

## Atonal, Rough and the resolution of proneural clusters in the developing *Drosophila* retina

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### SUMMARY

In the developing *Drosophila* retina, the proneural gene for photoreceptor neurons is *atonal*, a basic helix-loop-helix transcription factor. Using *atonal* as a marker for proneural maturation, we examine the stepwise resolution of proneural clusters during the initiation of ommatidial differentiation in the developing eye disc. In addition, evidence is provided that *atonal* is negatively regulated by *rough*, a homeobox-containing transcription factor expressed exclusively in the retina. This interaction leads to the refinement of proneural clusters to specify R8, the first neuron to emerge in the retinal neuroepithelium. Ectopic expression of *atonal* or removal of *rough* results in the transformation of a discrete 'equivalence group' of cells

into R8s. In addition, ectopic expression of *rough* blocks *atonal* expression and proneural cluster formation within the morphogenetic furrow. Thus, *rough* provides retina-specific regulation to the more general *atonal*-mediated proneural differentiation pathway. The opposing roles of *atonal* and *rough* are not mediated through the *Notch* pathway, as their expression remains complementary when *Notch* activity is reduced. These observations suggest that homeobox-containing genes can provide tissue-specific regulation to bHLH factors.

Key words: *atonal*, *rough*, proneural genes, bHLH, homeobox, *Drosophila*, photoreceptors, neural differentiation

### INTRODUCTION

The *Drosophila* retina has proven an especially useful model system for examining cell fate induction. It is a simple micro-nervous system consisting of several hundred identical 'ommatidia', each of which contains eight photoreceptor neurons. Most ommatidial cell fates are thought to arise by short-range cell-cell inductive signaling. In the larva, precursor cells are recruited into well-spaced ommatidial clusters through local cues provided by their previously differentiated neighbors (Tomlinson and Ready, 1987; Cagan and Ready, 1989a; Krämer et al., 1991; Zipursky and Rubin, 1994). The R8 photoreceptor neuron, in contrast, is the first terminally differentiated cell type in the neuroepithelium and therefore must arise by a different mechanism. Mutations in several different genes give rise to multiple R8s within a single ommatidium (Cagan and Ready, 1989a; Baker et al., 1990; Heberlein et al., 1991; Van Vactor et al., 1991; Cagan, 1993), suggesting R8 emerges from a larger group of 'R8 competent' cells. A process of selection between initially equivalent cells leads to the formation of only a single R8 neuron in each ommatidium.

During interactions among cells within an epithelium, establishing the first cell fate presents a special problem, namely creating differences within a seemingly naïve group of cells. Studies in other *Drosophila* tissues have identified a basic unit

of neuronal competence defined as the 'proneural cluster' (reviewed in Posakony, 1994; Jan and Jan, 1995). This group of typically 15-20 cells presages the emergence of one or a few neurons within a particular neuroepithelial region. Proneural clusters are defined by the expression of 'proneural basic helix-loop-helix' (bHLH) transcription factors, whose activity provides temporary neuronal competence to all cells within the cluster. For example, members of the *achaete-scute* complex (AS-C) have been identified as the proneural bHLH factors in several regions of the embryonic and adult nervous systems. The position of the proneural group defined by AS-C expression is determined by 'negative bHLH' factors such as *hairy* and *extramacrochaete*, and subsequent resolution to a single neuron or neuronal precursor requires activity by members of the *Notch* signal transduction pathway. These factors are required for the development of many different types of neurons in the developing fly. The factors that provide tissue-specific regulation to proneural cluster development have been more elusive.

R8 specification occurs in the larval eye imaginal disc. Formation of ommatidia commences in the posterior region and then recruitment of additional ommatidia occurs as a wave of differentiation which moves anteriorly. The front of this wave is defined by the morphogenetic furrow (MF; reviewed in Heberlein and Moses, 1995). The process of R8 selection begins within the MF. *atonal* has been identified as

a proneural gene required for R8 specification: genetic mosaic experiments indicated *atonal* activity was required in R8 for normal ommatidial assembly (Jarman et al., 1994) and complete loss of *atonal* activity results in a failure in R8 as well as ommatidial differentiation (Jarman et al., 1995). Interestingly, the initial expression pattern of *atonal* was found to define well-spaced proneural groups within the MF, which give way to R8-specific expression (Jarman et al., 1994). Thus, *atonal* provides the earliest known marker for both patterning of ommatidia and specification of R8. In this paper, we use *atonal* expression and the unique properties of the *Drosophila* retina to further examine the dynamics of proneural maturation. We provide evidence for distinct and stereotyped steps during proneural cluster maturation that lead to R8-specific expression.

Similar to other proneural bHLH factors, *atonal* requires the *Notch* pathway for proper resolution to R8. Loss of *Notch* activity expands neuronal and R8 specification in addition to *atonal* expression (Dietrich and Campos-Ortega, 1984; Cagan and Ready, 1989a; Lee et al., 1996). However, *Notch* activity is required for every cell fate decision in the developing retina (Cagan and Ready, 1989a) as well as for specification of cell fates throughout fly development. To determine if the *Notch* pathway alone provides regulation of the dynamic and patterned expression of *atonal* unique to the MF, we screened through mutations in many genes known to affect retinal development to identify factors required for proper R8 specification (Cagan, 1993). Loss-of-function mutations in *rough*, which encodes a homeobox-containing transcription factor (Tomlinson et al., 1988; Heberlein et al., 1994), resulted in 1-2 additional R8 neurons in each ommatidium (Heberlein et al., 1991; Van Vactor et al., 1991).

Rough protein is expressed exclusively in the retina, first broadly within the MF (however, see Results) and later specifically in R2/R5 followed by R3/R4 (Tomlinson et al., 1988). Given its retina-specific expression pattern and its mutant phenotype, the *rough* locus was a candidate to provide a more specific regulation to proneural progression. This paper explores the relationship between Atonal and Rough during patterning of the proneural cluster. We identify Rough as a retina-specific negative regulator required for proper resolution of *atonal* expression and proneural maturation. Rough acts in conjunction with the more ubiquitously used *Notch* regulatory pathway.

