). These phenotypes are similar to those described when Dpp signalling is blocked in mutant clones of the Dpp receptor *thick vein* (*tkv*) (Greenwood and Struhl, 1999). We could find only one phenotype of med^{8} clones not accounted for by phenotypes caused by loss of other members of the pathway: in some clones (6/11) posterior to the morphogenetic furrow, Atonal is ectopically expressed, always in isolated cells (Fig. 4B,H). We do not understand the basis for this phenotype, but note that it does not affect the region under consideration here – anterior to the furrow.

Double Dl^{rev10} med^{8} mutant clones show a combination of the phenotypes observed in independent mutant clones of *Delta* and *med*. Thus, all internal clones analysed (n=17) show *Delta*-like reduction of Atonal expression (Fig. 4D,J). In some of these clones (4) there are regions where Atonal expression is totally lost, a phenotype observed in *med* clones (Fig. 4F,L). Also as in *medea* clones, we also find posterior Dl^{rev10} Med^{8} clones that express Atonal ectopically. However, in this case, the Atonal expression is in clusters of cells (Fig. 4E,K), reflecting the fact that lateral inhibition is blocked in the absence of Delta.

Our results indicate that the initial expression of Atonal can be induced in the absence of Notch and Dpp signalling, implying that Hh signalling can, directly or via yet another intermediate, overcome the loss of function of both pathways.

Hairy and Emc expression are regulated by Notch signalling

The progression of the morphogenetic furrow correlates with the modulated expression of the negative regulators of Atonal expression, Emc and Hairy (Brown et al., 1995). Hairy is expressed in a broad stripe anterior to the furrow and rapidly switched off in the furrow. Emc protein is present in all cells but the highest levels are present in a dorsoventral stripe of cells anterior to the domain of Hairy expression, whereas the lowest levels are observed in the furrow (Brown et al., 1995). Thus, the increase of Atonal expression in the proneural groups within the furrow is associated with the downregulation of both Emc and Hairy (Fig. 5; Brown et al., 1995). We have tested whether this downregulation of Emc and Hairy is mediated by Notch by analysing the expression of Emc and Hairy when Notch signalling is blocked and when it is ectopically activated.

In mitotic clones of the *Notch* null allele $N^{54/9}$, we observed that the expression of Hairy is displaced posteriorly extending behind the morphogenetic furrow. The consequent ectopic expression of Hairy within the furrow is accompanied by a

