The *Drosophila* proneural gene *amos* promotes olfactory sensillum formation and suppresses bristle formation

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Summary

Proneural genes encode basic-helix-loop-helix (bHLH) transcription factors required for neural precursor specification. Recently *amos* was identified as a new candidate *Drosophila* proneural gene related to *atonal*. Having isolated the first specific *amos* loss-of-function mutations, we show definitively that *amos* is required to specify the precursors of two classes of olfactory sensilla. Unlike other known proneural mutations, a novel characteristic of *amos* loss of function is the appearance of ectopic sensory bristles in addition to loss of olfactory sensilla, owing to the inappropriate function of *scute*. This supports a model of inhibitory interactions between proneural genes, whereby *ato*-like genes (*amos* and *ato*) must suppress sensory bristle fate as well as promote alternative sense organ subtypes.

Key words: Proneural, bHLH, *Drosophila*, *amos*, Neurogenesis, Gene regulation

Introduction

The sequence and structure of the bHLH domain is highly conserved, and yet transcription factors of this family play a variety of roles in neurogenesis in a range of organisms (Bertrand et al., 2002). These roles include conferring neuronal competence, directing neural precursor specification, directing neuronal subtype specification and triggering neuronal differentiation. Dissecting bHLH gene functions and interactions is an important and challenging task, and the *Drosophila* PNS provides a good model in which to do this. Here, proneural bHLH genes are required for sense organ precursor (SOP) specification (Hassan and Bellen, 2000). These genes include *achaete* (*ac*) and *scute* (*sc*), from the *Achaete-scute Complex* (*ASC*), and *atonal* (*ato*), as well as the candidate proneural gene *amos* (Hassan and Bellen, 2000). These proneural proteins seem to combine two functions: promoting SOP specification, and providing these SOPs with information concerning neuronal subtype (Jarman and Ahmed, 1998). It is thought that vertebrate proneural gene homologues also have functions in neural progenitor specification and neural subtype identity (Hassan and Bellen, 2000; Bertrand et al., 2002).

bHLH functions depend on both intrinsic protein properties and extrinsic factors (Bertrand et al., 2002). Comparisons of protein capabilities, particularly by assaying the effect of misexpression on neural development, have shown evidence for intrinsic differences between closely related bHLH proteins, suggesting that they regulate distinct target genes (Jarman and Ahmed, 1998). However, bHLH protein specificity is also very dependent on extrinsic modifying factors. Ato has been well characterised and illustrates well the complexity of defining the intrinsic specificity of proneural proteins. In most of the developing ectoderm, Ato is required for chordotonal (stretch receptor) SOP specification. Ectopic expression of *ato* leads to ectopic chordotonal SOP formation (Jarman et al., 1993). In this property, it differs from Ac and Sc, which are necessary and sufficient for external sense organ (bristle) SOPs. This points to intrinsic differences in protein properties. However, the function of Ato is clearly also very context dependent. In addition to specifying chordotonal organs, Ato is also required for R8 photoreceptors in the eye (Jarman et al., 1994), and for one subset of olfactory sensilla (sensilla coeloconica) in the antenna (Gupta and Rodrigues, 1997). Moreover, in a group of CNS neurons, Ato regulates neurite arborization (Hassan et al., 2000). It is not known how the response to Ato is modified in these different regions.

We have argued for a specific mechanism by which proneural proteins specify neural subtype: SOPs may be biased to become external sense organs and, consequently, Ac/Sc promotes a default neural fate, whereas Ato must actively impose alternative neural fates (Jarman and Ahmed, 1998). This idea is based on two apparently paradoxical outcomes of misexpression experiments. Under certain very defined conditions, *ato* misexpression can transform *existing* bristle SOPs to chordotonal organs, thereby revealing an intrinsic ability of Ato (Jarman and Ahmed, 1998). However, in most contexts, *ato* misexpression induces a mixture of ectopic chordotonal and bristle SOPs (Jarman et al., 1993), suggesting that in many circumstances Ato can specify SOPs but may often fail to provide subtype information. This suggests that the two proneural roles are separable in misexpression studies, and it also gives the appearance that Ato function is more sensitive to cell context than is Ac/Sc function. Similar controlled misexpression data for vertebrate bHLH genes have recently been reported, which support an entirely analogous situation in which *neurogenin* (*ato* homologue) is more context

sensitive than *Mash1* (*ac/sc* homologue) (Lo et al., 2002) (see also Parras et al., 2002). But teasing out these functions is complicated and misexpression data could be misleading. There is no corroborative evidence from loss-of-function mutations in *Drosophila* as known proneural mutations always cause loss of SOP subsets, and so questions concerning the neural identity of SOPs are hard to approach through loss-of-function studies.

Recently, we and others described a new candidate proneural gene, *amos* (Goulding et al., 2000; Huang et al., 2000). Amos protein possesses a very similar bHLH domain to that of Ato, suggesting there may be functional similarities with Ato that set this gene pair apart from *ac/sc*. We provided strong but indirect evidence that *amos* is the proneural gene for the *ato*-independent classes of olfactory sensillum (sensilla basiconica and trichodea) (Goulding et al., 2000). Here, we report a detailed analysis of *amos* expression and function, including the first isolation and characterisation of specific *amos* mutations. We find that Amos protein is expressed in, and is required for, a late wave of olfactory SOPs in the antenna. These are the precursors for sensilla basiconica and trichodea, proving that *amos* is the proneural gene for these subtypes. However, an unexpected aspect of the mutant phenotype was the appearance of ectopic sensory bristles in place of the olfactory sensilla on the antenna. This replacement of sense organs rather than complete absence is unprecedented for a *Drosophila* proneural gene mutation. Our analysis suggests that loss of *amos* results in loss of olfactory sensilla and concomitant derepression of *ac/sc* leading to formation of external sense organ SOPs. This phenotype supports the argument that the *ato*-like proneural genes (*amos* and *ato*) suppress external sense organ fate as well as promote alternative neural fates.

Materials and methods

Fly stocks

Wild-type flies are generally Oregon R and pr^1, as appropriate. UAS-*amos* is described by Goulding et al. (Goulding et al., 2000). sc^{10-1}, ase^1 and lz^{34} are described by Lindsley and Zimm (Lindsley and Zimm, 1992). Deficiencies and mutants were obtained from the Umea stock centre. For *ato*/*amos* double-mutant analysis, $amos^1$ clones were induced in an ato^1 background by the FLP/FRT method using *eyelessFLP* (Newsome et al., 2000). The flies had the following genotype: *y w eyFLP; amos*^{1} *pr*^{1} *FRT40A/2×nlsGFP FRT40A; ato*^{1}. Clones were recognised by their sensillum phenotype.

Mutagenesis

$amos^1$ was isolated in an F2 screen for mutations that failed to complement a deficiency of the *amos* region [*Df(2L)M36F-S6* (Goulding et al., 2000)]. pr^1 male flies were mutagenised with 25-30 mM EMS. Mutagenised lines were collected over a CyO balancer and individually tested for complementation with *Df(2L)M36F-S6*. 4500 mutagenised lines were screened. $amos^2$ and $amos^3$ were isolated in a subsequent F1 screen of 25,000 flies using $amos^1$. PCR isolation of the ORFs and sequencing were by standard techniques.

