The control of cell fate in the embryonic visual system by \textit{atonal}, \textit{tailless} and EGFR signaling

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SUMMARY

We describe here the role of the transcription factors encoding genes \textit{tailless} (\textit{tll}), \textit{atonal} (\textit{ato}), \textit{sine oculis} (\textit{so}), \textit{eyeless} (\textit{ey}) and \textit{eyes absent} (\textit{eya}), and EGFR signaling in establishing the \textit{Drosophila} embryonic visual system. The embryonic visual system consists of the optic lobe primordium, which, during later larval life, develops into the prominent optic lobe neuropiles, and the larval photoreceptor (Bolwig’s organ). Both structures derive from a neuroectodermal placode in the embryonic head. Expression of \textit{tll} is normally confined to the optic lobe primordium, whereas \textit{ato} appears in a subset of Bolwig’s organ cells that we call Bolwig’s organ founders. Phenotypic analysis, using specific markers for Bolwig’s organ and the optic lobe, of \textit{tll} loss- and gain-of-function mutant embryos reveals that \textit{tll} functions to drive cells to optic lobe as opposed to Bolwig’s organ fate. Similar experiments indicate that \textit{ato} has the opposite effect, namely driving cells to a Bolwig’s organ fate. Since we can show that \textit{tll} and \textit{ato} do not regulate each other, we propose a model wherein \textit{tll} expression restricts the ability of cells to respond to signaling arising from \textit{ato}-expressing Bolwig’s organ pioneers. Our data further suggest that the Bolwig’s organ founder cells produce Spitz (the \textit{Drosophila} TGF\textit{\alpha} homolog) signal, which is passed to the neighboring secondary Bolwig’s organ cells where it activates the EGFR signaling cascade and maintains the fate of these secondary cells. The regulators of \textit{tll} expression in the embryonic visual system remain elusive, as we were unable to find evidence for regulation by the ‘early eye genes’ \textit{so}, \textit{eya} and \textit{ey}, or by EGFR signaling.

Key words: \textit{Drosophila} brain, Tlx, Optic lobe, Bolwig’s organ

INTRODUCTION

The development of the adult \textit{Drosophila} eye from the eye imaginal disc epithelium has been described in detail, starting with the posterior-to-anterior sweep of the morphogenetic furrow across the disc, followed by the sequential commitment of specific cell types to individual ommatidia (reviewed by Freeman, 1997). Prior to these steps, there is commitment of cells to form the eye primordium; this process is regulated by a group of transcription-factor-encoding genes, \textit{eyeless} (\textit{ey}; Quiring et al., 1994), \textit{sine oculis} (\textit{so}; Cheyette et al., 1994), \textit{eyes absent} (\textit{eya}; Bonini et al., 1993; Pignoni et al., 1997) and \textit{dachshund} (\textit{dac}; Chen et al., 1997; reviewed by Desplan, 1997). The best way to describe the function of these genes is that they render the expressing cells competent to become part of the compound eye with its structurally and functionally diverse cell types. The first gene expressed in the eye disc that conveys a specific cell fate is \textit{atonal} (\textit{ato}), expressed in the R8 precursor (Jarman et al., 1994; 1995). Subsequently, different signals emanating from R8 induce neighboring cells to become photoreceptors and support cells. Prominent among these signals is the \textit{Drosophila} TGF\textit{\alpha} homolog Spi, activating via EGFR the Ras signaling cascade, which is required for the differentiation of all ommatidial cells (reviewed in Dominguez et al., 1998; Freeman, 1997; Schweitzer and Shiloh, 1997). The proteins mediating EGFR signaling form a highly conserved molecular network. The proteins encoded by two genes, \textit{rhomboid} (\textit{rho}) and \textit{Star} (\textit{S}), which are expressed by the signaling cells, activate the signal, Spi (Schweitzer et al., 1995a; Golembo et al., 1996a). In cells receiving the Spi signal, the Ras pathway is activated and \textit{argos} (\textit{aos}), which encodes a secreted protein inhibiting further EGFR activation by Spi (Schweitzer et al., 1995b; Golembo et al., 1996b), is expressed. The Ras pathway activates the transcriptional regulators \textit{pointed} (\textit{pnt}) and \textit{inhibits yan} (Klaes et al., 1994; Gabay et al., 1996).

Little is known about the earliest steps in cell commitment in the \textit{Drosophila} visual system, i.e., the pathways functioning in the embryo to commit cells to different components of the visual system: eye imaginal disc, optic lobe (the target in the brain of axons arising in the eye disc) and Bolwig’s organ (the larval light-sensing organ). It has been shown previously that both \textit{so} and \textit{tll} (also a transcription factor encoding gene) are expressed in the embryonic optic lobe before it invaginates (Cheyette et al., 1994; Rudolph et al., 1997). While lack of \textit{so} expression has been shown to result in a failure of invagination of the optic lobe and formation of the Bolwig’s organ (Cheyette et al., 1994), the effect of lack of \textit{tll} activity on visual system development has not yet been investigated. The function of \textit{ato}
in the embryonic visual system, if there is any, has also not been analyzed.