## MATERIALS AND METHODS

### Fly stocks

All flies were maintained at 25°C on standard cornmeal-yeast-agar medium. Transgenic flies containing heat-shock-inducible constructs for *atonal* (*w*; P[*w*<sup>+</sup>, *hsp-GAL4*] P[*w*<sup>+</sup>, UAS-*ato*]/*CyO*) and for *rough* (*hs-ro-3/CyO*; *ry*<sup>506</sup>) were described in, respectively, Jarman et al. (1993) and Kimmel et al. (1990). *ro<sup>x63</sup>* was described by Heberlein and Rubin (1991). BBO2 was isolated in a P-*lacZ* enhancer trap screen in the laboratory of L. S. Zipursky. *ato<sup>1</sup>* is described in Jarman et al. (1995). *N<sup>ts1</sup>* is described in Lindsley and Zimm (1992).

### Immunohistochemistry and histology

$\alpha$ -atonal polyclonal serum (gift of A. Jarman and Y. N. Jan) was

raised in rabbits against a bacterially expressed fusion protein (Jarman et al., 1993) and was used at a 1:4000 concentration.  $\alpha$ bossNT1 is a mouse monoclonal antibody that recognizes an extracellular epitope (1:1000; Cagan et al., 1992).  $\alpha$ -rough antibody MAbro1 is a mouse monoclonal antibody (1:100; Kimmel et al., 1990).  $\alpha$ -E(spl) mouse monoclonal antibody (mAb 323; gift from S. Bray) was used at a 1:1 concentration with minimum time for washes after the primary and secondary antibody incubations as described in Jennings et al. (1994). Mouse  $\alpha$ - $\beta$ -galactosidase antibody is commercially available (1:1000; Promega Corporation).

Eye discs were dissected from third instar larvae grown at 25°C into PBS and fixed either in PLP (2% paraformaldehyde, 7.5 mM lysine, 10 mM sodium metaperiodate, 3.5 mM phosphate buffer, pH: 7.4) for the detection of boss and  $\alpha$ - $\beta$ -galactosidase or PEMP (100 mM PIPES at pH 7.0, 2 mM MgSO<sub>4</sub>, 2 mM EGTA, 4% paraformaldehyde) for the detection of Atonal and Rough. They were permeabilized by either 0.1% saponin in PBS (for Boss and  $\beta$ -galactosidase detection) or 0.3% Triton-X (for Atonal, Rough and E(spl) detection) in PBS (3 × 10 minutes). This was followed by primary antibody incubation overnight at 4°C in the PBS-detergent solution plus fetal calf serum (final concentration: 10%) as blocking agent. After three 10-minute washes in PBS-detergent, secondary antibody incubation was carried out 1-2 hours at 4°C. After three more washes and staining, discs were whole mounted in Crystal/Mount (Biomedica Corporation) for DAB stains or in 1:1 glycerol: PBS plus n-propyl gallate (95 mM) for fluorescent stains.

For HRP-DAB stains, the avidin-biotin complex (ABC) method was used (Vector Laboratories). These were observed and photographed on a Zeiss Axioscope microscope equipped with Nomarski optics. For fluorescent detection of primary antibodies, anti-mouse and anti-rabbit IgGs conjugated to Indocarbocyanine (Cy3) and Fluorescein Isothiocyanate (FITC) were used (both at 1:500 concentration). Fluorescent discs were observed and scanned on a Nikon Diaphot inverted microscope coupled to a Molecular Dynamics MultiProbe 2001 confocal laser scanning system. Images were collected and analyzed by ImageSpace software (Molecular Dynamics, Inc.) running on a Silicon Graphics Iris Indigo Workstation. Confocal projection images (Figs 3-5) were created by the superimposition of 10-20 sections using the lookthrough-extended focus feature of the ImageSpace software.

### Heat shocks

Several heat-shock regimens were used for the misexpression of Atonal (modified from Jarman et al. 1993). The phenotype in Fig. 2 was obtained with three 15-minute heat shocks at 39°C spaced by 45-minute recovery periods. Larvae were then left to develop at room temperature for 16 hours before dissection. Extensive time-course experiments were performed after two 15-minute heat shocks at 39°C spaced by a 1-hour recovery period. The percentage of transformed ectopic R8s were determined from video drawings by identifying and framing a maximally transformed region near the MF; the total number of ommatidia with additional R8s and the total number of ommatidia were then determined. Using both  $\alpha$ -GAL4 and  $\alpha$ -atonal antibodies, we observed that induction started 2 hours after the end of the second heat shock and lasted very strongly up to 18-24 hours. An intriguing finding was that ectopic expression was non-existent or very weak in the whole anterior region of the morphogenetic furrow and in a band of 4-5 columns posterior to the morphogenetic furrow. Very strong and stable expression was seen in the posterior of the disc. However, this expression pattern was neuronal. This could be due to a positional effect in this fly line or due to robust post-translational downregulation of the protein in the undifferentiated and non-neuronal cells.

Heat-shock induction of Rough was carried out at 37°C for 90 minutes (Kimmel et al., 1990). At 3, 12, 18, 24 and 36 hours after the heat shock, larvae were collected and dissected. *N<sup>ts1</sup>* flies were incubated at 32°C for 6 hours and were immediately dissected.

## RESULTS

### Proneural maturation proceeds in a stepwise fashion

In the developing larval retina, the MF contains an anterior-to-posterior gradient in which rows of ommatidia closer to the morphogenetic furrow (anterior) are developmentally less mature than those progressively further away (more posterior). Hence, each row represents approximately a 2 hour step in maturity (Campos-Ortega, 1980). In addition, a second finer gradient exists within each row of ommatidia along its equatorial-polar axis (Wolff and Ready, 1991; this paper). Ommatidia closer to the periphery ('pole') of a row are less mature than those progressively closer to the center ('equator'). We estimate that each ommatidial step toward the equator represents an approximately 10-15 minute step forward in developmental maturity.