Amos enhancer construct

A 3.6 kb fragment upstream of the *amos* start site was amplified by PCR and cloned into the transformation vector pTLGal4 (a gift of B. Hassan). Transformant flies were made by microinjection into syncytial blastoderm embryos. These were crossed to UAS-*GFP* or UAS-*nlsGFP* lines for assessment of enhancer activity.

Immunohistochemistry

Antibody staining of pupal antennae was carried out as previously described (Goulding et al., 2000). Pupae were staged by collecting at the time of puparium formation and then ageing on moist filter paper at 25°C before dissection. Antibodies used were: Cut (1:100), Ac (1:50), 22C10 (1:200) and Elav (1:200) (all from the Developmental Biology Hybridoma Bank, Iowa); Sens (1:6250) (Nolo et al., 2000); and Pros (1:200). Anti-Amos antibodies were raised in rabbits, using full-length His_6-tagged Amos protein expressed in *E. coli*, and purified by adsorption to nickel-agarose under denaturing conditions. Anti-Amos antibodies were used at 1:1250 after pre-adsorption against wild-type embryos. RNA in situ hybridisation was done according to standard protocols using digoxigenin-labelled *sc* cDNA. RNA/protein double labellings were carried out by initially detecting RNA using anti-digoxigenin-POD and an Alexa Fluor 488 tyramide substrate (Molecular Probes), followed by antibody staining. Microscopy analysis was carried out using an Olympus AX70 or Leica LCS-SP system.

Results

amos mutations result in loss of olfactory sensilla and the appearance of mechanosensory bristles

We generated three mutant alleles of *amos* in an EMS screen (Table 1). $amos^1$ is predicted to result in a protein truncation that removes the second half of bHLH helix 2 and the C-terminal region thereafter. $amos^2$ is a missense mutation that changes a Ser to an Asn in helix 1 of the bHLH domain. This position is not part of the bHLH core consensus sequence and is not predicted to affect directly DNA binding or dimerisation. Moreover, Asn is found in this position in the *ato* bHLH domain, and so the effect of this mutation would be predicted to be mild. $amos^3$ contains a 230 bp deletion within the ORF, which also causes a frame-shift that brings a spurious downstream stop codon in frame. This allele gives a predicted peptide of 74 amino acids, of which only the first 30 are shared with *amos*. It therefore lacks the entire bHLH domain and is likely to be a null.

Consistent with previous RNAi experiments (Huang et al., 2000), $amos^1$ mutant embryos lack two dorsal sensory neurons per segment, including the dorsal bipolar dendritic neuron (D.R.A.P. and A.P.J., unpublished). Nevertheless, all *amos* alleles are adult viable as homozygotes and hemizygotes. The antennae of mutant adult flies were mounted and examined by light microscopy in order to quantify the number and type of olfactory sensilla. Compared with wild-type (Fig. 1A and Table 2) (Carlson, 1996), *amos* mutant antennae carried dramatically reduced numbers of sensilla and, as a consequence, the third segment is significantly smaller (Fig. 1C,D). In particular,

Table 1. Molecular basis of *amos* mutations

Allele	DNA	Protein	Predicted effect	Phenotype
$amos^1$	$C_{550}>T_{550}$	$Q_{184}>$Stop	Truncates the bHLH domain	Strong hypomorph/null
$amos^2$	$G_{458}>A_{458}$	$S_{153}>N_{153}$	Substitution in bHLH domain	Moderate hypomorph
$amos^3$	230 bp deletion+frameshift		Severely truncated protein	Null?

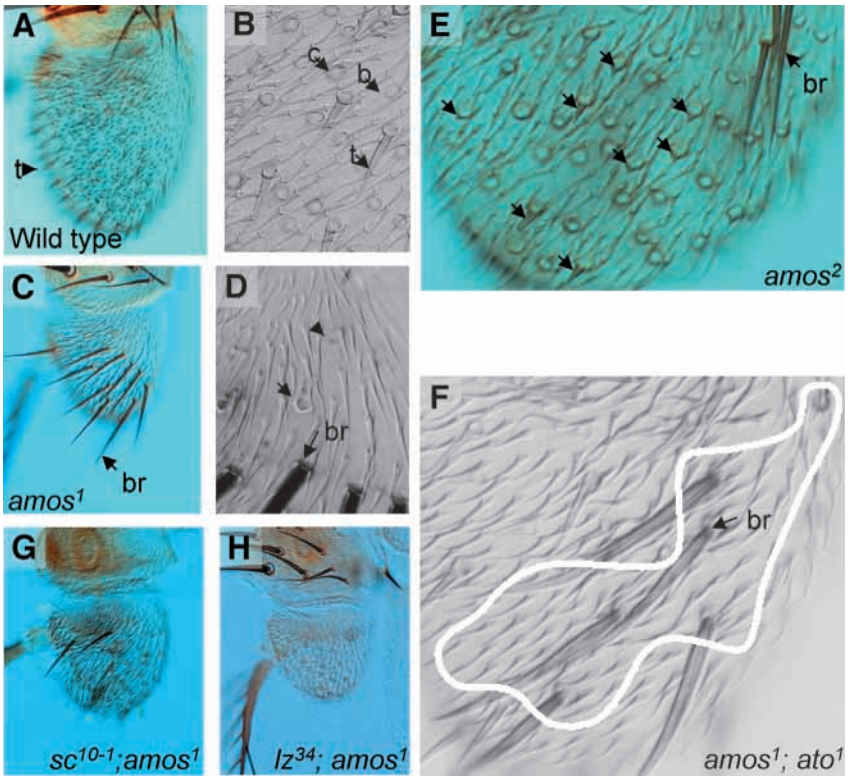

Fig. 1. Antennal defects in *amos* mutants. Third antennal segments are shown. (A) Wild-type antenna, with trichodea indicated (t). (B) Higher power view with examples marked of sensilla coeloconica (c), basiconica (b) and trichodea (t). (C) *amos*^{1}*/Df(2L)M36-S6*. Third segment is reduced because of missing basiconica and trichodea. Ectopic mechanosensory bristles are indicated (br). (D) Higher power view showing an abnormal domed sensillum (arrow) and a normal sensillum coeloconicum (arrowhead). Sensilla are very sparse. (E) *amos*^{2} with numerous abnormal sensilla (arrows). (F) Double mutant for *amos* and *ato* (clone of *amos*^{1} tissue in *ato*^{1} fly: *eyFLP; FRT-amos*^{1}*/FRT-nlsGFP; ato*^{1}). The clone patch contains no sense organs except bristles. (G) Double mutant for *amos* and *sc*$^{10-1}$ (which removes both *ac* and *sc* function). The extra bristles of *amos*^{1} are largely dependent on *ac/sc* function. (H) Double mutant for *amos* and *lz*. The extra bristles of *amos*^{1} are absent and therefore depend on *lz* function.

sensilla basiconica and trichodea were completely absent in the probable genetic nulls, *amos*^{1} and *amos*^{3}, whereas sensilla coeloconica appeared unaffected (Table 2). These phenotypes support the assertion that *amos* is the proneural gene for sensilla basiconica and trichodea, whereas *ato* is the proneural gene for sensilla coeloconica. However, mutant antennae exhibit a further unexpected phenotype. In wild type, the third segment bears only olfactory sensilla; in *amos* mutant flies, this segment bears a number of ectopic external sensory bristles and other abnormally structured sensilla (Fig. 1C,D and Table 2). These bristles do not have bracts (unlike bristles on the leg), and so this phenotype does not represent a transformation of antenna to leg (c.f. Johnston et al., 1998). These phenotypes are highly unusual as a characteristic of all other loss-of-function proneural gene mutations is that they cause the loss of sense organ subsets without the concomitant appearance of new or abnormal sensory structures. Therefore, the *amos* null phenotype is unique for a *Drosophila* proneural gene.