The investigation of the interrelationships among these genes and their relative roles in development of the *Drosophila* visual system is of general interest, given that vertebrate homologs (or families) are known for each of the *Drosophila* genes ey, eya, so, *tll*, and *ato*, namely Pax 6 (small eye, aniridia; Quiring et al., 1994), *Eya* (Xu et al., 1997), *Six* (Oliver et al., 1995), *Tlx* (Yu et al., 1994; Monaghan et al., 1995), and *Math5/Math5* (Gradwohl et al., 1996; Takeyabashi et al., 1997; Kanekar et al., 1997; Brown et al., 1998). All of these vertebrate homologs are expressed during embryogenesis in parts of the brain and the eye primordium. It seems likely that networks of gene activity regulating cell commitment in the visual system are conserved between arthropods and vertebrates; thus further elucidation, using genetic analysis in *Drosophila*, of the functions and interactions of these four genes might contribute to an understanding of eye development generally.

We have carried out an investigation into the role of *tll* and *ato* in development of the *Drosophila* embryonic visual system. We describe here the expression of these genes in the embryonic head region and describe the effect of loss of *tll* function, gain of *ato* function, and loss of *ato* function on development of the optic lobe and Bolwig’s organ. Our results indicate that *ato* acts as the proneural gene for a subset of photoreceptors, which we call Bolwig’s organ founders. These cells signal adjacent cells to also become part of the Bolwig’s organ. As stated above for the compound eye, part of the signaling mechanism originating from Bolwig’s organ founders is Spi and the EGFR cascade. In the cells that are in reach of the signal, there is a Bolwig’s organ versus optic lobe cell fate choice that crucially depends on *tll*. Thus, *tll* expression drives cells toward the optic lobe fate, and loss of *tll* function results in a strong increase in the number of Bolwig’s organ cells.

**MATERIALS AND METHODS**

**Fly stocks**

Oregon R flies were used as the wild-type stock. The following mutations, which are described in Lindley and Zimm (1992), if not otherwise indicated, were used in this study: *tll* 

1. **FGF** and hs-*tll* (Steingrimsson et al., 1991); *EGFR*1 (fgf17) kindly provided by Dr U. Banerjee; *spf* (spf1A, kindly provided by Dr U. Banerjee); the *argos* mutant *aso1* (Freeman et al., 1992; kindly provided by Dr C. Klambt); *rho* (kindly provided by Dr E. Bier); hs-*rho* 1c (kindly provided by Dr E. Bier); a PlacZ insertion in *pnr* (Klaes et al., 1994; kindly provided by Dr C. Klambt); a PlacZ insertion in *sine oculis* (so; Cheyette et al., 1994); the deficiency *Df(3L)H99* (White et al., 1994); the atonal mutant *ato*1 (Jarman et al., 1994; kindly provided by Dr Y. N. Jan). The *sine oculis* mutant *so* (Cheyette et al., 1994) and *eyes absent* mutant *eya* (Pignoni et al., 1997) were kindly provided by Dr F. Pignoni. The following driver lines and UAS constructs were kindly provided by Dr U. Banerjee: *rho-Gal4* (Schweitzer et al., 1995a); *sea-Gal4* (Bloomington stock center); UAS-*Spi* (Schweitzer et al., 1995a) and UAS-activated *EGFR* (Queenan et al., 1997). Egg collections were done on yeasted apple juice agar plates. Embryonic stages are given according to Campos-Ortega and Hartenstein (1997).

**Immunohistochemistry**

Embryos were dechorionated and fixed in 4% formaldehyde containing PT (1% PBS, 0.3% Triton X-100) with heptane. Embryos were then deviulinized in methanol and stored in ethanol prior to labeling with antibody, following the standard procedure (Ashburner, 1989). Expression of β-galactosidase (β-Gal) in enhancer trap lines was detected with a polyclonal anti-β-galactosidase antibody (Cappel) at a dilution of 1:2000. A monoclonal anti-FasII (Grenningloh et al., 1991) antibody was used at 1:80 dilution to detect FasII. A commercial monoclonal antibody against activated MAPK (dp-ERK, available through Sigma), used at a dilution of 1:1000, was used to visualize the embryo domains in which the Ras signaling pathway was activated (Gabay et al., 1997a,b). Anti-Elav (Robinow and White, 1991) antibody was used at a dilution of 1:100 and monoclonal antibody mAb22C10 (Zipursky et al., 1984; kindly provided by Dr S. Benzer) was used at a dilution of 1:200.

