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Antagonism of EGFR and Notch signalling in the reiterative recruitment of *Drosophila* adult chordotonal sense organ precursors

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SUMMARY

The selection of *Drosophila melanogaster* sense organ precursors (SOPs) for sensory bristles is a progressive process: each neural equivalence group is transiently defined by the expression of proneural genes (proneural cluster), and neural fate is refined to single cells by Notch-Delta lateral inhibitory signalling between the cells. Unlike sensory bristles, SOPs of chordotonal (stretch receptor) sense organs are tightly clustered. Here we show that for one large adult chordotonal SOP array, clustering results from the progressive accumulation of a large number of SOPs from a persistent proneural cluster. This is achieved by a novel interplay of inductive epidermal growth factor-receptor (EGFR) and competitive Notch signals. EGFR acts in opposition to Notch signalling in two ways: it promotes continuous SOP recruitment despite lateral inhibition, and it attenuates the effect of lateral inhibition on the proneural cluster equivalence group, thus maintaining the persistent proneural cluster. SOP recruitment is reiterative because the inductive signal comes from previously recruited SOPs.

Key words: *Drosophila melanogaster*, Neurogenesis, Proneural, Signalling, EGFR, Notch

INTRODUCTION

In many developmental processes, cell signalling plays a central role in assigning fates among groups of cells with equivalent developmental potential (equivalence groups) (Greenwald and Rubin, 1992; Simpson, 1997). This signalling can be from a source outside the group (inductive), or competitive inhibition among the cells themselves. The latter often takes the form of lateral inhibition, a process that requires Notch-Delta (N-Dl) signalling and acts to partition a pool of competent cells into two distinct fates (Sulston and White, 1980; Doe and Goodman, 1985). In general, one or more cells are selected to take on the primary fate. These cells then inhibit their neighbours, which consequently adopt the default secondary fate.

The selection of SOPs for *Drosophila melanogaster* external sense organs exemplifies this process of fate partitioning (Simpson, 1997). Groups of ectodermal cells (proneural clusters, PNC) acquire the potential to become neural, a potential correlated with expression of proneural genes of the *achaete-scute* complex (AS-C) (Romani et al., 1989; Cubas et al., 1991; Skeath and Carroll, 1991). Typically, only one cell of a PNC will attain SOP fate. Proneural gene expression triggers competitive N-Dl inhibitory signalling between the PNC cells, which in turn activates the expression of inhibitory transcription factors encoded by the *enhancer of split* complex [E(SPL)-C] (Jennings et al., 1995). Such signalling seems to have two sequential functions (Ghysen et al., 1993). First, mutual inhibitory signalling between the PNC cells maintains them in a continued state of competence by preventing commitment. In this process, E(SPL)-C proteins partially inhibit AS-C function such that proneural activity is high enough for competence, but not high enough for SOP commitment. Later, one cell becomes committed to an SOP fate. Once selected, this SOP completely inhibits the neural potential of the remaining cells of the PNC (lateral inhibition). This partition of cell fates is correlated with cessation of proneural gene expression in the PNC and upregulation in the SOP (Cubas et al., 1991; Skeath and Carroll, 1991). What determines which cell is chosen is not clearly established. In some cases, the future SOP is biased by a slightly higher initial level of AS-C products, and so is more inhibitory to surrounding cells. This bias is then amplified by N-Dl signalling (Simpson, 1997). In other cases, it appears that processes other than N-Dl signalling render the future SOP immune to lateral inhibition (Seugnet et al., 1997; Vervoort et al., 1997; Ligoxygakis et al., 1998; Rooke and Xu, 1998). Either way, the cells of the cluster are in communication to ensure that only a single SOP is selected.

This picture has emerged from studies of one type of peripheral nervous system (PNS) sense organ, the external sense organs, as exemplified by the sensory bristles. N signalling has similar roles to play in other processes involving restriction of fates in equivalence groups, such as wing vein formation (de Celis et al., 1997) and development of the compound eye (Cagan and Ready, 1989; Baker and Zitron,

1995). Often, it is not clear how the partitioning of cell fates relates to the paradigm of SOP formation. Indeed, even within the *Drosophila* PNS, SOP formation for other sense organs cannot be explained by the same model. Whereas external sense organs are widely spaced in the cuticle as a result of lateral inhibition, internal stretch receptors known as chordotonal organs are characteristically arranged in large arrays of closely aligned unit organs (scolopidia), which arise from clusters of SOPs (Brand et al., 1993). Despite this, chordotonal SOP selection in the embryo seems superficially to have much in common with the selection of bristle SOPs, including the expression of a proneural gene (*atonal*, *ato*) in PNCs and the refinement of these PNCs by N-Dl lateral inhibition (Goriely et al., 1991; Jarman et al., 1993b). In the embryo, SOP clustering results from the addition of a subsequent step of local recruitment (Okabe and Okano, 1997; zur Lage et al., 1997). Thus there are two distinct stages of chordotonal SOP formation. In the proneural stage, *ato* expression becomes refined to separate SOPs by lateral inhibition. After this, these founding precursors recruit further SOPs from surrounding uncommitted cells by EGFR signalling.

Embryonic chordotonal arrays contain at most five scolopidia, whereas adult chordotonal organs contain much larger clusters of neurons. The femoral chordotonal organ, for example, contains some 70-80 scolopidia. It is unclear how the two-stage mechanism can apply in these extreme cases. Indeed, although *ato* is also required for chordotonal precursor selection in the imaginal discs (Jarman et al., 1995), nothing is known of the requirement for N signalling here. To investigate this problem, the dynamics of femoral chordotonal organ SOP formation were analysed in relation to proneural and neural markers. This revealed a novel process of continuous accumulation of SOPs from a persistent proneural cluster, a process that differs strongly from bristle and embryonic chordotonal organ formation. We show that this process requires the opposing activities of N and EGFR signalling, but not in two sequential stages as described for embryonic chordotonal organs. Instead, N and EGFR are antagonistically interlinked in a continuous mechanism of reiterative recruitment.