Proneural groups elsewhere in the fly, defined by proneural gene expression, have been described as shifting from a larger (~15 cell) group to a single neuronal precursor (Skeath and Carroll, 1991; Cubas et al., 1991). The higher temporal resolution afforded by the equatorial-polar gradient allows for a uniquely detailed view of proneural development as it progresses toward R8 specification. We studied this process by examining *atonal* expression immunohistochemically as observed along the equatorial-polar axis. The details of this study are presented below and for purposes of reference are divided into three stages.

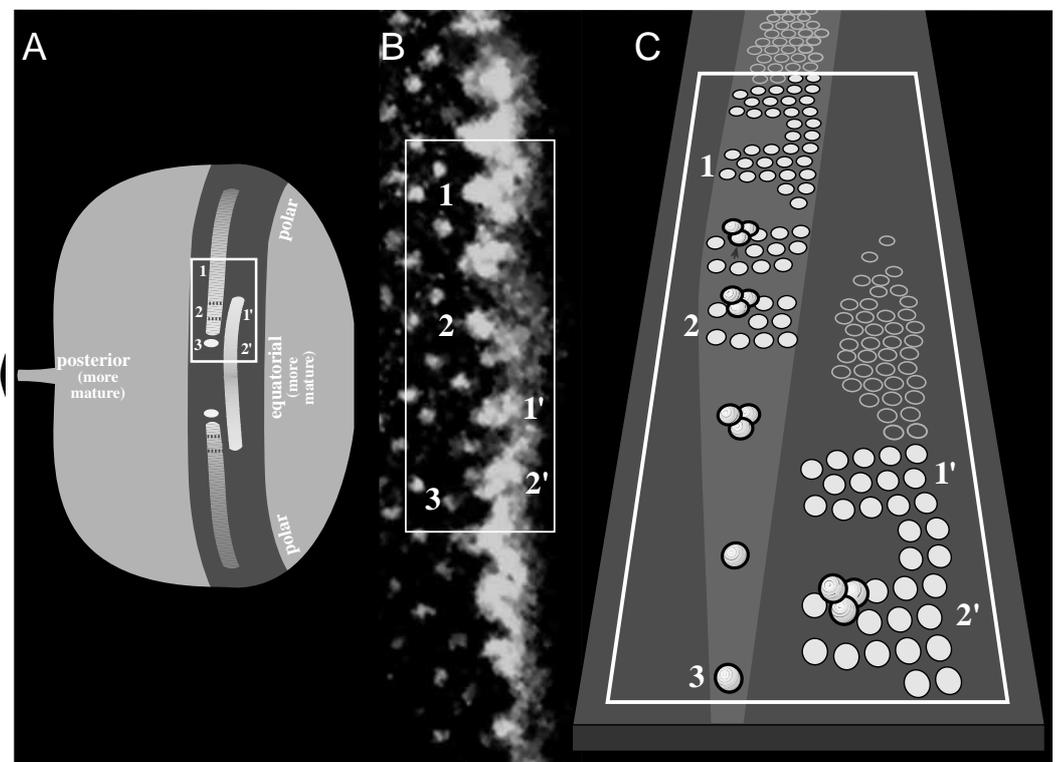
#### Stage 1

In the retina, *atonal* is first expressed within the MF (Jarman et al., 1993). Its expression pattern was analyzed in detail by exploiting the equatorial-to-polar gradient of developmental maturity within the MF (Fig. 1). In stage 1, diffuse *atonal* expression within the nuclei of all cells resolves to an approximately 15-cell cluster.

Initially, adjacent clusters are attached by a 3- to 4-cell 'bridge' of *atonal*-expressing nuclei, but this bridge is rapidly lost to create separate proneural clusters. Interestingly, this bridge represents the only cells within the MF to express both *atonal* and members of the *E(spl)* complex (see below). Initially, the nuclei of all cells within the MF are localized basally and remain so through stage 1. Within the cluster of basal *atonal*-expressing nuclei, the nuclei of 2-3 adjacent cells migrate apically. This step presages stage 2. These apically migrating nuclei belong to cells that are invariably positioned near the posterior of the cluster. Interestingly, apical nuclear migration typically signifies the initiation of a cell's differentiation in the retina (Tomlinson, 1985; Cagan and Ready, 1989b).

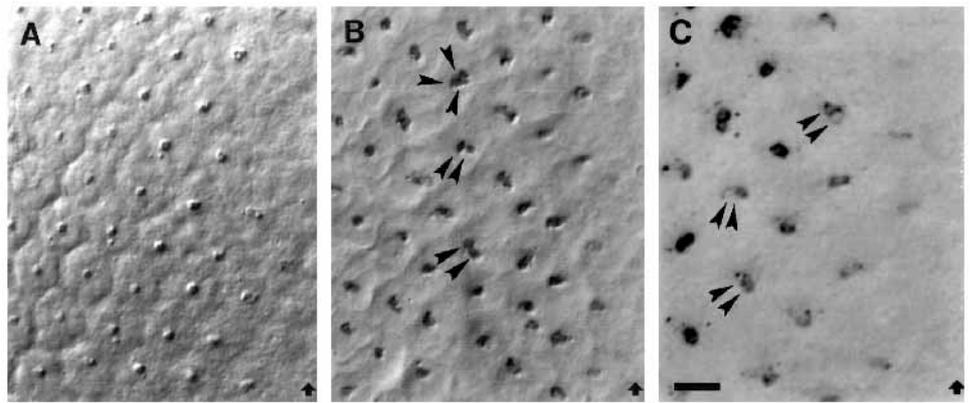
#### Stage 2

Within two rows of this apical migration, the remaining basal nuclei of the cluster lose *atonal* expression (stage 2).