**In situ hybridization**

Embryos were dechorionated and fixed in PBS containing 5% formaldehyde and 50 mM EGTA and stored in ethanol. They were then treated with xylene and fixed for a second time in PBS containing 0.1% Tween-20 and 5% formaldehyde. The embryos were then hybridized with probes synthesized using digoxigenin-labeled UTP (Boehringer) according to standard protocol (Tautz and Pfeifle, 1989). Embryos labeled with DNA probes were treated with Proteinase K (50 μg/ml) followed by glycine (2 mg/ml) prior to hybridization. The digoxigenin-labeled probes described below were hybridized to fixed embryos in buffer containing 50% formamide at 55°C. Anti-digoxigenin antibody (Boehringer) was used according to the manufacturers’ instructions to detect hybridized probe, following which the embryos were dehydrated in ethanol and embedded in Epon. Alternatively embryos were washed and incubated with a second antibody for immunohistochemistry following in situ hybridization.

**Probes**

tc1 pBS containing *tll* (Pignoni et al., 1990, Steingrimsson et al., 1991) was digested with EcoRI to make DNA probe and linearized with SalI to make the RNA probe. pBS containing *argos* was used to make DNA probe (Freeman et al., 1992; kindly provided by Dr M. Freeman). Plasmid pBS-pF3k (Cheyette et al., 1994) linearized with BamHI and used as template to synthesize the *so* RNA probe. pBS:eya I (Bonini et al., 1993; kindly provided by Dr F. Pignoni) plasmid linearized with SalI and used as template to make the *eya* RNA probe. cDNA containing *ey* (kindly provided by Dr F. Pignoni) was linearized with SalI and used as template to make the *ey* RNA probe. cDNA containing *rho* (Bier et al., 1990; kindly provided by Dr U. Banerjee) was linearized with HindIII and used as a template to make *rho* RNA probe. pBS containing *ato* (kindly provided by Dr Y. N. Jan) was linearized with BglII and transcribed to make the *ato* probe.

**RESULTS**

*tlI* is dynamically expressed in the embryonic visual system

The *Drosophila* visual system comprises the adult compound eye, the larval eye (Bolwig’s organ) and the optic lobe. All of these components are recognizable as separate primordia during late stages of embryonic development (Green et al., 1993; Younossi-Hartenstein et al., 1993). Previous analysis of their early development, however, reveals that they originate from a small, contiguous region in the dorsal head ectoderm. During the extended germband stage, the individual components of the visual system can be distinguished morphologically as well as by spatially localized expression of the homeobox gene *so* (Cheyette et al., 1994) and the adhesion molecule Fas II (Grenningloh et al., 1991). Initially centered as an unpaired, oval domain straddling the dorsal midline (Fig. 1A), the anlage of the visual system subsequently elongates in the transverse axis and narrows in the anteroposterior axis. By late gastrulation (stage 8), the
anlage occupies two bilaterally symmetric stripes anteriorly adjacent to the cephalic furrow (Fig. 1B). The domain of so expression at this stage contains two regions with a high expression level (ol\textsubscript{ex} and ol\textsubscript{in} in Fig. 1). Only these two regions will ultimately give rise to the optic lobe and Bolwig’s organ; the so-positive cells dorsally and posterior to these domains will form part of the dorsal posterior head epidermis (dph in Fig. 1A,B) or undergo apoptotic cell death.

During the extended germband stage, the anlage of the visual system expands further ventrally until, around stage 10, it reaches the equator (50% in the dorsoventral axis) of the embryo (Fig.1D). Shortly thereafter, ol\textsubscript{in}, the portion of the anlage that will give rise to most of the optic lobe and Bolwig’s organ, reorganizes into a placode of high cylindrical epithelial cells that differ in shape from the surrounding more squamous cells of the head ectoderm (Fig. 1F). During stage 12, this placode starts to invaginate, forming a V-shaped structure with an anterior lip (ol\textsubscript{a}) and a posterior lip (ol\textsubscript{p}). Bolwig’s organ, which consists of a small cluster of sensory neurons, derives from the basal part of the posterior lip and can be recognized during stage 12 as a distinct, dome-shaped protrusion (Fig. 1G, see also Fig. 3A). During stage 13, invagination of the optic lobe separates it from the head ectoderm; only the cells of Bolwig’s organ remain in the ectoderm (Fig. 1G). The ectodermal region ol\textsubscript{ex} (Fig. 1F) is also internalized and forms an external ‘cover’ of the optic lobe; many cells of this population undergo apoptosis.