MATERIALS AND METHODS

Immunohistochemistry

RNA in situ hybridisation was done according to standard protocols using digoxygenin-labelled cDNAs of *E(spl) mγ*, *Dl* and *rho*. Protein/RNA double labellings were carried out beginning with RNA in situ hybridisation followed by antibody staining. For confocal microscopy (Leica TCS-NT microscope), RNA was detected using anti-digoxygenin-POD and an FITC-tyramide substrate (TSA direct, NEN Life Sciences) (Wilkie and Davis, 1998), and protein using Cy5-conjugated secondary antibodies. Antibodies used were anti-Ato (1:2000; Jarman et al., 1994), anti-Asense (Ase) (1:500; Brand et al., 1993), and anti-dpERK (1:200, Sigma). Bromo-deoxyuridine (BrdU) incorporation was carried out with a 2 hour incubation time as described (Jarman et al., 1995), except that anti-Ato primary incubation and a post-fixation were carried out before DNA denaturation for BrdU detection. For Fig. 4, immunofluorescence was captured by a cooled CCD camera (Photometrics) on an Olympus IX70 inverted microscope, and out-of-focus light was reassigned using Sedat/Agard 3-D deconvolution algorithms (DeltaVision Software).

Gal4/UAS misexpression

Most experiments used a *Gal4* driver line that promotes expression in the chordotonal PNC and SOPs (*109-68Gal4*, obtained from Y. N. Jan) (Jarman and Ahmed, 1998). Larvae were raised at 29°C for most experiments. Some experiments used a heat shock-inducible *Gal4* driver; misexpression was induced by incubating at 39°C for 30 minutes. For the *ase-Gal4* driver construct, a 2-kb fragment upstream of the *ase* open reading frame (Jarman et al., 1993a) was isolated by PCR and inserted into the *Bam*HI site of pCaSpeR-Gal1. Flies were transformed by microinjection. Crossing of transformant flies to a UAS-GFP stock allowed the demonstration of expression driven in the mature chordotonal SOPs.

Stocks

N^{ts1} and N^{55e11} stocks were obtained from the Bloomington Stock Centre, USA. Flies containing UAS-*pnt-P1*, UAS-*m8*, UAS-*mγ*, UAS-*rho*, UAS-$N^{\Delta E}$ and UAS-$EGFR^{DN}$ were obtained from the Bloomington stock centre, F. Tata, C. Delidakis, M, Golembo, H. Ruohola-Baker and M. Freeman, respectively.

RESULTS

Development of the SOP cluster for the femoral chordotonal organ

The adult *Drosophila* femoral chordotonal sense organ (Fig. 1A) arises from a group of some 70-80 SOPs. A developmental analysis of Ato expression revealed that these SOPs accumulate over an extended period of time in the dorsal region of each leg imaginal disc during the third larval instar and early pupa (Fig. 1B-H). The continued expression of Ato implies a sustained requirement for proneural function throughout the process of SOP accumulation. Unusually, Ato is persistently expressed in a group of ectodermal cells, which we identify as the PNC (Fig. 1I,J). From this PNC, cells are funnelled inwards into a cavity formed by the folding of the disc. This invagination later becomes visible as a distinctive 2-cell wide intrusion, which we refer to as the ‘stalk’. Cells at the deepest end of the stalk undergo shape changes to form an amorphous inner SOP mass. Invaginating cells are characterised by upregulation of Ato expression, a characteristic of SOP commitment (Cubas et al., 1991). Surprisingly, SOP markers (Ase protein and the A101 enhancer trap line; Huang et al., 1991) are not expressed in all the stalk SOPs. Instead, these markers are only apparent in older cells, particularly at the time when they become part of the inner mass (which we therefore refer to as mature SOPs) (Fig. 1I,J). Despite this, entry into the stalk seems to mark SOP commitment, since both the stalk and the mature SOPs are absent in discs from *ato* mutant larvae (not shown). This apparent intermediate stage may not have a counterpart in external sense organ precursor formation, although there is some evidence for multiple steps between the uncommitted cell and the SOP (Huang et al., 1991) (the so-called pre-sensory mother cell state; Cubas et al., 1991; Skeath and Carroll, 1991).

Initially, Ato remains activated in all invaginated SOPs. This extended period of proneural gene expression is unusual since AS-C proneural expression is typically switched off in SOPs shortly after commitment (Cubas et al., 1991). Later, at approximately 6 hours BPF, Ato expression is switched off