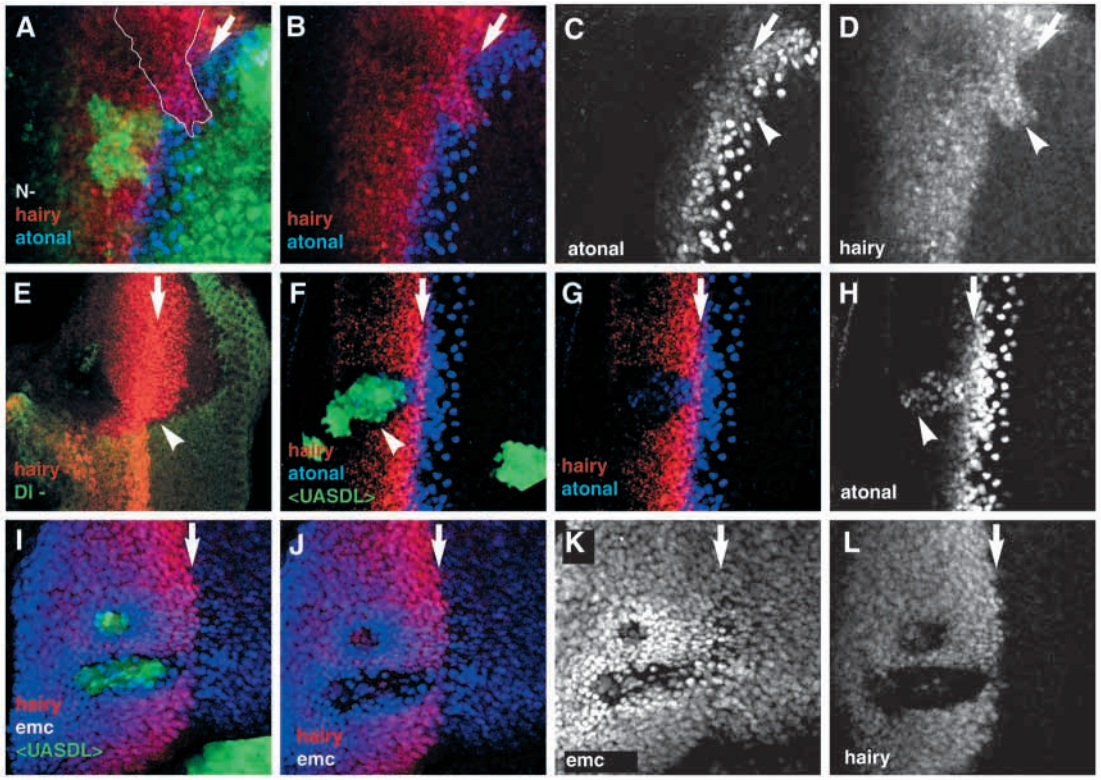

Fig. 5. Notch signalling regulates the expression of Emc and Hairy. (A-D) Clone of $N^{54/9}$ mutant cells. (E) Clone of Dl^{rev10} mutant cells. (F-L) Clones of *Dl*-expressing cells. (A-D) In clones of $N^{54/9}$ mutant cells (which lack green GFP, outlined in white), Hairy (red) is upregulated, and the sharp border between Hairy expressing and non-expressing cells is broken (B, and arrowhead in D). Conversely, in these mutant cells Atonal (blue staining in A,B) is maintained at low levels and not upregulated (white arrowhead in C). (E) Large $Minute^{+}$ Dl^{rev10} clone (which lacks red β-galactosidase staining) shows increased Hairy expression behind the morphogenetic furrow. Note that in the mutant cells adjacent to the clone (arrowhead) Hairy is not expressed. This non-autonomous rescue is characteristic of *Dl* mutant clones. (F-H) Clones of *Dl*-expressing cells (green), cause the downregulation of Hairy (red) expression, autonomously, as well as in the wild-type cells surrounding the clone (arrowhead). The downregulation of Hairy is correlated with increased levels of Atonal (blue staining in F,G and arrowhead in H). (I-L) The expression of Emc (blue β-galactosidase staining in the emc^{P5c} strain in I,J) and Hairy (red in I,J) are reduced in *Dl*-expressing cells (green). Note that whereas the expression of Hairy disappears in all the cells surrounding the clones and in most of the cells within the clones, the levels of Emc are downregulated within the clones but not in the adjacent wild-type cells. The white arrows indicate the approximate position of the morphogenetic furrow.

reduction in Atonal expression: Atonal levels remain at the low level normally observed anterior to the furrow (Fig. 5A-D). Similar results were obtained with *Delta* clones (Fig. 5E). Reciprocally, when Notch signalling is ectopically activated in clones of *Delta*-expressing cells, Hairy is downregulated, both within the clone and in the cells immediately surrounding it (Fig. 5F-L). In these clones we observed that Emc is also downregulated within the clone (Fig. 5I-K), although for reasons we do not understand, Emc levels are unusually high in the wild-type cells that border the clone. The downregulation of Emc and Hairy caused by the ectopic expression of Delta correlates with increased expression of Atonal ahead of the furrow (Fig. 5F-H). We conclude from these results that Delta/Notch signalling promotes Atonal activation and neural differentiation by downregulating the repressors Hairy and Emc.

DISCUSSION

The most well characterised role of Notch signalling in R8 photoreceptor determination is mediating the process of lateral inhibition, which refines Atonal expression from a small group of cells to a single cell (Cagan and Ready, 1989; Baker et al., 1990; Baker and Zitron, 1995; Baker et al., 1996; Baker and Yu, 1997). However, an earlier and opposite role for *Notch*, this time promoting neural determination, has also been recognised, although how this ‘proneural’ function integrates with other pathways necessary for neural differentiation has been unclear (Baker and Yu, 1997; Ligoxygakis et al., 1998; Li and Baker, 2001). In this work, we have shown that in normal eye development the proneural function of Notch signalling depends on prior Dpp signalling. We have also found that Emc and Hairy, two negative regulators of Atonal expression, mediate the proneural function of Notch signalling in the eye. Thus, we propose a model that links the upregulation of Atonal in the proneural groups with the downregulation of Hairy and Emc through the activation of Delta/Notch signalling.