The \textit{tll} gene is expressed in a dynamic pattern in the protocerebral neuromere (Pignoni et al., 1990; Younossi-Hartenstein et al., 1996a; Rudolph et al., 1997). Posteriorly, this region overlaps part of the anlage of the visual system (red outlining of cells in Fig. 1A-D), in particular that part that will give rise to the anterior lip of the optic lobe. The anterior lip of the optic lobe upregulates expression of \textit{tll} during stage12 (Fig. 1E,F). In addition, the posterior lip of the optic lobe, which had not expressed \textit{tll} at an earlier stage, now switches on this gene (Fig. 1E,F). Expression of \textit{tll} in the posterior lip is patchy, with some cells expressing the gene at a higher level than others. The Bolwig’s organ primordium does not express \textit{tll}. During later embryonic stages (13-16) and during larval development (Fig. 1H), \textit{tll} expression remains strong in the optic lobe, but is never detected in the Bolwig’s organ. Also the primordium of the eye disc, which expresses \textit{tll} during larval stages (Fig. 1I), is devoid of this expression during embryonic development.

\textit{tll} controls a switch between optic lobe and Bolwig’s organ cell fate

Loss of zygotic \textit{tll} activity results in an absence of most of the protocerebrum of the brain (Younossi-Hartenstein et al., 1996a). In addition, the visual system of the late \textit{tll} embryo shows a dramatic phenotype, namely the transformation of optic lobe into Bolwig’s organ (Fig. 2G). In wild-type embryos, the neuronal marker 22C10 labels 12 neurons and their axons that project towards the optic lobe. In \textit{tll} mutant embryos, the number of cells in the Bolwig’s

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Fig. 1. \textit{tll} expression in the developing visual system. The different domains of the visual system at different stages are shown schematically as blue and green shaded areas in A (stage 7), B (stage 8), D (stage 10), and F (stage 12). All panels show lateral views of the posterior part of the head ectoderm; the apical outline of ectodermal cells is indicated by grey or red lines. The boundaries of the domains of the visual system are based upon the expression pattern of \textit{sine oculis} and other genes expressed in the visual system, in particular Fas II. Red outlines reflect cells expressing \textit{tll} detected by in situ hybridization. (C,E) Photographs of embryos double labeled with \textit{tll} probe (blue) and anti-β-Gal to detect galactosidase driven in the \textit{sine oculis} pattern (brown). (C,D) During stage 10, \textit{tll} expression overlaps with the anterior part of the visual system that later gives rise to the anterior lip of the optic lobe. (E,F) At stage 12, \textit{tll} is also turned on in the posterior lip of the optic lobe (ol\textsubscript{p}; in E and F), but not the Bolwig’s organ (Bo; green in F). All embryos are shown with anterior to the left and dorsal up. (G) In the third larval instar, \textit{tll} is expressed in the optic lobe (ol) of the larval brain. At that stage, \textit{tll} is also expressed in the eye antennal disc (I), both in the antennal (an) and in the eye portion. (G) Diagram showing the development of the visual system during stages 12-15 (from Cheyette et al., 1994). The optic lobe placode (red shading; ol) invaginates from the head ectoderm to form a vesicle that attaches to the brain (grey shading). Bolwig’s organ (yellow; Bo) remains in the head epidermis. The eye disc primordium (blue; eye) forms part of the head epidermis anteriorly adjacent to the invaginating optic lobe.

Other abbreviations: cf, cephalic furrow; dph, dorsal posterior head epidermis; ol\textsubscript{ex}, external fold of optic lobe.
organ is dramatically increased (by a factor of 2-3, Fig. 2H,G), while the optic lobe, marked by anti-Crumbs (Fig. 2J) or a P\textsubscript{lac}Z insertion in so (data not shown), is absent. Use of antibodies to FasII and Crb, which label the apical surface of the optic lobe, and not that of the Bolwig’s organ, allowed us to analyze how the phenotype unfolds in the \textit{tll} mutant embryo. Abnormalities first become apparent during stage 11, when FasII expression increases strongly in the domain of the anterior lip of the optic lobe placode (Fig. 2F), which normally ceases to express FasII (Fig. 2A). In spite of this abnormal expression, the optic lobe placode appears to invaginate normally. As a result, in stage 13 \textit{tll} mutant embryos, a Crb-positive vesicle can be seen subjacent to the head epidermis (Fig. 2I, compare to the wild type in Fig. 2D). During stage 14, all cells of this aberrant vesicle activate expression of the neuronal marker 22C10 (Fig. 2H) and lose Crb expression (Fig. 2J), revealing that these cells are Bolwig’s organ cells. Overexpression of \textit{tll} under the control of a heat-shock promoter has an effect opposite to that seen in the absence of \textit{tll} activity. In stage 11 embryos pulsed with ectopic \textit{tll}, no Bolwig’s organ developed, as shown by the absence of 22C10 expression (Fig. 2N). In these ectopic \textit{tll} embryos, strong expression of FasII (Fig. 2M) and \textit{so} P\textsubscript{lac}Z (data not shown) indicates that cells of the optic lobe placode are present and have an optic lobe (as opposed to Bolwig’s organ) fate. An additional consequence of \textit{tll} overexpression is that the optic lobes become located more dorsally and fused in the dorsal midline (Fig. 2L). This ‘cyclops’ phenotype most likely arises because the dorsomedial cells (\textit{dph} in Fig. 1), which normally die or form part of the head epidermis, now express optic lobe markers and become an integral part of the optic lobe. This interpretation is supported by the fact that the stage during which ectopic \textit{tll} has a maximal effect, 5-7 hours after fertilization, is the time at which the dorsomedial cells normally turn down \textit{so} expression and start to undergo cell death.