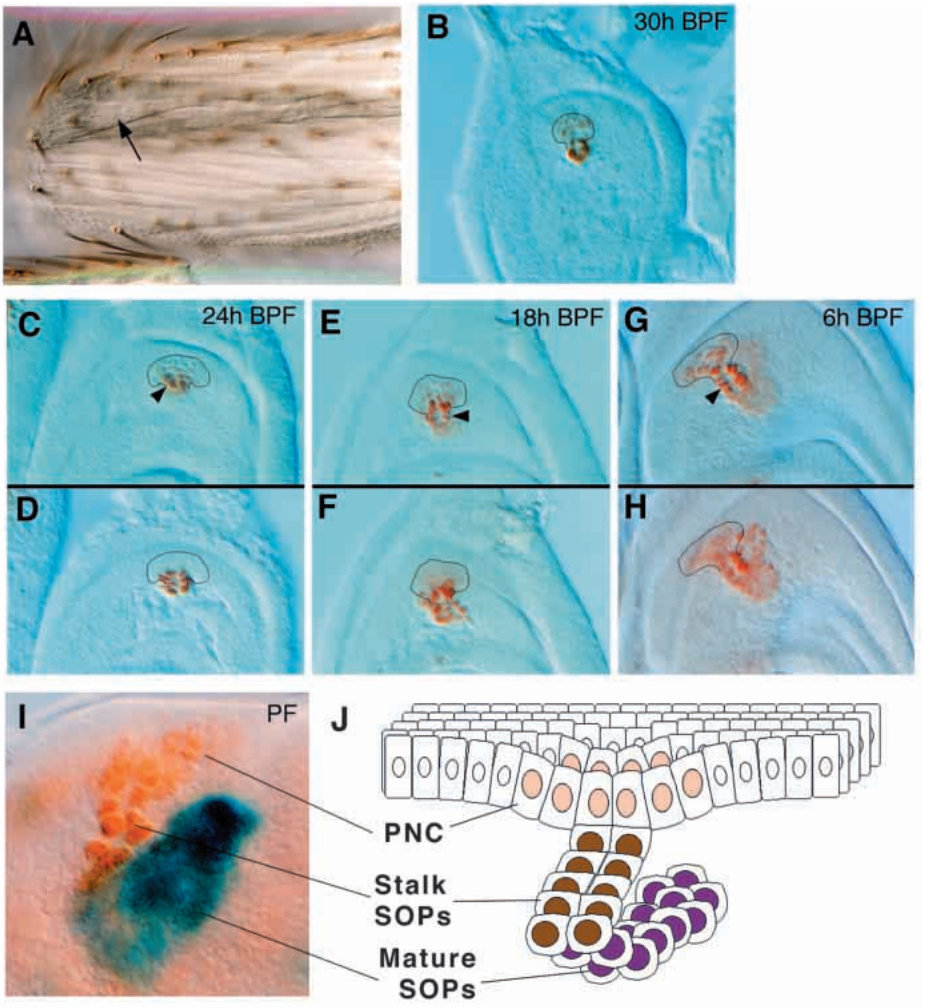

Fig. 1. Structure and development of the femoral chordotonal organ. (A) The proprioceptive chordotonal organ array (arrow) embedded among muscles in the base of the adult femur; optical cross-section. (B-H) Time course of Ato expression in dorsal portion of third instar leg imaginal disc. In each case the location of the PNC is outlined and the stalk SOPs indicated by an arrowhead. (B) 30 hours before puparium formation (BPF). Ato is weak in the PNC and strong in the first approx. 5 SOPs that appear ventral to the PNC (and below the epithelium). (C,D) 24 hours BPF. Two focal planes showing PNC and incipient stalk of stronger Ato-staining SOPs in (C) and the internal mature SOPs in (D). (E,F) 18 hours BPF. Accumulation of mature SOPs continues. The mature SOPs in (F) are directly under the stalk. (G,H) 6 hours BPF. The stalk is more prominent (G), and Ato expression is beginning to disappear from the mature SOPs (below the PNC, and slightly to the right in H). (I) Same region of leg disc at puparium formation from enhancer trap line, A101, doubly stained to detect Ato protein (brown, marking PNC and young stalk SOPs) and X-gal (blue, marking mature SOPs). Ato is no longer expressed in the mature SOPs and there is little overlap with the A101 marker. (J) Schematic representation of (I) with the disc epithelium overlying the SOPs 'removed'. In reality, the disc epithelium covers the whole developing SOP cluster and would be approximately in the plane of the page.

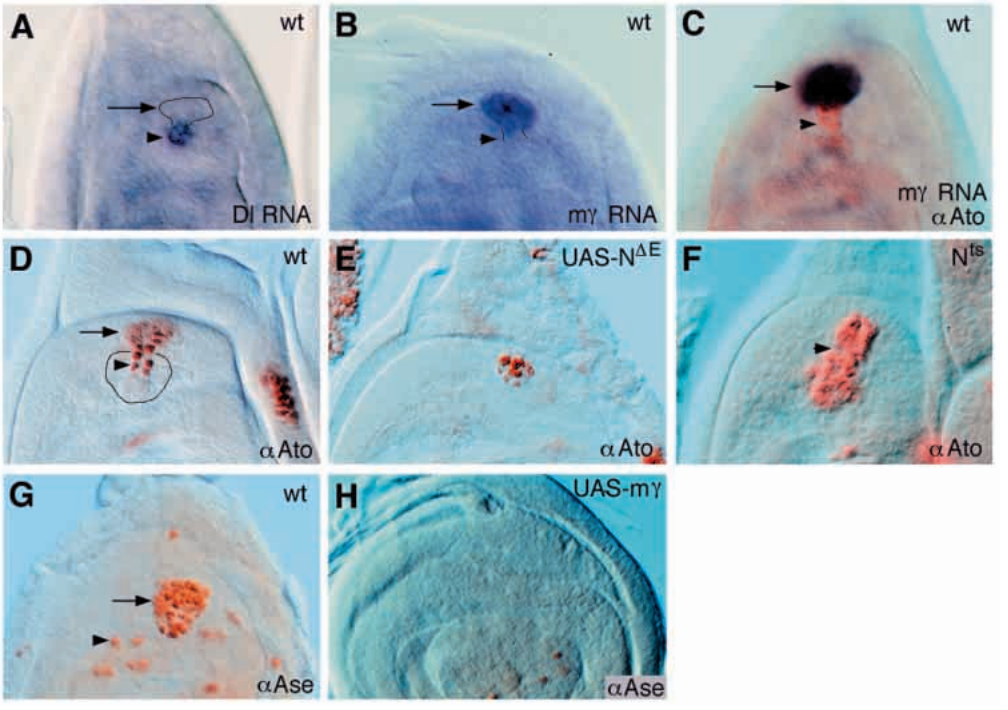

Fig. 2. N-Dl signalling. (A-C) Expression of signalling components. Arrow, PNC; arrowhead, stalk SOPs. (A) Expression of *Dl* RNA in the chordotonal SOPs of a young third instar larval leg disc. The approximate position of the PNC is outlined. (B) Expression of *mγ* RNA in the PNC. (C) Expression of *mγ* RNA (purple) in PNC compared with Ato protein (brown) in PNC and stalk SOPs. (D-I) Effect of altering N signalling. (D) Wild-type expression of Ato at around puparium formation (the mature SOPs are unstained but outlined). (E) Effect of expressing a constitutively active N (UAS-$N^{\Delta E}$) on Ato expression. A few cells remain in the epithelium that express Ato (but all mature SOPs are absent). (F) Effect of removing N activity (N^{ts}) on Ato expression. All cells seem to be stalk SOPs. (G) Wild-type expression of Ase in SOPs. (H) Ase expression in UAS-*mγ* disc, showing suppression of SOPs.

synchronously in the mature SOPs, although expression remains in the stalk SOPs and the PNC (cf. Fig. 1H,I). At this point there is very little overlap between Ato and Ase or A101.