Notch triggers the transition from pre-proneural to proneural cells: a model

Our results allow us to extend the model of Greenwood and

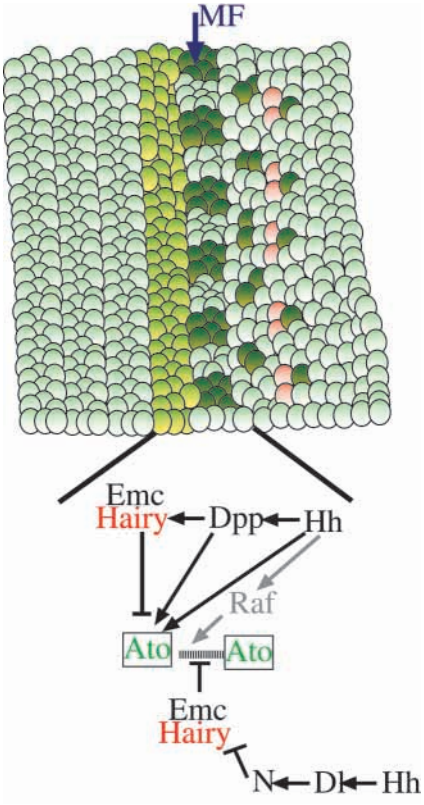

Fig. 6. During the progression of the morphogenetic furrow, Hh signalling activates at least two different signals. One is Dpp which, at long range, primes cells to adopt a 'pre-proneural' state. These cells are ready to initiate neural differentiation, because they express Atonal and Daughterless, but are held in check because they also express the inhibitors Hairy and Emc, which keep the Atonal expression and activity at low levels. Delta/Notch activation provides a second Hh-dependent signal, which only works at short range (within the morphogenetic furrow), as Delta is a membrane-bound ligand. The activation of Notch causes the downregulation of Hairy and Emc, and consequently the upregulation of Atonal expression and activity. A different short range signal, unidentified but transduced by Raf, has also been proposed to upregulate Atonal (Greenwood and Struhl, 1999) (here shown in grey, see text).

Struhl (Greenwood and Struhl, 1999), specifically to integrate proneural Notch signalling into the concept of a progression of cell states, from undetermined to pre-proneural to proneural. We will first outline the full model (Fig. 6) and then discuss aspects of the evidence supporting it and its implications.

Hh in the cells posterior to the morphogenetic furrow activates the expression of Dpp in the furrow (Blackman et al., 1991; Heberlein et al., 1993; Ma et al., 1993). Our data support the proposal of Greenwood and Struhl (Greenwood and Struhl, 1999) that as Dpp acts at a longer range than Hh, this relays a signal to a zone extending about 15 cells anterior to the furrow, priming these cells for differentiation. This makes cells competent to receive a later signal that upregulates Atonal expression, thereby initiating overt neural differentiation. This second signal is also dependent on Hh, but operates only much closer to the furrow: our evidence implies that it consists of Delta activating Notch signalling. The initial 'pre-proneural' state is molecularly defined by the accumulation of the repressors of *atonal* transcription Hairy and Emc (see below), as well as by the positive regulator of Atonal, the HLH transcription factor Daughterless (Brown et al., 1995). Therefore, although Atonal and Daughterless are both expressed in this pre-proneural zone, neural differentiation is not initiated, as Hairy and Emc ensure that Atonal activity remains below a threshold. We have shown that the Hh-dependent activation of Delta/Notch signalling triggers the transition from this pre-proneural state to the proneural state by downregulating both Hairy and Emc. This negative regulation of the Atonal repressors is sufficient to allow the accumulation of active Atonal in the proneural groups to a level where R8 determination is initiated. Interestingly, Greenwood and Struhl (Greenwood and Struhl, 1999) proposed another Hh-dependent short-range signal, unidentified, but transduced by Raf. We do not know how this putative Raf-mediated signal relates to the Notch signal we propose but this is discussed below.

Pre-proneural state and Notch signalling

We have shown that Notch can only trigger Atonal upregulation in a zone extending 12-15 cells anterior to the furrow, and that this zone is defined as the cells that receive the diffusible factor Dpp, whose source is in the furrow. We therefore endorse the proposal of Greenwood and Struhl (Greenwood and Struhl, 1999) that Dpp acts to define a 'pre-proneural' state that prepares cells for the imminent initiation of neural determination. This pre-proneural state was defined previously as the zone of cells that initiate Hairy and Atonal expression in response to Dpp signalling. We can now add a functional definition to this state: all these cells are primed for neural differentiation because all can respond to Notch activation by upregulating Atonal levels.