We have shown that \textit{tll} is expressed during specification of different fates within the anlage of the visual system. The results of both loss and gain of \textit{tll} expression are consistent with the interpretation that \textit{tll} is required to drive cells of the anlage, which would otherwise become photoreceptor neurons of Bolwig’s organ, to develop as optic lobe cells.

\textit{ato} specifies Bolwig’s organ fate
\textit{at}onal (\textit{ato}) encodes a bHLH transcription factor expressed in
and required for the development of the chordotonal organs (Jarman et al., 1993) and the compound eye (Jarman et al., 1994). In the primordia of these organs, *ato* is expressed in only a subset of the neuronal precursors: in the embryo, *ato* mRNA is detected in three of the five cells that will form the lateral chordotonal organ of each abdominal segment; in the eye disc, *ato* is expressed exclusively in R8, the first of the eight photoreceptors to be established during eye development. Inductive cell-cell interactions between the *ato*-expressing cells and their neighbors later recruit additional chordotonal neurons and photoreceptors, respectively (Jarman et al., 1995; Lage et al., 1997; Okabe and Okano, 1997).

A similar requirement for and expression pattern of *ato* is observed in Bolwig’s organ. *ato* is expressed in the head in several small cell clusters, one of which is a group of three to four cells that is part of the Bolwig’s organ primordium. Expression of *ato* in this domain begins during stage 11 (Fig. 3E) and continues until stage 12 (Fig. 3B). Initially, a group of 6-8 cells faintly expresses *ato*. By stage 12, their number has gone down to 3 cells. During this period, *ato*-expressing cells can be seen as a small group of cells within the dome-shaped Bolwig’s organ primordium. Loss of *ato* function results in the absence of Bolwig’s organ (Fig. 3D). Thus, similar to what has been demonstrated for the compound eye, even though only a small subset of photoreceptors actually expresses *ato*, lack of *ato* function results in absence of all photoreceptors.

Since Bolwig’s organ is enlarged in a *ttl* mutant background, we asked whether *ttl* inhibits *ato* expression. We found, however, that the number and pattern of *ato*-positive cells in *ttl* mutants is normal (Fig. 3F). These results suggest that *ttl* functions in parallel with, or downstream of *ato* in the development of the Bolwig’s organ/optic lobe primordium. We present in the Discussion a model for how *ttl* might act downstream of *ato* to affect the commitment of cells in this primordium.

**EGFR signaling is involved in the maintenance of the optic lobe placode and inductive signaling in Bolwig’s organ**

We showed previously that EGFR is activated in midline regions of the head neurectoderm, in particular in the anlage of the visual system (Dumstrei et al., 1998). Moreover, increased and/or ectopic activation of EGFR results in a ‘cyclops’ phenotype very similar to what we describe here for ectopic *ttl* expression. EGFR signaling has been shown to be required in both chordotonal organs (Lage et al., 1997; Okabe et al., 1997) and compound eye (Tio and Moses, 1997; Freeman, 1996; Dominguez et al., 1998; Spencer et al., 1998) for the inductive signaling triggered by *ato* expression. We investigated two questions raised by these observations.

(I) Is EGFR signaling required for *ttl* expression in the optic...
lobe? We examined 
tll expression in embryos either lacking 
EFGR or in which EGFR was ectopically activated by means of 
a rho Gal4 and sca Gal4 driver line (Brand and Perrimon, 1993). 
In EGFR− embryos, tll expression is unaltered, indicating that 
EGFR signaling is not required to either activate or maintain tll 
expression in the optic lobe (data not shown). Consistent with 
this result, ectopically activated EGFR in the dorsal head does 
ot result in ectopic expression of tll in the posterior 
position where the cyclopic optic lobe will appear; a 
small patch of tll-expressing cells does appear, 
however, further anteriorly in the dorsal midline (data 
not shown). We conclude that EGFR signaling does not play a role in regulation of tll expression in the 
optic lobe.