**Fig. 1.** Atonal expression delineates proneural development in the larval eye disc. The developmental maturation of cell clusters forming the morphogenetic furrow progresses in two dimensions: anterior-posterior and equatorial-polar. (A) A schematic of an eye disc demonstrating the difference in Atonal expression pattern between the equatorial and polar (distal) regions of the morphogenetic furrow (dark gray). The boxed region is magnified in B and C, and contains two adjacent rows (two 'proneural cassettes'; see text) (B) A fluorescent microscopic view of Atonal expression within the morphogenetic furrow. The broader *atonal* expression found in the earliest (polar and anterior) expression, composed of approximately 15-cell 'proneural clusters', gives way to a smaller *atonal*-expressing R8 equivalence group and eventually a single *atonal*-positive cell (equatorial and posterior). This transition can be seen along the equatorial-to-polar axis of the two adjacent rows of Atonal expression. The rectangle encompasses a region of transition in both rows and is schematized in C. (C) A more detailed schematic of the three-step gradual maturation of Atonal expression based on direct fluorescent and confocal images. The plane of MF is rotated on the A-P axis to give a pseudo-3-D vision of the displacement of nuclei described below. Portions of two 'rows' of *atonal* expression are presented; only nuclei are shown for simplicity. After initial light, diffuse expression, Atonal becomes localized to a broad group of nuclei in the anterior most portion of the furrow (stage 1). Next, the nuclei of 2-3 adjacent cells displace apically; these cells form a proposed R8 equivalence group (stage 2). Finally, expression of Atonal becomes restricted to the future R8 photoreceptor (stage 3). Anterior in all panels is to the right.

**Fig. 2.** Boss expression as a marker of R8 photoreceptor neuron specification. Third instar larval eye discs stained with  $\alpha$ bossNT1 monoclonal antibody. Arrows mark the morphogenetic furrow in each panel. (A) In the wild-type disc, each ommatidium has a single R8 photoreceptor cell expressing the Boss protein in its apical region. In more mature, posterior rows this is accompanied by a small stained vesicle within the R7 photoreceptor cell precursor. (B) When flies carrying a heat-inducible *atonal* construct receive varying degrees of heat shock, 2-3 cells per ommatidium stably express Boss (arrowheads), indicating the higher vulnerability of the R8 equivalence group to ectopic *atonal* expression. This is unlikely to be the result of transient gene expression because a very late marker, BBO2, also displays a multiple R8 phenotype after ectopic *atonal* expression. Typically, one cell expresses the boss epitope more strongly than the other 1-2 stained cells; this difference is stable for at least 24 hours. Ommatidial patterning is slightly irregular compared with wild type. (C) A more penetrant, but similar phenotype is observed in flies lacking *rough* activity. Most ommatidia in *ro<sup>x63</sup>* flies contain multiple R8s. Boss staining is typically observed in one cell in the anterior-most ommatidia and then 1-2 more cells commence expression in the following rows (arrowheads). Ommatidial arrangement is much more aberrant in these flies than that in the transiently heat-shocked *hs-atonal* flies. Anterior is to the right. Bar in C, 5  $\mu$ m.



Expression remains in the 2-3 cells containing the apically migrating nuclei. These apical nuclear movements occurred prior to narrowing of *atonal* expression, suggesting *atonal* expression is not likely to define this group. Based on their posterior position within the proneural group, the 2- to 3-cell cluster observed in stage 2 appears to correspond to our previously postulated 'R8 equivalence group' of cells competent to differentiate as R8 (Cagan, 1993; see below). In the remainder of this paper, these cells will be referred to as the 'R8 equivalence group'.

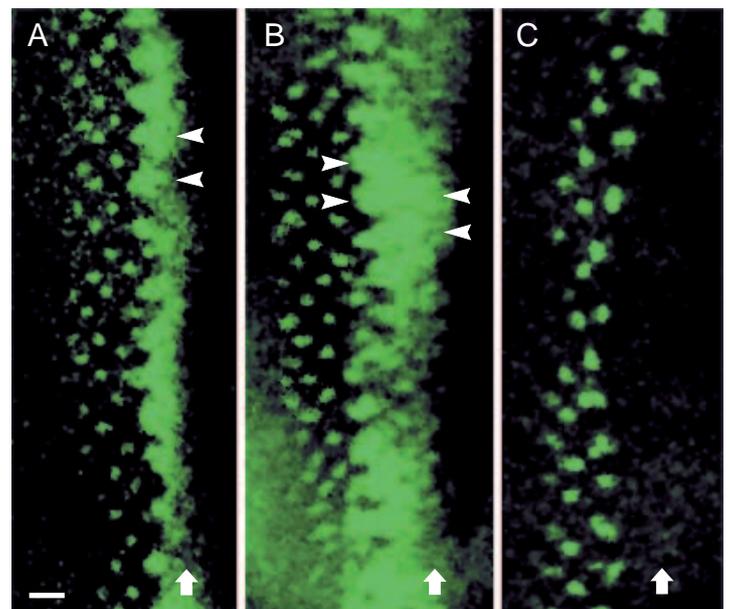
### Stage 3

Finally, of the 2-3 *atonal*-expressing nuclei observed in stage 2, 1-2 nuclei lose *atonal* expression and a single R8 precursor retains expression for several subsequent rows (stage 3). Stage 1 to stage 3 encompasses approximately 7 ommatidial units along the equatorial-to-polar axis, defining a 'proneural cassette'. Each proneural cassette is a patterned strip of maturing proneural clusters that has a symmetric counterpart across the equatorial midline (Fig. 1). A typical MF exhibits at least one pair of proneural cassettes containing a complete complement of stage 1-3 proneural clusters; this row is generally flanked by a more anterior, less mature row containing e.g., only stage 1 clusters.

### Ectopic *atonal* expression directs all cells of the equivalence group into the R8 pathway

The evolving expression pattern of *atonal* revealed a dynamic view of proneural maturation. To determine if this expression pattern is required for selection of a single R8 neuron, ectopic expression was induced throughout the developing eye disc. A single 15 minute pulse of *atonal* in *hsp70-GAL4; UAS-atonal/CyO* (*hs-atonal*) flies was sufficient to induce ectopic R8s in several ommatidia, as assessed by the R8-specific markers *boss* and *lacZ* expression in the BB02 enhancer trap line (not shown). Three 15 minute pulses of *atonal* resulted in 2-3 R8s in 46% of ommatidia scored near the MF ( $n=183$  ommatidia;

see Materials and Methods) (Fig. 2B, compare to wild-type in 2A). Multiple R8s were predominantly observed in ommatidial rows that were within the MF at the time of heat shock, as