Given its subtle molecular basis, the putative hypomorph *amos*^{2} has a surprisingly strong phenotype: it has no sensilla trichodea, and sensilla basiconica are reduced very substantially (Table 2). There are also fewer ectopic bristles than in the null alleles, but there are many sensilla of unusual morphology. In the case of this allele, these seem to represent intermediates between sensilla basiconica/trichodea and external sense organs (Fig. 1E).

Late pupal antennae were stained with a sensory neuron marker, MAb22C10, to visualise olfactory receptor neurons (ORNs). Olfactory sensilla are innervated by multiple sensory neurons (Shanbhag et al., 1999), which can be seen as groups in the wild-type antenna (Fig. 2A). *amos* mutant antennae have many fewer neuronal groups, corresponding in number to the sensilla coeloconica and the bristles (Fig. 2B). There are instances of sensilla innervated by a single neuron, which appear to correspond to the ectopic bristles (Fig. 2C,D). In wild-type flies, ORN axons form three olfactory nerves leading to the antennal lobe of the brain (Jhaveri et al., 2000b) (Fig. 2E). In *amos* mutant antennae, all three antennal nerves are still

Table 2. Sensillum numbers on adult antennae

Genotype	Basiconica	Trichodea	Coeloconica	Mechanosensory bristle	Mixed*
Wild type	177.5±8.6	114.5±3.5	70.3±1.5	0	0
amos^{1}	0	0	63.8±9.3	14.4±4.2	7.0±2.1
amos^{1}*/Df(2L)M36F-S6*	0	0	53.5±5.0	14.5±3.0	2.2±2.2
amos^{2}	9.6±5.6	0	62.8±7.1	13.3±4.5	15.2±3.8
amos^{2}*/Df(2L)M36F-S6*	5.8±3.4	0	52.0±1.4	12.5±3.9	21.5±2.6
amos^{3}*/Df(2L)M36F-S6*	0	0	72.6±6.4	20.8±0.8	3.6±2.2
lz^{34}; *amos*^{1}*/Df(2L)M36F-S6*	0	0	56.7±3.8	0	0
sc$^{10-1}$; *amos*^{1}	0	0	83.5±0.7	6.0±1.4	10.5±2.2
ase^{1}; *amos*^{1}*/Df(2L)M36F-S6*	0	0	58.2±8.3	14.8±2.2	6.8±2.9

Values are sensilla per antenna±s.d. (number of antenna scored, *n*=4-11).
'Mixed' refers to sensilla of undefinable morphology (*amos*^{1} and *amos*^{3}) or intermediate olfactory/bristle morphology (*amos*^{2}).

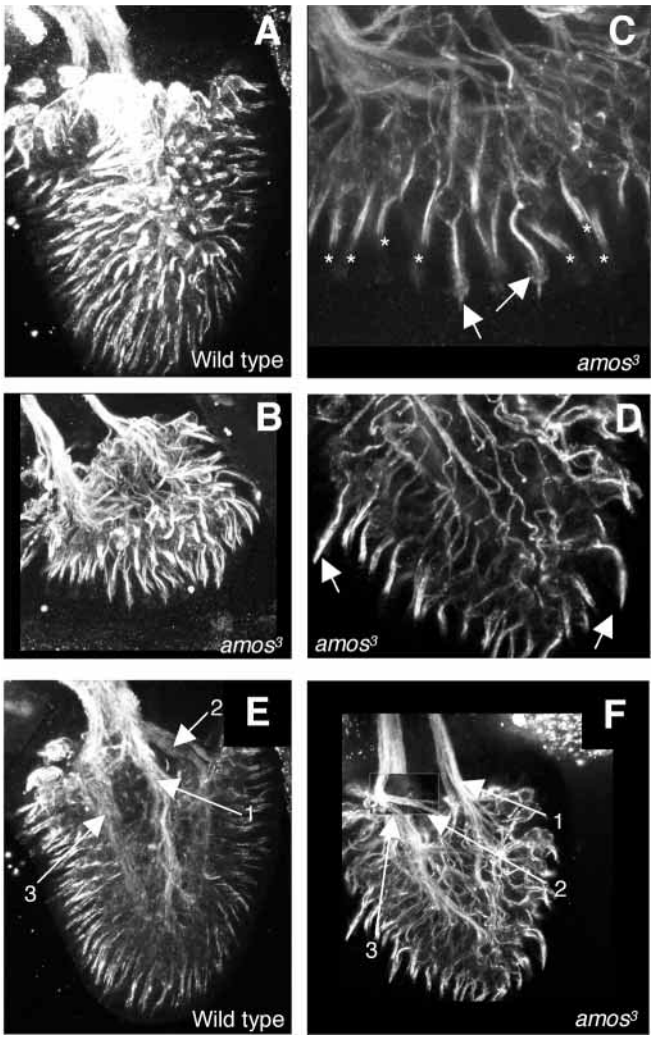

Fig. 2. Olfactory receptor neurons in *amos* mutants. Confocal projection images of late pupal antennae stained to detect sensory neurons. (A) Wild type. Clusters of cell bodies and their dendrites can be seen. (B) *amos*^{3} mutant showing far fewer clusters. (C,D) Higher magnification views. Although most ORNs are clustered, as seen by the multiple dendrites (*) (representing sensilla coeloconica), some sensilla appear to be mono-innervated (arrows) and may represent the bristles. (E) Wild-type confocal section showing the three olfactory nerve bundles. (F) Confocal section of *amos* mutant, with the three bundles labelled (a small section has been pasted in from another confocal plane to show clearly the second bundle).

present, although consisting of fewer axons as expected (comprising the axons of *ato*-dependent ORNs) (Fig. 2F). Although thinner, the fascicles appear normal in structure and location. Thus, in contrast to *ato* (Jhaveri et al., 2000b), mutations of *amos* do not cause defects in routing or fasciculation of the olfactory nerves. This supports the conclusion that the *ato*-dependent sensory lineage provides the information for fasciculation of these nerves.

amos expression prefigures a late, *ato*-independent subset of olfactory precursors

Olfactory precursors arise in the pupal antennal imaginal disc over an extended period of time (Ray and Rodrigues, 1995). Given their high density, the appearance of olfactory precursors is complex and incompletely characterised. We initially characterised the evolution of this pattern by studying Senseless (Sens; Lyra – FlyBase) expression, which is a faithful indicator of proneural-derived sensory precursors and is probably a direct target of proneural proteins (Nolo et al., 2000). We found that precursor formation occurs in three waves. First, Sens expression begins a few hours before puparium formation (BPF) in an outer semicircle of cells (Fig. 3A). A second wave begins at 0-4 hours after puparium formation (APF) to give a very characteristic pattern, including three semicircles of precursors (Fig. 3B). After this, a third wave appears over an extended period of time, with increasing numbers of cells appearing intercalated between the early precursors until no spatial pattern features can be observed (Fig. 3C,D).