(ii) Is EGFR signaling involved in the recruitment of the secondary (non-atonal-expressing) Bolwig’s organ cells? To investigate this question, we tested for 
the presence of EGFR-relevant mRNAs or proteins: 
rho mRNA, which would be expected to be present only 
in the signaling cells, and phosphorylated 
MAPK, pointed and argos mRNAs, which would be 
expected to be expressed in all cells receiving an 
EGFR-mediated signal. In stage 12 embryos, rho is 
expressed only in the small group of Bolwig’s organ 
founder cells (the same cells expressing ato; Fig. 4C). 
In contrast, activated (phosphorylated) MAPK is 
present in a larger cluster of cells including the entire 
Bolwig’s organ and part of the adjacent optic lobe 
(Fig. 4A). Consistent with this, pnt and aos, both 
known to be switched on in cells receiving the Spi 
signal, are expressed at the same stage throughout the 
entire Bolwig’s organ primordium (data not shown).

These gene expression and MAPK activation 
patterns are consistent with the idea that the Spi signal 
is activated by rho in the Bolwig’s organ founders and 
passed to the neighboring secondary Bolwig’s organ 
cells where it activates the EGFR signaling cascade. 
Supporting this view, only 3–4 photoreceptor neurons 
are found in the Bolwig’s organ of embryos lacking 
rho or spi (Fig. 4D); furthermore, the size of the 
posterior lip of the optic lobe is reduced in such 
embryos (data not shown). The fact that absence of 
secondary Bolwig’s organ cells in rho or spi mutant 
embryos can be rescued by blocking cell death in the 
background of a deficiency that takes out the reaper 
complex of genes (Df(3L)H99; White et al., 1994; 
Fig. 4E) indicates that the Spi signal is not necessary 
for the specification of secondary Bolwig’s organ 
cells, but for their maintenance.

**Interaction between tll and the ‘early eye 
genes’ so, eya and ey in the embryonic 
visual system**

While the maternal patterning systems that regulate 
tll during its blastoderm expression have been 
determined (Pignoni et al., 1992), the genes required 
to turn on tll at a later stage in the visual system are 
not known. Candidates are the ‘early eye genes’ so, 
eya and ey, which encode transcription factors 
expressed in the embryonic visual system and in the 
larval eye disc in front of the morphogenetic furrow.

We analyzed expression of these genes in the visual system 
anlage (Fig. 5), and examined tll expression in embryos mutant 
for each of these genes. Our results reveal that tll expression 
in the optic lobe does not depend on any of these three genes. 
Further, we conclude that ey and so, which have been shown 
to interact with each other during eye disc determination 
(Pignoni et al., 1997), must act independently in embryonic
visual system development, since they are expressed in those primordia in non-overlapping patterns (Figs 3A, 5A-C).

Although so is expressed initially in the entire visual system anlage (Cheyette et al., 1994), during later stages its expression becomes increasingly restricted to subsets of visual system progenitors. Thus, during stage 11, when a morphologically distinct optic lobe placode first becomes visible, the domain of so expression retreats to the posterior lip of this placode; slightly later its expression is limited to only the Bolwig’s organ, where it is maintained until stage 13 (Fig. 3A). eya expression is initiated during the late blastoderm stage in a trapezoidal field in the dorsomedial head region that includes the visual system anlage, as well as progenitors of the medial brain (Bonini et al., 1998). Beginning during gastrulation (stage 6/7), the eya domain becomes divided into an anterior stripe and a narrow posterior stripe immediately anterior to the cephalic furrow that widens laterally; this posterior domain will become part of the posterior lip of the optic lobe, including Bolwig’s organ (Fig. 5I). eya expression continues in the optic lobe until stage 12 and in Bolwig’s organ until stage 13 (Fig. 5J,K).

Embryos that lack either so or eya exhibit defects in the portions of the visual system where these genes are expressed. In both mutants, development proceeds normally until stage 11, when the posterior lip of the optic lobe (olp) would normally start to invaginate. In eya and so embryos, invagination of the optic lobe placode does not take place and differentiation markers characteristic of the lobe (such as 22C10 for Bolwig’s organ) are not expressed (Cheyette et al., 1994; Bonini et al., 1998; data not shown). To investigate a possible role of so and eya in regulating tll, we labeled so (Fig. 6) and eya (data not shown) mutant embryos with a tll probe and anti-FasII antibody. At late stage 11 in both mutants, tll is expressed in both the anterior and posterior lips of the optic placode, but not the Bolwig’s organ (Fig. 6). At later stages, tll expression ceases in the posterior lip, as this structure fails to differentiate/dies. By contrast, tll expression in the anterior lip continues at a high level (Fig. 6). These results indicate that tll expression in the visual system does not depend on so or eya.