Notch signalling prevents premature SOP commitment, but does not completely inhibit multiple SOP formation

The process of chordotonal SOP formation described above is at odds in several respects with the well-known paradigm of SOP selection for sensory bristles. In the latter, the solitary SOP expresses Dl, which triggers expression in the PNC of genes of the E(SPL)-C (Jennings et al., 1995), thereby preventing further SOP commitment and forcing loss of AS-C expression and neural competence. In the case of the femoral chordotonal organ, newly committed cells from the PNC are in contact with previously committed SOPs in the stalk, but are apparently not receiving (or not responding to) lateral inhibition signals from these to prevent their commitment. Likewise, the presence of committed SOPs does not switch off *ato* expression in the PNC. Nevertheless, components of the N-Dl pathway are expressed in patterns consistent with lateral inhibition. The newly formed SOPs express *Dl*, suggesting that they send inhibitory signals (Fig. 2A), while the PNC expresses *mγ*, a member of the E(SPL)-C (Fig. 2B), suggesting that these cells are responding to the N-Dl signal (de Celis et al., 1996). Indeed, *mγ* is coexpressed with *ato* in the PNC throughout the development of the SOP cluster (Fig. 2C).

To determine whether chordotonal SOP formation is insensitive to N inhibitory signalling, we examined the effect of strong activation of N signalling by *Gal4*/UAS misexpression of a constitutively active form of N (UAS-$N^{\Delta E}$) (Larkin et al., 1996). For these experiments we used a *Gal4* line that drives expression in the chordotonal PNC, stalk, and mature SOPs, as well as many of the PNCs for bristles (*109-68Gal4*) (Jarman and Ahmed, 1998). In *109-68Gal4*/UAS-$N^{\Delta E}$ discs, formation of bristle SOPs was strongly suppressed as expected (not shown). In addition, chordotonal SOPs were also very strongly suppressed, as judged by Ase (not shown) and Ato expression (Fig. 2D,E). UAS-$N^{\Delta E}$ results in *mγ* upregulation (not shown), and a similar inhibition of SOP formation was achieved when a member of the E(SPL)-C was misexpressed directly (UAS-*mγ* or UAS-*m8*) (Fig. 2G,H). Thus, strong activation of N signalling or its effectors can inhibit chordotonal SOP formation.

In a complementary experiment, we disrupted N signalling by analysing imaginal discs from larvae carrying a temperature sensitive allele of *N* (N^{ts}/N^{55e11}) that had been reared at the restrictive temperature for 4-16 hours before dissection. Ato expression showed severe alteration (Fig. 2F): all Ato-expressing cells formed a large, disorganised mass of SOPs below the epithelium. We interpret this as consistent with a neurogenic phenotype. That is, a release of inhibition allows uncontrolled commitment and ingress of all cells of the PNC. Thus, N signalling has an important role to play: it acts to limit the process of SOP selection from the PNC. Some mechanism, however, must prevent N signalling from completely inhibiting multiple SOP formation.

EGFR signalling is required for chordotonal SOP clustering in the leg disc

The progressive accumulation of chordotonal SOPs suggested that a recruitment mechanism could explain the clustering of SOPs. The *Drosophila* EGFR signalling pathway is involved in a number of recruitment processes in development, and we have previously uncovered a role for EGFR signalling in the induction of embryonic chordotonal precursors (zur Lage et al., 1997). Although there appear to be significant differences in the process of SOP formation in imaginal discs compared with the embryo, we asked whether EGFR signalling is also involved in forming the femoral chordotonal cluster. To address this question, the pathway was conditionally disrupted by expressing a dominant negative form of EGFR protein (*109-68Gal4*/UAS-$EGFR^{DN}$; Freeman, 1996). Expression of UAS-$EGFR^{DN}$ resulted in a dramatic loss of chordotonal SOPs in late third instar imaginal leg discs (as judged by Ase protein expression or the A101 enhancer trap line) (6±0.5 Ase-expressing cells, *n*=20 compared with 27±0.9, *n*=20 for wild type) (Fig. 3A,B). This demonstrates that EGFR signalling is required for the process of femoral chordotonal SOP formation. In contrast, the appearance of bristle SOPs was unaffected, arguing against the possibility of a nonspecific effect on SOPs in general.

To determine whether EGFR signalling controls SOP number, we forced expression of components of the EGFR pathway that determine the level of signalling, thus resulting in hyperactivation of the pathway. *rhomboid* (*rho*) encodes a transmembrane protein (Bier et al., 1990) required to activate EGFR signalling; it is possibly involved in processing of the EGFR ligand, Spitz (Spi), from its inactive membrane-bound form to the active secreted form (Golembo et al., 1996). *pointed* (*pnt*) is an effector gene that encodes a transcription factor (Klämbt, 1993) and is activated in cells responding to EGFR signalling. Both *rho* (see below) and *pnt* (not shown) are expressed during chordotonal SOP formation. In discs from *109-68Gal4*/UAS-*rho* larvae, the number of SOPs in the femoral cluster was doubled (53±2.4, *n*=23) (Fig. 3C). *109-68Gal4*/UAS-*pntP1* flies showed an even greater increase in chordotonal SOP hyperplasia (76±2.9, *n*=6) (not shown).

EGFR promotes commitment of PNC cells as SOPs

EGFR could promote SOP formation by stimulating the commitment of PNC cells or by stimulating proliferation of SOPs. Both functions would be consistent with known EGFR roles (Baumann and Skaer, 1993; Domínguez et al., 1998), but our investigations favour the former. Analysis of Ato expression in leg discs in which *rho* had been misexpressed revealed a large invagination of cells and a smaller PNC (Fig. 3D,E). Shrinking of the PNC was confirmed by the reduced extent of *mγ* expression (Fig. 3G,H). These observations are consistent with an increased rate of SOP commitment upon EGFR hyperactivation. Moreover, this effect is reminiscent of the effect of N loss of function on Ato expression, suggesting that EGFR signalling supplies the mechanism that interferes with lateral inhibition of SOP commitment.