Initiation of Atonal expression

The ectopic expression of *dpp* does not reproduce the effects caused by the overexpression of Hh, indicating that at least one other signal mediates the effects of Hh. Our results imply that Notch is one such signal. In support of this, the ectopic expression of both Dpp and Delta mimic the effect cause by the misexpression of Hh signalling. As described above, a similar short-range proneural function has been proposed for Raf by Greenwood and Struhl (Greenwood and Struhl, 1999), who showed that Atonal expression was abolished in *raf*$^{-}$ clones. The significance of this result is made unclear by the report of Yang and Baker (Yang and Baker, 2001) that clones of another well-characterised null allele of *raf* initiate Atonal expression normally. Our results focus on the role of Notch and do not directly address the question of a Raf-mediated signal, but they do not rule it out. However, as there is no known connection between Notch and Raf, the relationship (if any) between these two proposed proneural signals is currently obscure. In fact, our results do point to the existence of at least one proneural signal other than Dpp and Notch: the initial expression of Atonal can be induced even when both Dpp and Notch signalling are simultaneously blocked. On the basis of current evidence, we favour the idea that this other signal may be Hh itself, acting directly to activate Atonal expression, as has been proposed by Domínguez (Domínguez, 1999).

Hairy and Emc regulation by Notch signalling

Simultaneous loss of Hairy and Emc activity leads to the

precocious differentiation of photoreceptors in a competent region ahead of the morphogenetic furrow (Brown et al., 1995), a phenotype that resembles that caused by ectopic expression of Delta. In addition, we have shown that ectopic Notch signalling downregulates Hairy and Emc ahead of the morphogenetic furrow, causing the accumulation of Atonal at high levels; conversely, loss of function of Notch signalling increased the levels of Hairy. We conclude that Delta/Notch signalling regulates the expression of these negative regulators in the eye. Consistent with this proposal, Emc is also regulated by Notch in the developing wing disc (Baonza et al., 2000).

Although Notch signalling negatively regulates both Hairy and Emc, the ectopic expression of Delta does not affect both genes identically. Thus, whereas Hairy is removed both within the clone and in the neighbouring cells, Emc is only downregulated autonomously within the clone. This distinction could be an artefact caused by the perdurance of β-galactosidase. Alternatively, these differences may reflect a different requirement for Notch signalling in the regulation of both genes. Furthermore, the expression pattern of Hairy and Emc is different during the normal progression of the morphogenetic furrow. Hairy is precisely regulated, being expressed only in the cells anterior to the furrow, and is rapidly downregulated in the furrow (Brown et al., 1995). This precise regulation is crucial as shown by the ectopic expression of *hairy* (Brown et al., 1991). Emc has a much broader expression pattern in the eye disc, although it shows a similar upregulation followed by downregulation in the zone immediately anterior to the furrow (Brown et al., 1995).

It is also worth pointing out that not only does the expression pattern of Emc and Hairy differ, but their exact mechanism of repression is also distinct. Hairy regulates bHLH proteins by a mechanism of direct DNA binding and transcriptional repression. Emc, however, forms complexes with bHLH proteins, preventing their DNA binding (Ellis et al., 1990; Garrell and Modolell, 1990; Van Doren et al., 1991; Van Doren et al., 1992). Thus, Emc can antagonise the proneural function of Atonal by two distinct mechanisms. First, Emc presumably binds to Atonal, rendering it incapable of activating its targets. Second, we have shown that Emc controls the levels of Atonal. By analogy to its regulation of two other bHLH transcriptional regulators, Achaete and Scute (Cubas et al., 1991; Cubas and Modolell, 1992; Van Doren et al., 1992), we expect that Emc interferes with the autoregulatory upregulation of *atonal* expression. This positive autoregulation is an essential component of its accumulation in cells within the morphogenetic furrow (Sun et al., 1998). In conclusion, the proneural action of Notch signalling increases Atonal activity by two mechanisms: *atonal* is transcriptionally upregulated, and at the same time a repressive co-factor is removed. These concerted actions lead to the accumulation of active Atonal and thereby the initiation of neural differentiation.

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