eya is expressed in the eye imaginal disc primordium. In contrast to previous reports (Quiring et al., 1994), we find that eya is not expressed in the optic lobe or Bolwig’s organ at any stage of embryogenesis. During early stages, eya is expressed only in groups of neuroblasts in the ventral nerve cord and brain (Fig. 5A,B); in the latter, the eya-positive neuroblasts include those that will form the mushroom body (A. Noveen, A. Daniel and V. H., unpublished data). Starting at late stage 12, eya is expressed in the primordium of the eye imaginal disc (Fig. 5C). At this stage, the eya-expressing disc primordium forms a stripe in the head ectoderm anteriorly adjacent to Bolwig’s organ. Double labeling with anti-Fas II and eya cDNA clearly demonstrates that eya is not expressed in Bolwig’s organ (Fig. 5D). eya continues to be expressed in the eye disc primordium throughout the remainder of embryonic and larval development (data not shown).

In conclusion, we can show that so and eya, although expressed coincidental with tll, are not required for its activation. eya plays no role in the embryonic visual system.

DISCUSSION

Specification of the larval and adult eye

We have analyzed the genetic mechanism that specifies the embryonic visual system of Drosophila. Between the onset of gastrulation (stage 6) and the late extended germ band stage (stage 11), the complex and partially overlapping expression patterns of transcriptional regulators (so, eya, tll, ato) and the EGFR signaling pathway define several distinct domains with different morphogenetic behavior and differentiative capacities. These domains are the anterior, posterior and external lip of the optic lobe and the Bolwig’s organ. In this paper, we have focussed upon the function of tll, ato and the EGFR signaling pathway in the specification of Bolwig’s organ. Our results are consistent with, and can be integrated into the following model for the establishment of this larval light sensing organ (Fig. 7).

Fig. 6. Expression of tll in a so mutant embryo (stage 12, lateral view). Note invagination defects of optic lobe (dotted line demarcates apical surface of optic lobe placode that should form a V-shaped invagination by this stage; compare with Fig. 3B). tll is expressed in the anterior (olA) and defective posterior lip (olp) of the optic lobe.

Fig. 7. Proposed model of Bolwig’s organ specification. ato-expressing Bolwig’s organ pioneer cells (dark green) signal to neighboring cells (medium and light green) to become Bolwig’s organ cells. The signaling is partly mediated by Spi (black arrows), which acts as a maintenance signal. Blue arrows indicate another, unknown component of the signal responsible to convey a specific (Bolwig’s organ) fate onto the receiving cells. Although within reach of the signals emanating from the Bolwig’s organ pioneers, those cells expressing Tll (light green) are restricted from responding to the signal and do not become part of Bolwig’s organ.
Expression of *ato* in a small cluster of cells at the posterior boundary of the optic lobe placode specifies these cells as Bolwig’s organ founders. These cells signal to neighboring cells of the placode to become secondary Bolwig’s organ cells, and thus to join the primordium. At least part of the signal emanating from the Bolwig’s organ founders is the *Drosophila* TGFα homolog, Spi. This signal is counteracted by Tll. *tll* expression in the optic lobe placode restricts cells from taking on the Bolwig’s organ fate; only the handful of cells that are the immediate neighbors of the Bolwig’s organ founders become secondary Bolwig’s cells. In the absence of *tll* activity, all cells of the optic lobe placode develop as Bolwig’s organ photoreceptors. Conversely, if *tll* is expressed ectopically in all cells, no Bolwig’s organ develops.

This model in many ways resembles the current model for ommatidial cell specification in the compound eye. In the eye disc, *ato* is expressed throughout the morphogenetic furrow and subsequently restricted primarily to the R8 cells, which are the first ommatidial cells to differentiate (Jarman et al., 1995). It is not well understood how *ato* expression is regulated in the eye disc (the same is true for *ato* expression in the embryo); what is known is that *ato* is subject to autoregulation and that Notch signaling, as well as the genes *emc* and *l*, act as negative regulators of *ato* (Brown et al., 1995; Baker et al., 1996). Loss of *ato* function results in the absence of R8 photoreceptors, as well as all other photoreceptors, attesting to the crucial inductive signaling between R8 and later differentiating cells.

The inductive mechanism which, starting with R8, leads to the successive determination of the different photoreceptors involves at least two signaling steps, one non-specific and one specific. The first, non-specific step requires the TGFα homolog Spitz (Tio and Moses, 1997; Freeman, 1994). Spi is secreted by R8 and, subsequently, by other photoreceptors as well. Spi activates the *Drosophila* EGF Receptor in the receiving cells; this triggers a signal transduction cascade involving the Ras pathway. This pathway in itself does not convey photoreceptor specificity, since reduction in Ras signaling leads to a variable reduction in all photoreceptors. As reported here for the Bolwig’s organ photoreceptors, the EGFR loss-of-function phenotype may be due to cell death. To account for the specificity of photoreceptors induced by R8, there must be another, specific signaling step. Nothing is presently known about such signaling molecules, their receptors or the signal transduction cascades specifying photoreceptor fate. The existence of specific signaling systems is inferred from the identification of a group of transcription factors required in (and expressed in) particular subsets of photoreceptors (reviewed by Dickson, 1995). In the absence of any of these molecules, the corresponding set of photoreceptors is missing; overexpression of these molecules, in some cases, lead to the conversion of other cell types into the corresponding photoreceptors.