Since EGFR signalling affected the PNC and stalk SOPs, we determined whether cell proliferation occurs in these locations by BrdU incorporation experiments. Scattered S-phase (proliferating) cells were detected throughout the leg disc, but not among the Ato-expressing cells of the chordotonal PNC or stalk (Fig. 4A,B). Consistent with the BrdU incorporation pattern, staining for β-tubulin, phosphorylated histones or Hoechst revealed a lack of mitoses in the PNC and stalk (data

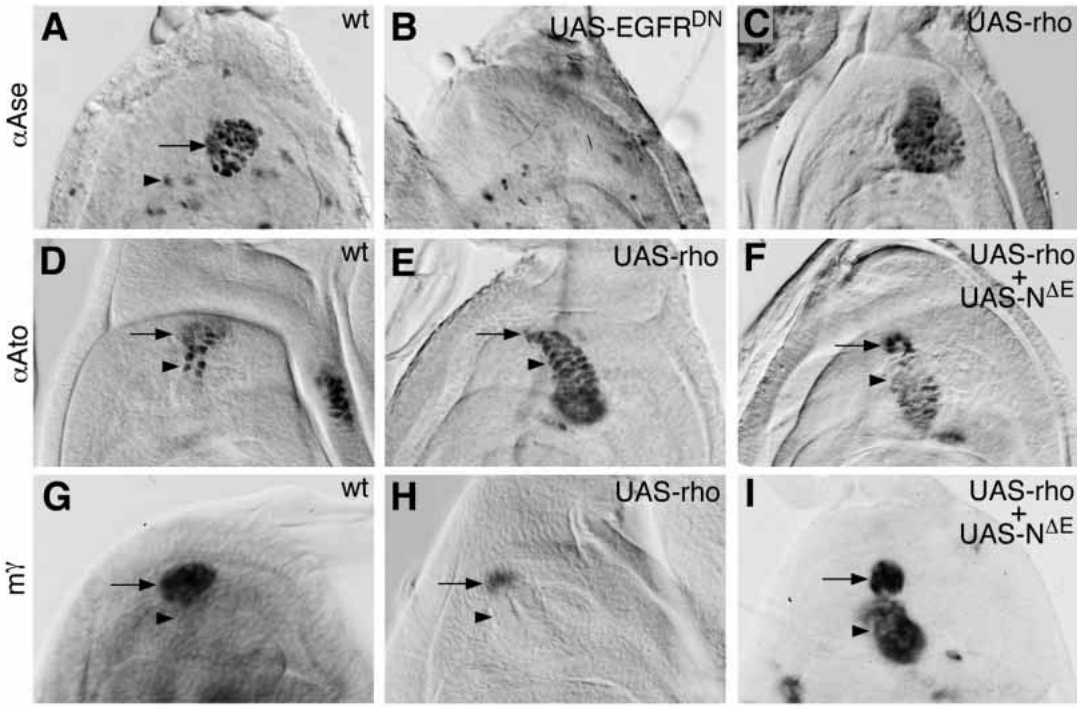

Fig. 3. EGFR signalling is required for chordotonal cluster formation. (A-C) Ase expression in SOPs. (A) Wild type, with clustered chordotonal SOPs (arrow) and isolated bristle SOPs (arrowhead) marked. (B) UAS-*EGFR*^{DN}. Chordotonal SOPs are missing, but bristle SOPs appear unaffected. (C) UAS-*rho*, with many more SOPs in the chordotonal cluster (even though the disc is younger than that in A. (D-F) Ato expression. Arrow, PNC; arrowhead, stalk SOPs. (D) Wild type, showing PNC and stalk SOPs. (E) UAS-*rho*. The PNC is reduced or absent and the stalk is much thicker. (F) Leg disc from larvae containing both UAS-*N*^{ΔE} and UAS-*rho*. SOP formation resembles that for UAS-*rho* alone rather than UAS-*N*^{ΔE} (see Fig. 2E), although Ato is weak in some of the SOPs. (G,H) *mγ* expression. (G) Wild type, in PNC. (H) UAS-*rho*, showing reduction of PNC size. (I) UAS-*rho* + UAS-*N*^{ΔE}, showing strong *mγ* activation despite lack of SOP suppression.

not shown). Thus it seems that cells of the PNC and stalk are held in a state of mitotic quiescence throughout the time that SOP fate decisions are being made, so that SOP commitment occurs in the absence of cell division. Furthermore, this quiescence suggests that the PNC expression of Ato must expand over time to include further ectodermal cells in order to replace those cells that leave the PNC as SOPs. Cell cycle arrest appears to be a consistent feature of neural fate selection, since AS-C-expressing PNCs are also located in quiescent zones in the developing wing disc (Usui and Kimura, 1992). In the eye, cells in the morphogenetic furrow, in which *ato* is required for the selection of R8 photoreceptors, are arrested in G_1 phase (Thomas et al., 1994).

In contrast to the PNC, we observed strong BrdU incorporation in the older (mature) SOPs (Fig. 4C). It is likely that this correlates with the cell divisions that each SOP undergoes to generate the component cells of each scolopidium (Bodmer et al., 1989), but we considered whether it might also represent EGFR-dependent SOP proliferation by determining whether EGFR was required in the mature SOPs. For this, UAS-*EGFR*^{DN} was expressed using a *Gal4* driver line that restricts expression to mature SOPs only (*ase-Gal4*). Such expression had no effect on the number of Ase-expressing SOPs (not shown). This suggests that EGFR-dependent SOP proliferation is not the basis for clustering. The result, together with the observation that bristle SOPs are unaffected by EGFR disruption, also argues against a cell survival function for EGFR in SOPs (cf. the eye: Domínguez et al., 1998).