**Fig. 3.** *Rough* negatively regulates *atonal* expression. Confocal projection images demonstrating *atonal* expression in (A) wild type, (B) *rough* and (C) *hs-rough* eye discs. (A) A wild-type eye disc for comparison. See legend for Fig. 1B. (B) *atonal* expression in a *ro<sup>x63</sup>* disc. *Atonal* staining expands both in the cluster and single-cell stages. Note that multiple clusters from adjacent rows overlap at certain regions, giving rise to extended regions of *atonal* expression (arrowheads). Although apparent in only some ommatidia in this image, *atonal* is expressed within 2-3 cells in nearly all mature ommatidia. (C) A view of *atonal* expression 3 hours after ectopic expression of *rough* in a *hs-rough* eye disc. Expression of *atonal* in stage 1 was completely blocked, though the single-cell expression of *atonal* remained in more mature clusters. This expression will be lost as the ommatidial clusters mature. Arrows mark the furrow. Anterior is to the right. Bar in A, 6  $\mu$ m.

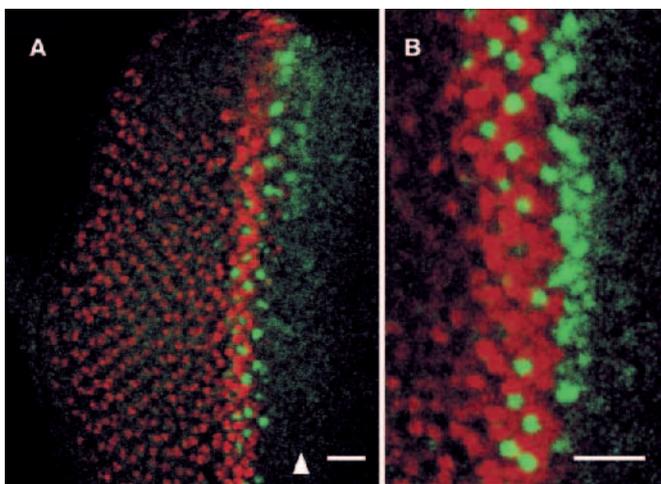
determined by their distance from the MF and time from the last heat-shock pulse to the fixation of tissue. No more than three R8s per ommatidium were ever observed even after ten 15-minute pulses. Based on the number and position of these transformed R8s near the posterior of the ommatidial cluster, they appear to correspond to the 2- to 3-cell R8 equivalence group observed in stage 2 (see above). Therefore, ectopic *atonal* expression blocked stage 3 in the selection of a single R8, indicating that the transition from stage 2 requires a narrowing of *atonal* expression. Surprisingly, cells outside the R8 equivalence group remained refractory to ectopic *atonal*, suggesting that changes in *atonal* expression alone may not be sufficient to narrow the larger proneural cluster.

#### Rough is a negative regulator of R8 specification

Transformation of all 2-3 cells within the R8 equivalence group into R8s by ectopic *atonal* expression is a phenocopy of loss-of-function mutations in several loci (Cagan, 1993). One notable example is *rough*. Previous work demonstrated that most *rough* ommatidia contain 2-3 R8 neurons as assessed with R8-specific markers (Fig. 2C, compare to wild-type in Fig. 2A). Using immunoelectron microscopy to assess their position within the early ommatidial cluster, these ectopic R8s were determined to arise from the R8 equivalence group (not shown). Thus, *rough* acts to inhibit the emergence of ectopic R8s during early ommatidial differentiation. This activity appears to be in opposition to the positive regulation of R8 specification provided by *atonal*.

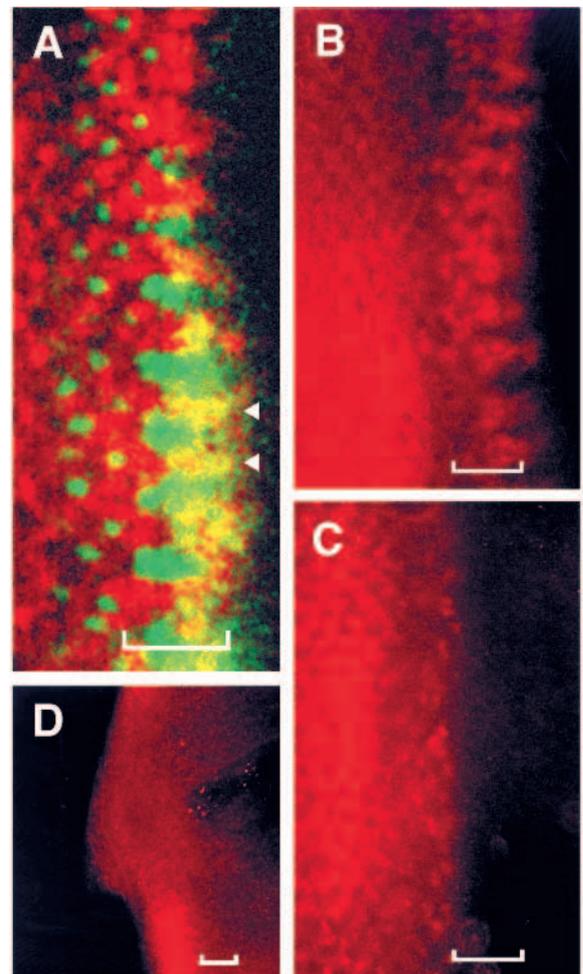
#### Rough is a negative regulator of *atonal* expression

To determine the earliest developmental step requiring *rough*



**Fig. 4.** Atonal and Rough are expressed in a non-overlapping, exclusive pattern within the MF. Confocal images of eye discs (A,B) double-labeled for Rough (red) and Atonal (green) demonstrate that each nucleus within the morphogenetic furrow expresses one or the other but never both (yellow). Atonal is expressed first within the furrow; when its expression narrows to a single cell, this cell is surrounded by Rough-expressing cells (*rough* is expressed further anterior than can be seen in this focal plane). Rough expression continues posteriorly beyond the furrow, eventually including photoreceptor neurons R2 and R5 and later R3 and R4.  $\alpha$ -*rough* antibody MAbr01 is a mouse monoclonal antibody (1:100). Arrowhead marks the furrow. Anterior is to the right. Bars 10  $\mu$ m.

activity, *atonal* expression was examined in *ro<sup>x63</sup>* (a null *rough* allele) eye discs. Loss of *rough* activity did not affect the initial formation of the proneural clusters during stage 1 (Fig. 3B, compare to wild-type expression in Fig. 3A); however, transition to the 2- to 3-cell stage (stage 2) was delayed 2-4 hours. The result of this delay was the presence of adjacent stage 1 proneural clusters in neighboring rows (arrowheads in Fig. 3B), an occurrence never observed in wild-type eye discs.