Using a polyclonal antibody raised against the entire Amos protein, we determined that *amos* expression begins at puparium formation in three distinct semicircles and then continues for the next 16 hours, with the semicircles becoming indistinct by around 8 hours APF (Fig. 3E-H). The characteristic early waves of SOPs arise between the Amos domains of expression and do not show overlap with Amos expression (Fig. 3I-L). However, the third wave of SOPs appears to arise from the Amos expression domains. These late SOPs co-express Amos, and their nuclei lie beneath the Amos expression domains, consistent with these cells being olfactory SOPs (Fig. 4E,F and data not shown). Unusually, overlying *amos* proneural cluster expression is evidently not affected by lateral inhibition upon the appearance of the SOPs. *ato* is expressed much earlier than *amos*. All wave 1 and 2 SOPs appear to express Ato or to have arisen from Ato-expressing cells (see also Jhaveri et al., 2000a) (Fig. 4A-D). Consistent with this, the entire early SOP pattern is missing in antennal discs from *ato*^{1} mutant pupae (Fig. 5A-C). SOPs only begin to appear between 4 and 8 hours APF, corresponding to the third wave of precursors. These coincide very precisely with Amos expression, which itself appears unaffected (Fig. 5D-F).

In summary, there are three waves of olfactory precursor formation (Fig. 4G). The first and second waves are well defined, giving rise to the sensilla coeloconica of the sacculus and the antennal surface, respectively. These precursors express and require *ato*. The third wave of precursors is much more extensive and has little obvious pattern, giving rise to the more numerous sensilla basiconica and trichodea. Amos is expressed in a pattern entirely consistent with it being the proneural gene for the late wave precursors. Expression of Amos is complementary with that of Ato and is independent of Ato function. Thus, Ato- and Amos-expressing SOPs show a degree of spatial and temporal separation.

amos appears to be expressed in proneural domains and then in SOPs. For *sc* and *ato*, these two phases of expression are driven by separate enhancers, and SOP-specific enhancers have been identified (Culí and Modolell, 1998; Sun et al., 1998). A 3.6 kb fragment upstream from *amos* was found to support GFP reporter gene expression in the pupal third antennal segment. Comparison with Amos and Sens expression showed that GFP coincides with the Amos but not Ato SOPs (Fig. 6A,B). This fragment therefore contains an *amos* SOP enhancer. Perduring GFP expression driven by the enhancer

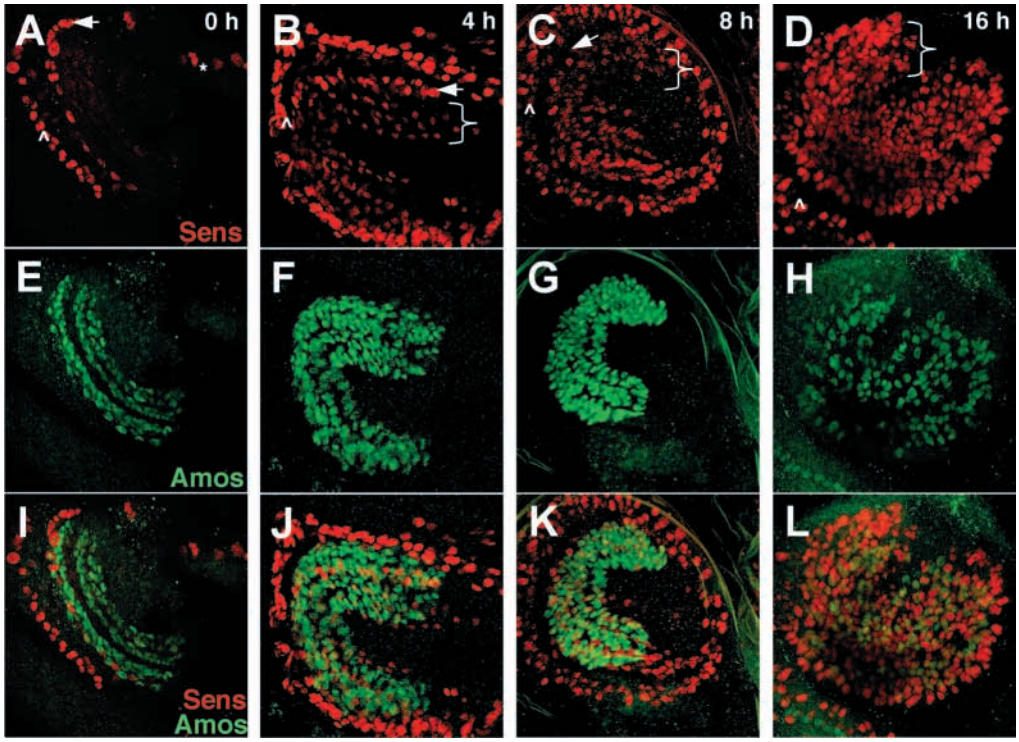

Fig. 3. Amos expression during olfactory precursor formation. (A-L) Time course of Amos protein expression relative to olfactory precursor formation. In all cases we concentrate on precursors in the third segment, although a large number of chordotonal precursors are also visible in the surrounding second segment (carets; see also the summary in Fig. 4). (A,E,I) At 0 hours APF, the first wave of precursors appear (arrow). (B,F,J) At 4 hours APF, the second wave of precursors appears in a highly characteristic pattern (bracket). (C,G,K) At 8 hours APF, the third wave of precursors accumulate between the rows of the second wave, eventually obscuring any clear pattern by 16 hours APF (D,H,L). (A-D) Amos expression is detected throughout this time, but the expression is ectodermal from 0-4 hours APF, and then it co-labels with some of the precursors between 4-16 hours APF. Amos continues to be expressed in some cells at 16 hours APF, and these cells seem to be a mixture of ectodermal cells and precursors.

can be observed in large numbers of sensilla on the maturing pupal antenna. From their morphology, it is clear that the GFP-expressing subset are the sensilla trichodea and basiconica (Fig. 6G). This confirms that early SOPs form sensilla coeloconica whereas late SOPs produce sensilla trichodea and basiconica. Interestingly, these GFP-expressing sensilla differentiate late, because there is no overlap with the 22C10 marker until late in development (Fig. 6C,D). Thus, the timing of neuronal differentiation reflects the timing of SOP birth. These findings correlate with the differing effects of proneural genes on fasciculation as described above: the first-born *ato*-dependent cells organise the nerves, and the later *amos*-dependent ORNs follow passively.

Loss of olfactory precursors in *amos* mutants

Loss of SOPs is one of the defining characteristics of proneural gene mutations. We examined SOP formation in *amos* mutants relative to wild type by examining Sens expression. As expected from the expression analysis described above, the first two waves of SOP formation show little discernible difference in pattern between *amos* mutant antennal discs and wild-type discs (Fig. 5G,H). This is consistent with these early SOPs expressing and requiring *ato*, and they indeed express *ato* in a pattern indistinguishable from wild type (Fig. 5K). This shows that *ato* expression does not depend on *amos* function. After this, the later arising SOPs do not appear to form between rows of *ato*-dependent SOPs, corresponding to those cells shown to express *amos* (compare Fig. 5K with Fig. 4D). A few precursors do not express *ato*, and these may represent precursors of the ectopic bristles (Fig. 5K). This is supported by an analysis of Cut expression, which is the key molecular switch that must be activated to allow SOPs to take a bristle fate (Bodmer et al., 1987; Blochlinger et al., 1991), and whose expression correlates with bristle SOPs (Blochlinger et al., 1990). In the wild-type antenna, Cut is not expressed during olfactory SOP formation (Fig. 5M), although later it is expressed in differentiating cells of all olfactory sensilla (Fig. 6I and data not shown). This expression normally appears after 16 hours and does not overlap with Amos. In *amos* mutant antennae, expression begins earlier than normal in a subset of SOPs that appear to correspond to the ones identified above (Fig. 5N).