Location of EGFR signalling: evidence for direct role in reiterative SOP recruitment

Our experiments so far have indicated that EGFR signalling affects SOP commitment from the PNC. To determine more precisely the spatial patterning of EGFR activity required for SOP clustering and N antagonism, we characterised the expression patterns of key components of the pathway. Localised expression of *rho* appears to play a central role in spatial restriction of EGFR activity in cases where Spi is the ligand (Golembo et al., 1996; Okabe and Okano, 1997; zur Lage et al., 1997; Wasserman and Freeman, 1998); in these cases it appears to mark the cells that are a source of signalling. During development of the femoral chordotonal organ, *rho* is expressed in a very restricted pattern: *rho* mRNA was only detected in the SOPs, becoming confined in the late third instar larva to the youngest SOPs at the top of the stalk (Fig. 5A).

To identify the cells responding to *rho*-effected signalling, we used an antibody that detects the dual-phosphorylated (activated) form of the ERK MAP kinase (dp-ERK) (Gabay et al., 1997), which is a component of the RTK signal transduction pathway and is encoded by the gene *rolled* (Biggs et al., 1994). In leg imaginal discs, we detected dp-ERK in a confined area corresponding to the uppermost (youngest) stalk SOPs. Thus, like *rho*, dp-ERK is expressed in the newly formed stalk SOPs. Double labelling for *rho* RNA and dp-ERK confirmed this (Fig. 5B), but also suggested that the overlap in expression is not complete: dp-ERK was detected above the uppermost *rho*-expressing cells of the stalk, probably in one or a few cells of the proneural cluster as they funnel into the stalk. This suggests that EGFR promotes SOP commitment as a consequence of direct signalling from previous SOPs to overlying PNC cells. Since *rho* expression is itself activated upon SOP commitment, this process occurs cyclically: the newly recruited SOPs are in turn able to signal to further overlying PNC cells. That is, recruitment is reiterative. Additionally, it seems that the committed SOPs transactivate each other or auto-activate, since dp-ERK remains on and is

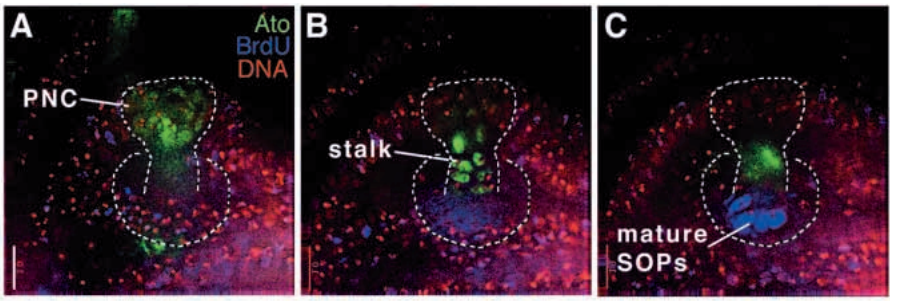

Fig. 4. Chordotonal SOPs are selected from a mitotically quiescent PNC. (A-C) Optical sections of wild-type dorsal leg disc after BrdU incorporation (blue), and anti-Ato (green) and DNA (Hoechst: red) labelling. Ato is in the PNC and stalk, but not the mature SOPs, while BrdU is present in some of the mature SOPs (heavily stained in C), a proportion of the ectodermal cells (seen in A), but not in the PNC or stalk. Sections are 5 μm apart. Bar, 10 μm.

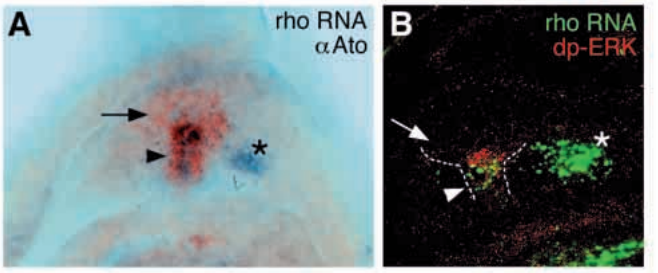

Fig. 5. Expression of EGFR components. (A) Expression of *rho* RNA (purple) in the stalk SOPs compared with Ato protein (brown) in stalk and PNC. Arrowhead marks nearby additional expression of *rho* in the developing non-neural apodeme. (B) Detection of *rho* RNA (green) and dp-ERK (red) in a projection of two consecutive confocal microscopy sections taken 2 μm apart. dp-ERK is both within and above the *rho*-expressing cells.

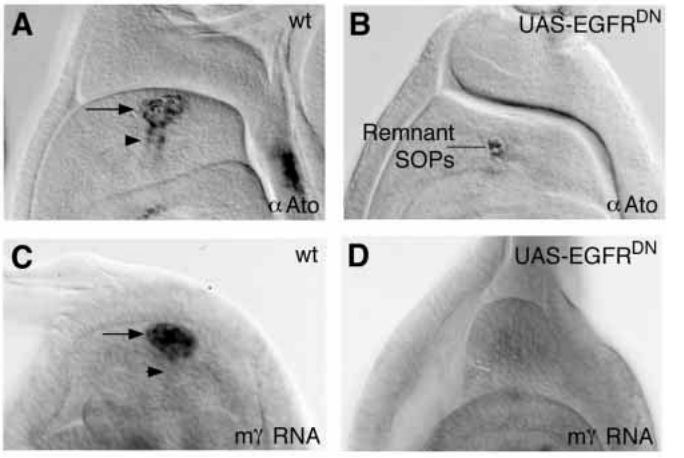

Fig. 6. Persistent PNC requires EGFR signalling. (A,B) Effect on Ato expression. (A) Wild type, in PNC and stalk SOPs. (B) UAS-*EGFR*^{DN}; Ato is lost from the PNC and only a few SOPs remain. (C,D) Effect on *mγ*RNA expression. (C) Wild type, in PNC. (D) UAS-*EGFR*^{DN}; *mγ*is lost from PNC. As a control, expression of Ato and *mγ*in the eye is largely unaffected (not shown).

coexpressed with *rho*. This suggests that in addition to providing a direct recruitment signal, EGFR signalling may also play some role in the newly formed SOPs themselves.