**Fig. 5.** Enhancer of split expression within the MF with relation to *atonal* expression (A) and in *hs-rough* (C) and *ato<sup>1</sup>* (D) backgrounds. (A) A confocal microscopic view of a wild-type MF double-labeled for Atonal (green) and E(spl) proteins (red) shows that, except for the 'bridges' (arrowheads) between early proneural clusters, the two expression patterns do not overlap. Posterior to the MF, E(spl) proteins are continued to be expressed in the 'lattice' region of the ommatidial clusters which is formed by the non-neuronal support cells. (B) Fluorescent microscopic view of E(spl) staining alone in the wild-type MF. (C) E(spl) expression three hours after ectopic Rough induction in the eye disc. The expression that corresponds to the earliest stage (within the MF) is abolished. (D) E(spl) expression in a homozygous *ato<sup>1</sup>* eye disc. The expression pattern is seen posterior to the furrow and is broad and homogeneous. The eye discs from these mutants are smaller and have a very rudimentary MF; the position of the MF was determined by *atonal* expression (not shown; note that *ato<sup>1</sup>* contains non-functional protein detectable by antibody). Brackets in all panels mark the approximate span of the MF and are 15  $\mu$ m long in A, B and C and 5  $\mu$ m long in D.

More dramatically, the 2- to 3-cell group failed to further resolve its *atonal* expression to a single cell. Hence, stage 3 did not occur, accounting for the additional R8s observed in *rough* mutants.

This delay in the resolution of *atonal* expression did not reflect a general delay in the rate of ommatidial maturation: other markers of differentiation such as *boss* and 22C10 (a neuron-specific antibody) were observed at their wild-type stage. Instead, overlap of *atonal* expression with *boss* expression increased from one row to three (not shown). Therefore, the additional R8s observed in a *ro<sup>x63</sup>* background is due to the failure to resolve *atonal* expression.

The expansion of *atonal* expression in a *ro<sup>x63</sup>* background suggests that *rough* is a negative regulator of *atonal*. To test this possibility further, ectopic *rough* expression was induced throughout the developing eye disc. Flies containing a *rough* cDNA fused to a heat-shock-inducible promoter received a single 90 minute pulse of *rough*. Ectopic *rough* expression prevented initiation of *atonal* expression (Fig. 3C, compare to wild type in Fig. 3A). Loss of *atonal* expression within the MF was apparent within 3 hours after induction of ectopic *rough*. Normal levels of Atonal protein were maintained in more mature clusters, suggesting either ectopic *rough* can block only initiation of *atonal* expression or that Atonal protein can perdure for several hours. Expression was not reinitiated in the MF for at least 24 hours (not shown). This is consistent with the previously described time course for reinitiation of the MF movement in eye discs receiving ectopic Rough (Kimmel et al., 1990). Interestingly, Atonal protein was detected at normal levels in the developing ocelli and antennal disc, indicating this block is retina-specific (not shown).

### Expression of Rough and Atonal is mutually exclusive

Previous histological work indicated *rough* and *atonal* were expressed broadly in the MF (Kimmel et al., 1990; Jarman et al., 1993). The antagonistic nature of their interactions raised the question as to whether their expression patterns overlapped. Fluorescent antibody double-labeling and confocal microscopy were used to compare expression in nuclei within the morphogenetic furrow (Fig. 4). The two patterns were found to be mutually exclusive: as *atonal* expression resolved from an initial ubiquitous stripe to individual proneural clusters, *rough* expression emerged in the intervening cells. As *atonal* expression further resolved to 2-3 cells and then to a single R8 precursor cell, *rough* expression concurrently expanded to include all *atonal*-negative cells. Eventually, *rough* was found in most or all cells within the posterior of the morphogenetic furrow except the single *atonal*-expressing R8 precursor cells. Although most nuclei within the furrow contained either *atonal* or *rough*, no nucleus was ever observed to contain both ( $n=5$  eye discs). These perfectly complementary expression patterns are also consistent with an opposing role for these two transcription factors.

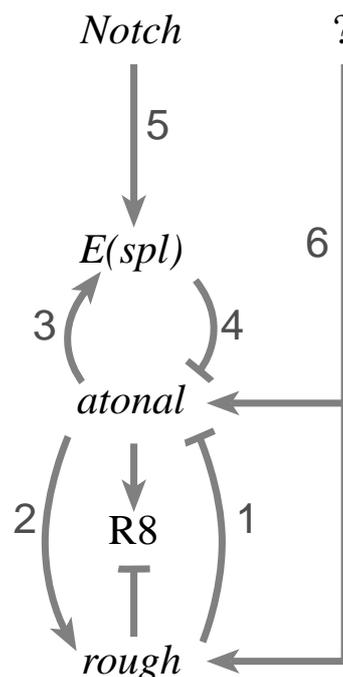
### *E(spl)* and *rough* expression are similar

One target of the proneural genes *achaete* and *scute* are members of the *E(spl)* locus, a complex of seven bHLH factors (Bailey and Posakony, 1995; Jennings et al., 1995; Heitzler et al., 1996). In turn, *E(spl)* genes act as negative regulators of

*achaete* and *scute* expression (reviewed in Campos-Ortega, 1993). Given its role in delimiting proneural gene expression, we examined the potential for *E(spl)* genes to also regulate *atonal*. Consistent with a role as a negative regulator of *atonal*, the expression pattern of several members of the *E(spl)* locus are complementary to *atonal* in the MF (Fig. 5A), a pattern similar to *rough* expression. The only difference between *rough* and *E(spl)* expression was a region of overlap between *E(spl)* and *atonal* at the 'bridge' between immature proneural groups (Fig. 5A).