By 16 hours APF, there is a large loss of Sens staining in *amos* mutants (Fig. 5I,L). The remaining cells tend to be in clusters as would be expected for the early *ato*-dependent sensilla, but otherwise the identity of these cells cannot be determined. Detection of Amos protein in *amos*^{1} mutant antennal discs also shows that although the Amos domains are still present, the deeper Amos/Sens-expressing nuclei are absent (Fig. 5J). Thus, at least a large number of *amos*-associated SOPs are not formed in the *amos* mutant.

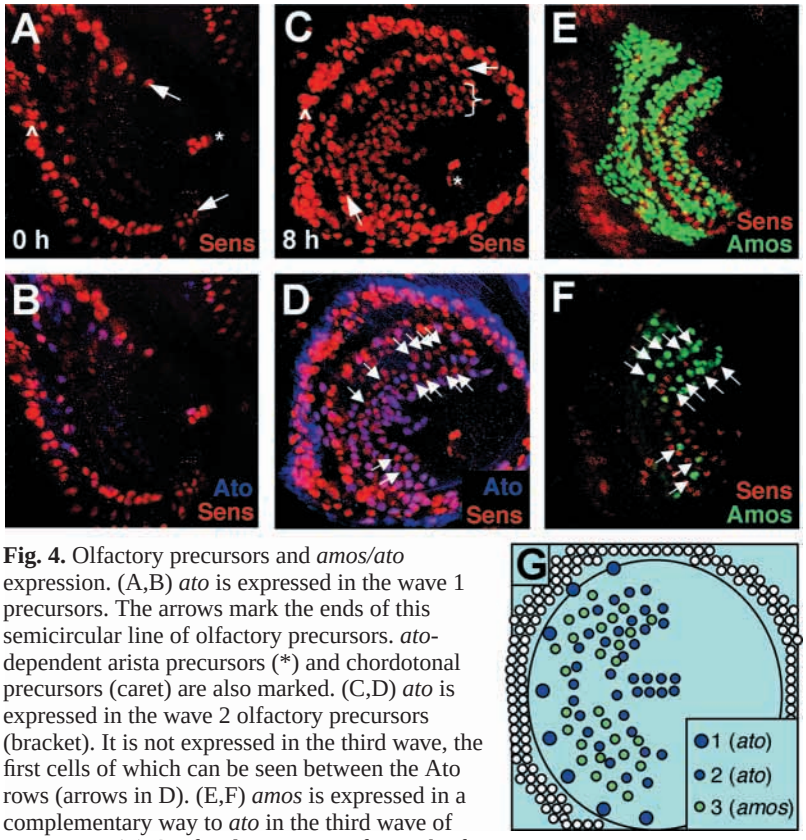

Fig. 4. Olfactory precursors and *amos/ato* expression. (A,B) *ato* is expressed in the wave 1 precursors. The arrows mark the ends of this semicircular line of olfactory precursors. *ato*-dependent arista precursors (*) and chordotonal precursors (caret) are also marked. (C,D) *ato* is expressed in the wave 2 olfactory precursors (bracket). It is not expressed in the third wave, the first cells of which can be seen between the Ato rows (arrows in D). (E,F) *amos* is expressed in a complementary way to *ato* in the third wave of precursors. (E) Confocal projection of a stack of images showing Amos detection in a similar disc to D. (F) Deep confocal section from E showing nuclei of *amos*-expressing precursors (arrows) underlying the main *amos* proneural expression domain. These correspond to the non-Ato-expressing precursors (arrows in D). (G) Schematic summary of *amos*- and *ato*-dependent precursor pattern at ~8 hours APF.

Expression of *amos* during sensillum development

The processes and lineages by which olfactory SOPs lead to the differentiated cells of the olfactory sensillum are not entirely known. The limited information available comes from analysis of the early wave of SOPs, which we have established represent the *ato*-dependent sensilla. After an SOP is selected there appears in its place a cluster of 2-3 cells expressing the A101 enhancer trap [the pre-sensillum cluster (PSC)]; this is apparently caused not by division of the SOP but perhaps by recruitment by the SOP (Ray and Rodrigues, 1995; Reddy et al., 1997), although the evidence for this is indirect. These PSC cells then divide to form the cells of the sensillum, including the outer support cells (hair and socket cells), inner support cells (sheath cells) and 1-4 neurons. For the early subset of SOPs, formation of the PSC occurs at a time in which *amos* is still expressed in the epithelial domains, and so *amos* could influence the development of these cells. Using A101 as a marker of the PSC cells, we determined that *amos* is not expressed in recognisable PSCs at 8 or 16 hours APF (Fig. 6E). Moreover, there is also no apparent co-labelling of Amos and Pros [a marker of one of the PSC cells (Sen et al., 2003)] (Fig. 6F). This suggests either that early PSC cells do not derive from *amos*-expressing cells or that *amos* is switched off rapidly when cells join a PSC.

The situation appears different for the cells derived from *amos*-dependent SOPs. Surprisingly at 24 hours and beyond, the *amos* enhancer drives GFP expression in most or all cells of the differentiating sensilla basiconica and trichodea (Fig. 6G): including most or all of the neurons (recognised by Elav expression; Fig. 6H); the sheath cell (recognised by Pros expression; Fig. 6H); and the outer support cells (recognised by the higher expression of Cut; Fig. 6I). This suggests that the late PSC cells do derive from *amos*-expressing cells and that activation of an enhancer within the 3.6 kb regulatory fragment (possibly separate from the SOP enhancer) is part of their specification process, although *amos* expression itself may not be long lived in these cells.

amos represses *scute* function

amos mutant antennae have Cut-expressing SOPs, but, although *cut* expression decides SOP subtype fate, it does not specify ectodermal cells as SOPs de novo. To investigate the involvement of other proneural genes, we first determined whether the bristles depended on *ato*, as it is expressed in close proximity to the emerging bristle SOPs. Clones of $amos^1$ mutant tissue were induced in ato^1 mutant antennae. In such clones, all olfactory sensilla were absent, as expected, but ectopic bristles were still formed (Fig. 1F). Therefore the bristles do not depend on *ato* function.

Cut expression normally follows from *ac/sc* proneural function, and so the ectopic bristle SOPs might depend on these proneural genes. Indeed, mutation of *ac* and *sc* greatly reduces the number of ectopic bristles in $amos^1$ flies ($In(1)sc^{10\text{-}1}/Y$; $amos^1/Df(2L)M36F\text{-}S6$ flies) (Fig. 1G and Table 2). By contrast, mutation of the non-proneural *ASC* gene *asense* (*ase*) had no effect alone (Table 2). This suggests that in the absence of *amos*, *ac/sc* function, to a large extent, causes the formation of bristle SOPs.