Previous studies have revealed that certain aspects of morphogenesis (Green et al., 1993) as well as Rhodopsin expression (Pollock and Benzer, 1988) are similar in Bolwig’s organ and in individual ommatidia. The present study adds a significant facet to this picture: like cell fate in the ommatidium, cell fate in Bolwig’s organ is determined primarily by inductive cell-cell interactions involving EGFR signaling. In view of the fact that 3-4 Bolwig’s organ pioneers express *atopal* and induce secondary cells, it is tempting to go one step further and proclaim these cells evolutionarily related to the R8 cells of the compound eye.

**function of *tll* in visual system cell fate determination**

We have shown that, in the embryonic visual system anlage, *tll* represses Bolwig’s organ fate and promotes optic lobe fate. Our data support a model in which expression of *tll* in the optic lobe placode inhibits the effect of the signal from the *ato*-expressing Bolwig’s organ founders to the cells of the optic lobe. Thus, in the wild type, only a small number of optic lobe cells (those not expressing *tll*) are able to react to the signal and become recruited as secondary Bolwig’s organ cells (Fig. 7). In the absence of *tll* function, signaling from the founders affects cells in a larger region of the adjacent optic lobe, resulting in a dramatic increase in Bolwig’s organ cells.

Since *tll* is a nuclear receptor transcription factor, it must function to block the effect of signaling from the founder cells (which we have shown is mediated at least in part by EGFR) at the transcriptional level. In the posterior of the blastoderm stage embryo, *tll* has been shown to function directly as both a repressor (of *Kruppel*, *knirps* and *Ubx*) and as an activator (of *hunchback*) (Hoch et al., 1992; Pankratz et al., 1992; Quian et al., 1993; Margolis et al., 1995). Additional activator effects of *tll* (not yet demonstrated to be direct) have been shown for *brachyenteron* at the posterior of the blastoderm embryo, and for the proneural gene *lethal of scute* in the brain (Singer et al., 1996; Younossi-Hartenstein et al., 1996b). Thus, in the optic lobe, *tll* could repress genes that would in its absence be activated by EGFR signaling, and/or activate genes that would block receptor, or execution, of the signal.

**Ontogenetic and phylogenetic significance of the ‘cyclops’ phenotype**

Overexpression of *tll* during the extended germband stage results in enlargement of the optic lobes and their dorsal fusion. The same ‘cyclops’ phenotype is produced by other genetic manipulations, such as the increased activity of the EGFR that occurs in *yan* and *aos* mutant embryos, or ectopic *rho* or *Spi* expression (Dumstrei et al., 1998; K. D., unpublished observations). The cyclops phenotype arises from the abnormal development of cells in the dorsomedial head ectoderm, which would normally become part of the head epidermis or undergo apoptosis. These cells are part of the early expression domain of *so* and *eya*; at a later stage, they downregulate these genes and many of them die. We have shown previously (Nassif et al., 1998) that the timely death of these cells is necessary for head involution: in embryos in which cell death does not occur, the dorsomedial head ectoderm cells persist and head involution is blocked. This same involution defect is seen in all situations (including the overexpression of *tll* or EGFR discussed here) where the dorsomedial cells persist. Ectopic and ubiquitous expression of *tll* apparently leads to an optic lobe fate only in the dorsomedial cells. The same is true for ectopic activation of EGFR: if expression of constitutively active EGFR or active *Spi* is driven throughout the entire head ectoderm, only dorsomedial cells express optic lobe markers (K. D., unpublished observation). These findings suggest that the dorsomedial cells form a population of cells that are particularly readily transformed into optic lobe by a variety of different genetic activities (in particular, *tll* and EGFR signaling).
An explanation for the ready transformation of this group of cells may be obtained from a consideration of phylogeny. There is evidence suggesting that the brain of the ancestral organism from which arthropods and other protostomes derived was an unpaired structure, which secondarily split into two hemispheres as a result of the suppression of neural fate in the dorsal midline. Thus, an unpaired apical brain primordium is typically seen in annelid larvae and even in primitive arthropods, such as myriapods (Anderson, 1973). In other words, the formation of two separate brain hemispheres is an evolutionarily more recent event. This might explain why a cryptic genetic mechanism specifying dorsomedial cells as optic lobe is still in place and becomes active in various mutant background of *Drosophila*.

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