Further evidence for direct EGFR signalling in recruitment comes from experiments in which N and EGFR signalling were simultaneously hyperactivated in all cells of the PNC. Leg discs from flies misexpressing both UAS-*rho* and UAS-$N^{\Delta E}$ exhibit a chordotonal hyperplasia similar to UAS-*rho* alone (Fig. 3F), demonstrating that the EGFR neuralising signal is dominant or epistatic over the N inhibitory signal. Since *mγ*is strongly activated in these discs (Fig. 3I), EGFR signalling antagonises lateral inhibition at a point downstream of *mγ* activation by N signalling. That is, EGFR signalling antagonises *mγ* regulation of target genes rather than N signalling per se. Consistent with this, a similar result was obtained in flies combining UAS-*rho* and UAS-*mγ* (not shown).

EGFR helps to maintain neural competence by attenuation of lateral inhibition

In bristle PNCs, coexpression of proneural AS-C genes and 'antineural' E(SPL)-C genes is transitory, since E(SPL)-C products rapidly inhibit the expression of AS-C genes. Thus, N-Dl signalling normally switches off PNC competence in addition to preventing further SOPs forming. A striking

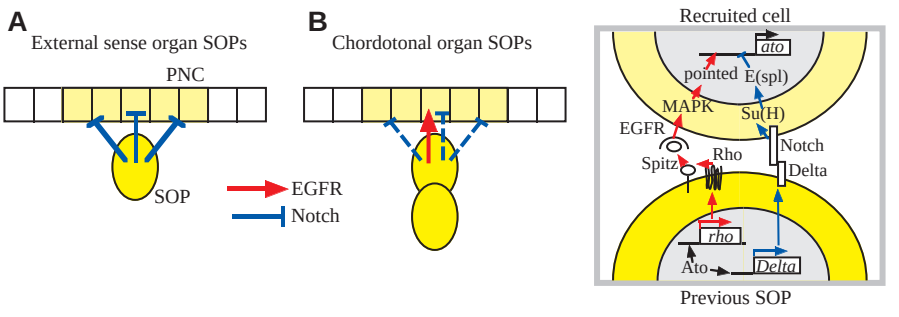

Fig. 7. (A) Model for selection of SOPs for the femoral chordotonal organ compared with the selection of external sense organ SOPs. External sense organ SOPs send a lateral inhibitory N signal to PNC to prevent further SOP formation and switch off proneural competence. Chordotonal SOPs also inhibit the PNC, but this N signal is at least partially attenuated by EGFR signalling within the SOPs, allowing maintenance of competence. In addition, chordotonal SOPs directly signal via EGFR to recruit further SOPs from the PNC. (B) Model of chordotonal SOP recruitment by previously committed SOPs, showing abbreviated EGFR and N pathways. Both signals may be activated by *ato* in the signalling cell. The EGFR signal predominates over the N signal in the recruited cell at a level downstream of *E(spl)* gene activation, suggesting that integration of the two signals may occur at the level of *ato* gene regulation. It should be noted that our experiments strongly suggest that Spi is the functioning EGFR ligand in this process, but this remains to be proved.

characteristic of chordotonal SOP formation is the persistence of the PNC, with prolonged coexpression of *ato* and *mγ*. This suggests that, in addition to local suppression of lateral inhibition to allow multiple SOP formation, N signalling to the PNC as a whole might also be attenuated. Our experiments suggest that this might be achieved as a second function of EGFR signalling, because persistence of the PNC depends on EGFR function (Fig. 6A,B). Interestingly, *mγ* expression is also lost upon prolonged EGFR disruption (Fig. 6C,D), although one might predict that it should be upregulated. It appears likely that loss of *mγ* expression is a secondary effect of loss of *ato* expression and SOP formation. Although EGFR is required for PNC persistence, its ability to attenuate lateral inhibition appears relatively weak, since a pulse of *rho* expression (*hsGal4*; UAS-*rho* flies) does not severely inhibit *mγ* expression (not shown). Similarly, transient loss of EGFR activity (*hsGal4*; UAS-$EGFR^{DN}$ flies) results in only a slight initial increase in *mγ* expression (derepression of lateral inhibition) before eventual loss of *mγ* and the PNC (not shown).

DISCUSSION

The femoral chordotonal organ arises from a cluster of SOPs. The development of this SOP cluster shows remarkable differences from the established paradigm of sensory bristle SOP formation. N signalling is required to limit SOP commitment, but does not prevent multiple SOP formation because of the opposing action of EGFR signalling. We have demonstrated that EGFR signalling is required for clustering; more specifically, our data suggest that it promotes SOP commitment rather than proliferation or protection from cell death. We show that N-EGFR antagonism is important for controlling the rate of SOP commitment, because this rate can be pushed in either direction by manipulation of either signalling pathway. Indeed, chordotonal cluster size is highly labile in insect evolution (e.g. Pringle, 1957); a fact that could be accounted for by changes in the relative strength of the N and EGFR signalling pathways.

Reiterative recruitment in SOP clustering

Our data suggest that, by virtue of co-expressing *Dl* and *rho*, SOPs are simultaneously the source of opposing signals: a 'global' inhibitory N signal to the whole PNC to check further neural commitment and a more localised EGFR signal to directly overlying PNC cells to promote SOP commitment (Fig. 7A). It seems that the localised EGFR signal countermands lateral inhibition by opposing the action of E(SPL)-C proteins on target genes, since misexpression of E(SPL)-C genes could not prevent hyperactivated EGFR signalling from causing hyper-recruitment. A likely target of EGFR signalling is the *ato* gene itself, because E(SPL)-C proteins normally switch off proneural gene expression (Heitzler et al., 1996) whereas proneural gene upregulation is the first sign of SOP commitment (Cubas et al., 1991; Skeath and Carroll, 1991; this work) (Fig. 7B). Interestingly, antagonism of EGFR and N at the level of proneural gene regulation has also been suggested to explain the formation of embryonic dorsal midline neural precursors (Dumstrei et al., 1998).