To determine how *amos* might normally repress bristle formation, we examined the pattern of *sc* mRNA in the pupal antenna. Significantly, a weak stripe of *sc* expression was observed in the wild-type antenna. (Fig. 7A). This stripe coincides with *amos* expression, and consists of ectodermal cells and SOPs (Fig. 7B,C). In the *amos* mutant antenna, *sc* mRNA expression was stronger and more clearly correlated with SOPs (Fig. 7C). This suggests that *sc* is expressed in olfactory regions of the wild-type antenna but that its function is repressed by the presence of *amos*. We therefore investigated *sc* functional activity in the antenna by analysing the expression of specific *sc* target genes as indicators of Sc protein function. Firstly, we examined Ac protein, whose expression is ordinarily activated by Sc function as a result of cross regulation (Gomez-Skarmeta et al., 1995). Ac protein is present in some SOPs in *amos* mutant antennae, but is not present in wild-type antennae (Fig. 7E,F). A similar result was observed for *sc-SOP-GFP*, which is a reporter gene construct that is directly activated by *sc* upon SOP formation (L. Powell and A.P.J., unpublished) (Culí and Modolell, 1998). This reporter showed GFP expression in some SOPs in *amos* mutant antennae but not in

wild-type antennae (data not shown). Finally, we examined *sc-E1-GFP*, a reporter gene construct comprising GFP driven solely by a *sc*-selective DNA binding site (L. Powell and A.P.J., unpublished) (Culí and Modolell, 1998). This reporter is invariably activated in all cells containing active Sc protein (including PNCs and SOPs) (L. Powell and A.P.J., unpublished). As with the other target genes, this reporter was only expressed in *amos* mutant antennae (Fig. 7G,H). Thus, we conclude that *sc* mRNA is expressed in the wild-type pupal antenna, and *amos* normally must repress either the translation of this RNA or the function of the Sc protein produced. This conclusion is supported by misexpression experiments. When *amos* is misexpressed in *sc* PNCs of the wing imaginal disc (*109-68Gal4*/UAS-*amos*) there is a dramatic reduction in bristle formation (Fig. 8A,B), even though endogenous *sc* RNA levels are unaffected (data not shown).

The transcription factor encoded by *lozenge* (*lz*) plays a number of roles in olfactory sensillum development, including activating *amos* expression (Goulding et al., 2000). Mutants therefore show a loss of many *amos*-dependent sensilla. Interestingly flies mutant for both *lz* and *amos* (lz^{34}; $amos^{1}/Df(2L)M36F\text{-}S6$) have third antennal segments that bear only sensilla coeloconica, and so the ectopic bristles of *amos* mutants are dependent on *lz* function (Table 2, Fig. 1H). Correlating with this, the expression of *sc* mRNA in the third antennal segment was much reduced in a *lz* mutant compared with wild type (Fig. 7D). Thus, *lz* appears at least partly responsible for the expression of *sc* in the antenna.

Discussion

We show definitively that *amos* is the proneural gene for the precursors of two classes of olfactory sensilla. These precursors are absent in *amos* mutants, resulting in highly defective antennae lacking all sensilla basiconica and trichodea. Unusually, this is not the only phenotype of *amos* mutants. Unique among *Drosophila* proneural genes, mutation of *amos* results in the appearance of new sense organs: mechanosensory bristles are now formed on the third antennal segment. We provide evidence that *amos* must normally repress *sc*-promoted bristle specification in addition to promoting olfactory neurogenesis. Significantly, inhibitory interactions between bHLH genes have recently been reported during mouse neurogenesis, where discrete domains of bHLH transcription factor expression are set up partly by mutual cross-inhibition combined with autoregulation (Gowan et