Given their similarity of expression, Rough could repress *atonal* expression indirectly by activating members of the *E(spl)* complex. However, ectopic *rough* expression blocked initiation of *E(spl)* in addition to *atonal* within the MF (Fig. 5C, compare to wild-type in 5B). Rough is not likely to be a direct negative regulator of *E(spl)* expression since their expression patterns extensively overlap. Instead, Rough-induced loss of *E(spl)* expression may be due to loss of *atonal* expression in a manner analogous to *E(spl)* requirement for *achaete* and *scute* activity. Nevertheless, requirement for *atonal* activity in the retina is not absolute: *E(spl)* expression is lost within the MF of homozygous *ato<sup>1</sup>* (an allele deficient in the DNA-binding domain) eye discs, but broad unpatterned expression was observed posterior to the MF (Fig. 5D, compare to wild-type in 5B), again similar to observations for *E(spl)* in other tissues (Bailey and Posakony, 1995; Jennings et al., 1995; Heitzler et al., 1996). Thus, *E(spl)* activity requires *atonal* activity only within the MF. In contrast, *rough* expression was completely lost in an *ato<sup>1</sup>* background (not shown). A model for the interaction of these three transcription factors is presented in Fig. 6. The seven bHLH members of the *E(spl)* complex exhibit extensive redundancy of function (Knust et al., 1992), and a better understanding of the role of *E(spl)* in R8 specification awaits analysis of broad deletions.

**Fig. 6.** A model for interactions between Atonal, Rough, *E(spl)* and Notch. Proposed interactions are based on: (1) complementary *atonal* and *rough* expression patterns, expansion of *atonal* expression in a *ro<sup>x63</sup>* background and loss of *atonal* expression in the presence of ectopic *rough*; (2) loss of *rough* expression in *ato<sup>1</sup>* mutant background; (3) loss of *E(spl)* in *ato<sup>1</sup>* MF; (4) previously demonstrated interactions between *E(spl)* and other bHLH factors; (5) expansion of *atonal* expression in *N<sup>ts1</sup>* flies and previously demonstrated requirement for *Notch* activity by *E(spl)* and (6) the factors that initiate expression of *atonal* are not known. Rough may also require other factors.



### The *Notch* pathway is not required for the downregulation of *atonal* by *rough*

Previous work has demonstrated a role for the *Notch* signaling pathway during cell fate choice in the developing retina. The inductive ligand Delta provides signals to neighboring cells through direct binding of the Notch receptor, which in turn regulates transcription factors such as *E(spl)* and proneural bHLHs (Artavanis-Tsakonas et al., 1995; Bailey and Posakony, 1995; Jennings et al. 1994, 1995). In the retina, loss of Notch function results in expansion of *atonal* expression and ectopic R8s (Dietrich and Campos-Ortega, 1984; Cagan and Ready, 1989a; Baker et al., 1990; Lee et al., 1996; unpublished data). To determine if downregulation of *atonal* by *rough* is mediated through the Notch pathway, Notch signaling was disrupted using a temperature-sensitive allele of *Notch* (*N<sup>ts1</sup>*). Larvae were placed at 32°C for 4-6 hours and *atonal* and *rough* expression were assessed. Expression of *atonal* expanded dramatically to include large contiguous areas within the MF but expression of *rough* remained complementary, outlining the large *atonal*-expressing groups (not shown). In no case were both *atonal* and *rough* expression observed in the same cell. Therefore, the complementary pattern of *atonal* and *rough* in the MF is not mediated through Notch, nor does it require Notch activity. One important caveat is that *Notch* signaling may not be fully attenuated in heat-shocked *N<sup>ts1</sup>* flies.

## DISCUSSION

The placement and specification of most neurons examined in the developing nervous system of *Drosophila* requires transition from the proneural group to a single neuronal precursor. Recently, the proneural gene *atonal* was identified as encoding a bHLH transcription factor required for specification of the R8 neuron in the developing *Drosophila* retina (Jarman et al., 1994). We extend these observations by using *atonal* expression and the stepwise nature of ommatidial development to provide a closer look at the dynamics of proneural group resolution. Our evidence indicates at least three distinct steps in proneural maturation. Proper resolution of the dynamic *atonal* expression pattern is required to define a single R8 neuron. We also present evidence that resolution of this expression pattern is influenced by Rough, a homeobox-containing transcription factor which plays an opposing role to Atonal during R8 specification. Rough provides retina-specific negative regulation which is required to delimit Atonal expression. Thus, this paper provides evidence to support a significant role by at least one homeobox-containing gene during the earliest steps of neuronal differentiation.

### Expression of *atonal* demarcates a rapidly evolving proneural cluster

The morphogenetic furrow (MF) contains an equatorial-to-polar gradient of developmental maturity, which allows for a detailed examination of proneural resolution. This gradient is dramatically revealed by *atonal* expression. Expression has previously been described as occurring first in a stripe, then in clusters, and finally in the R8 neuron (Jarman et al., 1995). We extend this description to include an earlier, broader expression of *atonal*, which precedes discrete proneural clusters, and a later intermediate step in which 2-3 cells distinguish them-

selves from the proneural group. This latter distinction is first revealed by the apical migration of their nuclei, followed by continued *atonal* expression after *atonal* is no longer detected in the remaining cells of the proneural cluster. Importantly, although proneural gene expression is thought to define and regulate proneural competence, the nuclei of the R8 equivalence group migrate apically before a corresponding restriction of *atonal* expression. This suggests that the R8 equivalence group may not be defined by the progressive maturation of *atonal* expression. Perhaps post-translational modification or an additional factor(s) provide the necessary restriction.