This localised inductive role of EGFR is reminiscent of the induction of primary vulval cell fate by the anchor cell (AC) in *Caenorhabditis elegans*, which is also achieved via an EGFR homologue (the product of the *let-23* gene; Aroian et al., 1990). In the latter case, however, the induction only happens once, partly because the signal is provided by a source that is completely extrinsic to the vulval cell equivalence group (Greenwald, 1998). In contrast, chordotonal SOPs accumulate over time because their induction is reiterative. Reiteration arises because the key activator of EGFR signalling, *rho*, is itself activated within SOPs as a response to signalling from previous SOPs (perhaps as a target of upregulated *ato*). Hence a cycle is formed in which the recruited become the recruiters. Strikingly, a reiterative model of recruitment involving EGFR has also been proposed for the progressive recruitment of cells in *Drosophila* eye development (Freeman, 1996). It is notable that the same proneural gene, *ato*, is involved in both cases, suggesting a causative role for this gene in triggering EGFR signalling. A key difference, however, is that *ato* is continuously required in adult chordotonal SOP formation, whereas it is only required to select the first cell (photoreceptor R8) in the eye (Jarman et al., 1994).

If SOP accumulation results from reiterative recruitment, what sets off the cycle? We imagine that the first SOP(s) appear from the early *ato* PNC by the same intrinsic resolution of unstable mutual inhibition that is thought to underlie sensory bristle SOP selection (Ghysen et al., 1993; Simpson, 1997). If *rho* expression is then activated in these first SOPs by *ato*, then it is clear that this alone could start the cycle of reiterative EGFR signalling and recruitment.

Maintenance of competence by antagonism of lateral inhibition

Reiterative recruitment alone cannot entirely explain the accumulation of SOPs. It also relies on the persistence of the competent pool of PNC cells from which SOPs can be recruited. For AS-C PNCs, this does not occur, because the mutual inhibition required for continued competence is unstable and resolves quickly to a state of lateral inhibition once the SOP emerges from the PNC (Ghysen et al., 1993). This results in rapid shutdown of AS-C expression and hence competence within the PNC. One possible explanation for the persistence of the *ato* PNC is that there is a low (undetected) level of Dl in the PNC which maintains a low level of N activity that is insufficient to repress *ato* completely. It is also possible that the members of E(SPL)-C that are expressed in the PNC (notably *mγ* and *mδ*) are less aggressive inhibitors of proneural gene expression than the E(SPL)-C members expressed in AS-C PNCs (*m5* and *m8*). Our results also suggest, however, that EGFR has a role to play in maintaining the PNC by partially attenuating lateral inhibition on a PNC-wide scale. Thus, the PNC is not completely shut off by inhibition from SOPs, but instead kept in check, allowing continued mutual inhibition and maintenance of competence but not allowing general SOP commitment. Since neither *rho* nor dp-ERK are detected in the PNC as a whole, this function of EGFR could be indirect and achieved through partial attenuation of Dl signalling from the stalk SOPs themselves. The trans- or auto-activation of EGFR signalling between the stalk SOPs (as suggested by the co-expression of dp-ERK and *rho*; Fig. 5B) might be an indicator of this function. It is also possible, however, that EGFR signalling is direct and that the dp-ERK antibody is not sensitive enough to detect expression in the PNC cells.

N-EGFR antagonism in development

Adult chordotonal SOP formation provides an example of an antagonistic relationship between N and EGFR that seems to be commonly repeated in development, such as in wing vein formation (de Celis et al., 1997), eye development (Verheyen et al., 1996; Price et al., 1997; Miller and Cagan, 1998) and *C. elegans* vulval induction (Greenwald, 1998). One emerging theme is that EGFR often induces commitment within competence groups while N inhibits commitment, either by maintaining competence or by locking in the alternative 'default' fate. These two roles of N signalling occur sequentially in AS-C proneural clusters, in which mutual inhibition (maintaining competence) gives way to lateral inhibition (locking in commitment). The result is a one-off partitioning of fates. Interestingly, chordotonal clustering is achieved because EGFR antagonises each of these N functions via different routes. This allows the processes of maintaining competence and commitment to occur simultaneously over a period of time. Such coexistence of competence and commitment is indicated elsewhere in development too, such as commitment of neuronal progenitors in vertebrate neuroepithelia. In the chick retina, for instance, the uncommitted progenitor state is maintained by partial lateral inhibition from committed neurons that arise continuously (Henrique et al., 1997), suggesting that chordotonal SOP formation may provide a model for such processes. There is evidence that EGFR-promoted progressive recruitment in the *Drosophila* eye also takes place in the background of N signalling required to maintain the plasticity of surrounding cells, although the details of any interplay are not clear (Fortini et al., 1993; Schweitzer and Shilo, 1997; Freeman, 1998). It has been proposed that differential diffusion of Spi and Argos proteins may control the rate of recruitment to the ommatidium (Freeman, 1996, 1997). Our results suggest that the rate of recruitment is also controlled by the relative strengths of N and EGFR signalling.

Other chordotonal organs

Expression of *rho* and dp-ERK is associated with all developing adult chordotonal organs (our unpublished data), suggesting that one or both levels of EGFR antagonism are also required for clustering of other chordotonal organs. None of these other SOP clusters seem to have as distinctive a structure as the femoral chordotonal organ, supporting the idea that the regulatory linkage between signalling pathways may be very changeable in evolution (Gerhart and Kirschner, 1997), even among different chordotonal organs in the same organism. Indeed, our present findings differ in a number of respects from the role that we had previously uncovered for EGFR in the induction of embryonic chordotonal organs (zur Lage et al., 1997). In the latter, only a single round of recruitment occurs because *rho* is not activated in the recruited cells, perhaps because the recruited cells do not express *ato* (i.e. they do not arise from a persistent PNC). It seems likely that signalling during neural precursor commitment has been simplified in the embryo to accommodate rapid development (see Seugnet et al., 1997). The limited requirement for EGFR in embryonic chordotonal clusters, along with their modest size, suggests that the embryo may be in the evolutionary process of dispensing with chordotonal recruitment altogether.

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