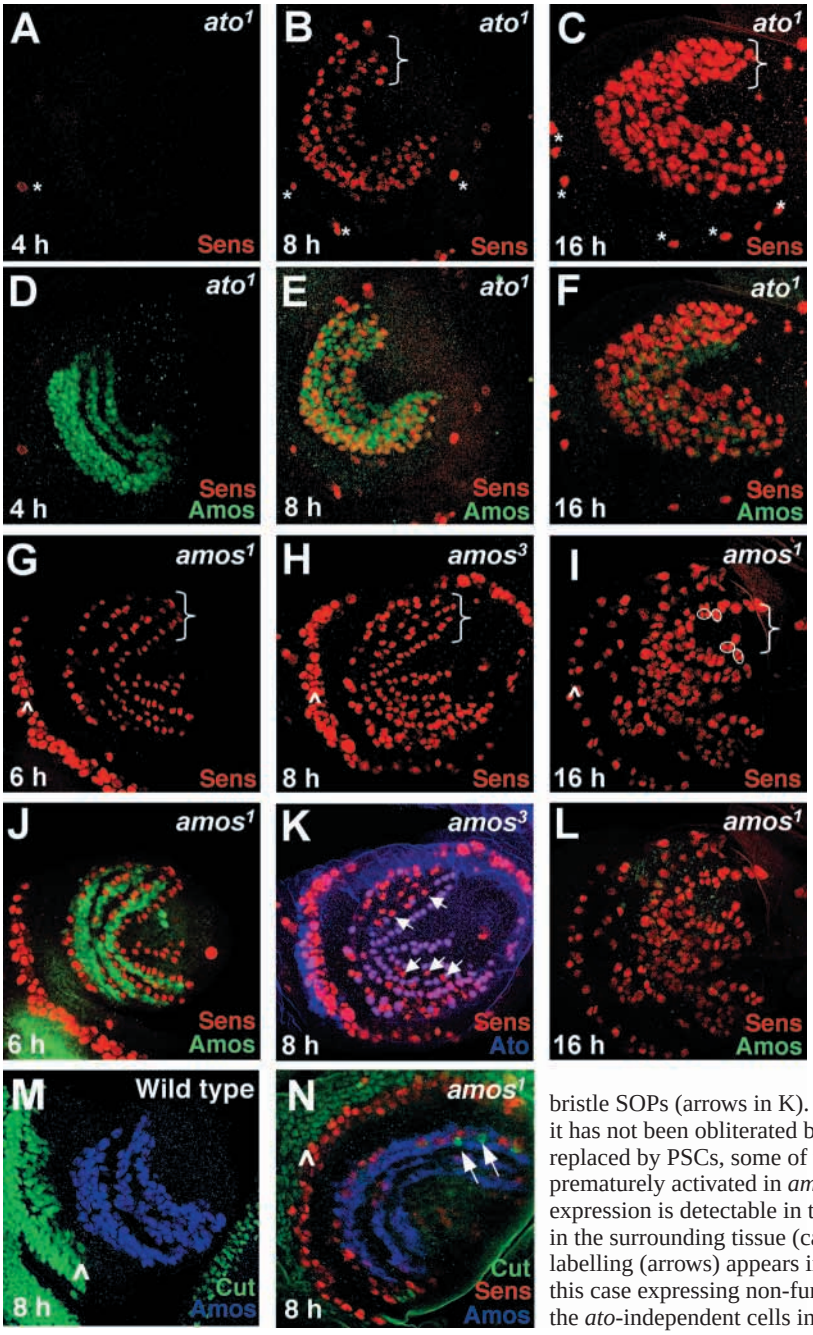

Fig. 5. Olfactory precursors in *amos* and *ato* mutants. These discs should be compared with the corresponding wild-type discs in Figs 3 and 4. (A,D) The early precursors are specifically lost in *ato* mutants. The remaining olfactory precursors correspond to the third wave (B,C) and align very closely with the *amos* expression domains (E,F). In the second segment, the chordotonal precursors are also missing and only a few bristle precursors remain (*, A-C). (G-L) The late precursors are specifically lost in *amos* mutants. (G,J) Early precursor pattern resembles wild type, with mutant $Amos^{1}$ protein detectable between the rows of precursors (brackets). Caret in G indicates chordotonal precursors. (H,K) At 8 hours APF, the pattern remains unchanged as the third wave SOPs are not formed (c.f. Fig. 4C). These early precursors mostly express Ato, although a number of non-Ato expressing SOPs appear between the early rows, which could correspond to the bristle SOPs (arrows in K). (I,L) The early pattern is still apparent at 16 hours APF as it has not been obliterated by the third wave of SOPs (the early SOPs have now been replaced by PSCs, some of which are ringed). (M,N) Cut expression appears prematurely activated in *amos* mutants. (M) Wild type at 8 hours APF. No Cut expression is detectable in the third segment SOPs; however, Cut stains very strongly in the surrounding tissue (caret). (N) $amos^{1}$ mutant at 8 hours APF. Some Cut labelling (arrows) appears in SOPs derived from the Amos-expressing domains (in this case expressing non-functional $Amos^{1}$ protein). These cells seem to correspond to the *ato*-independent cells in K.

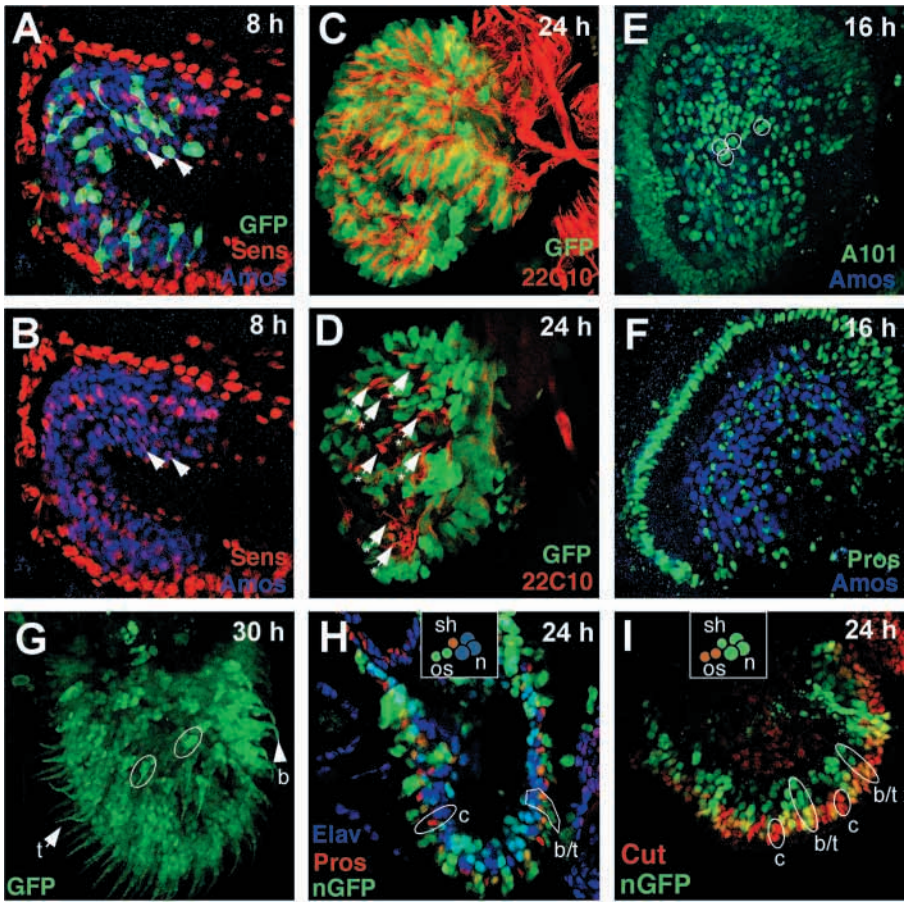

Fig. 6. Fate of *amos*-expressing cells during olfactory development. (A-D,G) Activity of an *amos* SOP enhancer driving GFP expression. (A,B) At 8 hours APF, GFP can be detected in the third wave of olfactory precursors, some co-labelled SOPs are indicated by arrows (co-labelled with Sens and Amos in the separation in B). (C,D) The *amos-GFP* expressing cells contribute to late differentiating sensilla, as shown by lack of co-labelling with a neuronal marker (22C10) at 24 hours APF. C is a projection of many sections whereas D is a confocal section with some of the differentiating neurons marked by asterisks, these do not express GFP. (E,F) Later expression of *amos* does not correspond to PSC cells. (E) At 16 hours APF, Amos expression is fading, but there is no overlap with A101 β-galactosidase expression in the PSCs, some of which are ringed. (F) There is no overlap of Amos expression with that of Pros, a marker of one of the PSCs. (G) *amos-GFP* construct at 30 hours APF: a large number of sensilla retain GFP. Protein appears to be in sensillar groups (as indicated by rings), and includes the outer support cells, so that sensilla trichodea (t) and basiconica (b) can clearly be discerned. (H,I) Analysis of *amos-GFP* in confocal sections of antennae at 24 hour APF relative to the component cells of the sensilla (see insets). n, neuron; sh, sheath; os, outer support cells. *amos-GFP* labels rows of cells corresponding to each sensillum basiconicum or trichodeum (some are ringed), whereas presumptive coeloconica (c) do not express GFP. (H) GFP is expressed in neurons (marked by Elav) and sheath cells (marked by Pros). (I) GFP is expressed in outer support cells (marked by stronger expression of Cut).

al., 2001; Nieto et al., 2001). As with *amos*, cross-inhibition occurs between members of different bHLH families: *Mash1* (ASC homologue), *Math1* (*ato* homologue), and *neurogenin1* (*tap* homologue).

How proneural genes determine neuronal subtype

On misexpression evidence, we have argued that neuronal subtype specification involves repression of bristle fate by *ato* during chordotonal SOP formation (Jarman and Ahmed, 1998) and by *amos* during olfactory precursor formation (Goulding et al., 2000). In this light, the ectopic bristles in *amos* mutants are of significant interest. They represent the first loss-of-function evidence that an *ato*-type proneural gene suppresses bristle fate during the normal course of its function. However, how this relates to *amos* function is complex. In misexpression experiments, bristle suppression by *amos* is most strongly observed using a PNC- and SOP-specific Gal4 driver line (Goulding et al., 2000) (this report). Yet paradoxically, misexpression of *amos* more generally in the ectoderm, but only weakly in SOPs, yields dramatically different results: in such cases *amos* produces ectopic bristles very efficiently (Huang et al., 2000; Lai, 2003; Villa Cuesta et al., 2003). This bristle formation does not require the function of endogenous *ac/sc* genes (Lai, 2003), but probably reflects the intrinsic SOP-specifying function of *amos* in situations that are not conducive to its subtype-specifying (and bristle suppressing) function. It appears therefore that bristle suppression particularly requires *amos* expression in SOPs.