Further evidence for the existence of the R8 equivalence group comes from the study of mutations in which cells of the R8 equivalence group are transformed into R8 neurons, including *scabrous* (Baker et al., 1990; Cagan, 1993), *Star*, *asteroid*, *Scutoid* (unpublished results) and *rough* (Heberlein and Rubin, 1991; Van Vactor et al., 1991; this paper). In addition, these cells are uniquely sensitive to ubiquitous *atonal* expression, which also resulted in a transformation limited to 2-3 R8 neurons. This suggests that neurons outside the R8 equivalence group are not sensitive to *atonal*. Either these cells lack a component of the *atonal* signaling pathway or, conversely, *atonal* activity is specifically blocked. Interestingly, *hairy* and *extramacrochaete*, implicated as negative regulators of *achaete* and *scute*, are also expressed in the retina. Expression of *extramacrochaete* occurs throughout the retina, whereas *hairy* expression is primarily just anterior to the MF. Loss of these two factors results in an anterior expansion of ommatidial development including expression of *atonal* (Brown et al., 1995). However, neither protein is expressed at appreciable levels within the MF (Brown et al., 1995) and therefore are not likely to contribute to the patterning of *atonal*. Perhaps the best candidate for an additional negative regulator within the MF is *E(spl)*, and we provide evidence in this paper that its expression pattern is complementary to *atonal* expression.

### Rough is a retina-specific negative regulator of *atonal*

Based on genetic mosaic results, *rough* has been implicated in specification of R2 and R5 (Tomlinson et al., 1988), the photoreceptor neurons that emerge immediately after R8. In this paper, we identify an additional earlier role for *rough* during R8 specification. Loss of *rough* activity results in ectopic R8s (Heberlein et al., 1991; Van Vactor et al., 1991) due to specific transformation of cells within the R8 equivalence group (this paper). Ectopic *rough* expression blocks R8 specification at least in part through suppression of *atonal*. Furthermore, its expression pattern is complementary to that of *atonal*. Therefore, *rough* is a negative regulator of *atonal* activity and R8 specification.

One critical question is the nature of the regulation between *rough* and *atonal*. The expression of *rough* was never observed to overlap *atonal*, consistent with non-autonomous regulation. The complementary patterns of *rough* and *atonal* expression were resistant to loss of *Notch* activity, which blocked the primary signaling pathway implicated in proneural resolution. Therefore, *rough* does not act on *atonal* through *Notch* signaling. Alternatively, *rough* may act by direct transcriptional regulation; assessing this possibility will require defining the *atonal* promoter followed by binding studies in vitro and

in vivo. Interestingly, ectopic *rough* expression results in a 'furrow stop' phenotype where the MF fails to progress anteriorly across the eye disc field (Basler et al., 1990; Kimmel et al., 1991). A similar 'furrow stop' phenotype occurs when *atonal* activity is lost in *ato<sup>1</sup>* mutants (Jarman et al., 1995), suggesting that one reason ectopic *rough* expression halts furrow progression may be its effect on *atonal*.

### Regulation of proneural bHLH factors by homeobox-containing genes

Rough expression and function are restricted to the developing eye (Tomlinson et al., 1988). In contrast, *atonal* is also expressed in the antennal and ocellar precursors (Jarman et al., 1995; unpublished results). Ectopic Rough does not affect *atonal* expression in these regions. This suggests the presence of another factor(s) that provides retinal specificity to *rough*. In addition, while *rough* expression is limited to the retina, *atonal* is also involved in proneural development of the chori-dotal organs in the embryo (Jarman et al., 1993). Therefore, Rough provides retina-specific regulation to the more extensive proneural function of *atonal*. Recently, the mammalian homeobox-containing protein Msx-1 was demonstrated to inhibit *myoD* transcription in cell culture (Woloshin et al., 1995). In addition, the expression patterns of the homeobox-containing factors Pax3 and Dlx-2 overlap with the bHLH factors MATH-1 (a mouse orthologue of *atonal*; Akazawa et al., 1995) and MASH-1 (a mouse orthologue of *achaete*; Porteus et al., 1994), respectively, in the mouse brain. Based on these findings, homeobox-containing and bHLH transcription factors have been proposed to interact during early neural and myogenic specification, possibly providing tissue-specific regulation. This paper provides the first in vivo evidence for such an interaction, suggesting that one role of homeobox-containing proteins is to delimit the more general bHLH pathway.

Another class of homeobox-containing genes have also been demonstrated to regulate bHLH factors in *Drosophila*. Members of the *Iroquois* locus directly regulate members of the *Achaete-Scute Complex (AS-C)* during proneural specification in the *Drosophila* wing disc (Gomez-Skarmeta et al., 1996). Unlike *rough*, these Hox factors act before initiation of *AS-C* expression and are direct positive regulators. This would appear to correspond to a developmental step before stage 1 in our description. Therefore, the *Iroquois* locus regulates establishment of the proneural region, while *rough* is involved in the later resolution process. It will be intriguing to determine if different classes of Hox genes provide regulation at separate steps during proneural maturation.

### Conclusion

In recent years, several important questions have emerged as to the mechanisms responsible for ommatidial establishment and maturation. Little is understood about the patterning by which clusters arise in well-spaced groups. Our evidence indicates that patterning begins at the equator and radiates distally; the factors responsible for this remarkable process are not known. Related to this is the question of which factors activate the patterned expression of *atonal* and *rough*. One candidate to activate *rough* expression is *atonal* itself (Fig. 6). *atonal* activity is required for *rough* expression (see above), but *rough* is not expressed in the one cell (R8) where *atonal*

activity is most stable. Therefore, *atonal* alone is not sufficient to fully regulate *rough*. Based on its similar expression pattern to *rough*, another candidate is *E(spl)*. One model suggests *atonal* activates *E(spl)*, which activates *rough*, which in turn regulates *atonal*. Alternatively, *atonal* may activate *rough* directly. Failure to activate *E(spl)* and *rough* may be sufficient to allow R8 differentiation.

Finally, a morphological observation raises an intriguing question about proneural maturation. Our studies provide evidence for a 2- to 3-cell R8 equivalence group; the earliest indication for segregation of this group is the apical nuclear migration observed within the MF. This apical movement is independent of *atonal* expression. What directs this nuclear movement in a manner independent of *atonal* expression? In studies of other proneural groups, stronger scute expression has been noted in the vicinity of the eventual sensory organ precursor (e.g., Cubas et al., 1991). Therefore, the equivalence group may prove a general feature of proneural cluster formation.

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