What does *amos* repress in the antenna? It appears that *sc* is expressed within the wild-type *amos* expression domain during olfactory SOP formation. Clearly *amos* must prevent the function of *sc*, as *sc* expression in ectoderm usually results in bristle specification. It may be significant that some of the *sc* RNA is in olfactory SOPs in the wild-type antenna, suggesting that the SOP may be a major location of repression by *amos*, as indicated by misexpression experiments. However, some bristle formation is maintained in *ac/sc; amos* mutants. This may be due to redundancy with other genes in the ASC: certainly wild-type bristle formation outside the antenna is not completely abolished in the absence of *ac/sc* (A.P.J., unpublished). An alternative possibility is that some bristle SOPs result from other proneural-like activity in the antenna. Direct proneural activity of *lz* is a possibility, although misexpression of *lz* elsewhere in the fly (using a *hs-lz* construct) is not sufficient to promote bristle formation (P.I.z.L., unpublished).

The *amos*^{2} hypomorph appears to represent a different situation. In such flies, a number of *amos*-dependent SOPs appear to have mixed olfactory/bristle fate. This suggests that on occasions the mutant Amos^{2} protein is able to specify SOPs, but is less able to impose its subtype function (and so this, to some extent, resembles more the outcome of some misexpression experiments). *amos*^{2} may therefore be a useful

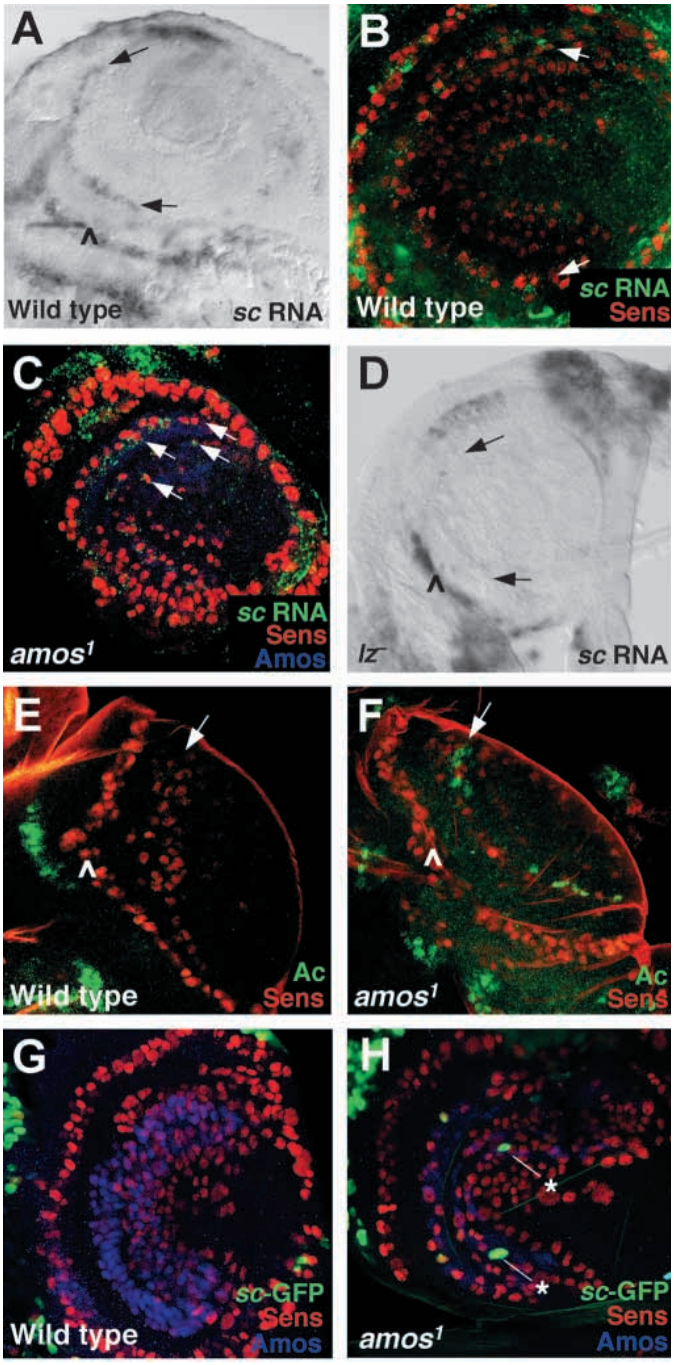

Fig. 7. Expression of *sc* and *sc* target genes in the antenna. (A-D) *sc* mRNA detected by in situ hybridisation. (A) Wild type, with *sc* expressed not only in the second antennal segment (caret) but also in the third segment (arrows). (B) Wild type, with sc RNA detected by immunofluorescence (green). (C) *amos*^{1} mutant. *sc* mRNA is increased and is present in SOPs (arrows). (D) The second segment *sc* expression is reduced in *lz*^{34} mutants. (E,F) Ac expression is present in some SOPs in *amos* mutants. (E) Wild type at 8 hours APF, showing very little Ac expression in the third segment (first precursor wave marked by arrow) (some is visible in the second segment; caret). (F) *amos*^{3} mutant at 8 hours APF, showing some Ac expression in second segment (arrow). (G,H) GFP expression from *sc-E1-GFP* reporter transgene. (G) Wild type, showing no expression in third segment. (H) *amos*^{3} mutant showing expression (*).

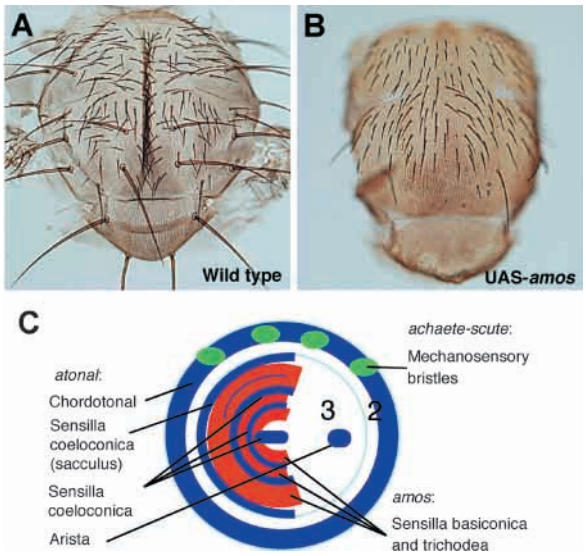

Fig. 8. *amos* misexpression represses bristle formation. (A) Wild type dorsal thorax. (B) Dorsal thorax from *109-68Gal4*/UAS-*amos* fly. *amos* misexpression driven in *sc* PNCs by this driver line results in loss of many bristles (mainly the large macrochaetae). (C) Summary of proneural functions in antenna. Diversity of sense organs laid down by function of three proneural gene systems. Blue, *atonal*; red, *amos*; green, *achaete/scute*.

tool for exploring these two functions. For example, if subtype specification requires interaction of Amos with protein co-factors (Jarman and Ahmed, 1998; Brunet and Ghysen, 1999; Hassan and Bellen, 2000), then these interactions may be specifically impaired in the *amos*^{2} mutant.

Because the proneural proteins are normally transcriptional activators, it is unlikely that Amos/Ato proteins directly inhibit gene expression during bristle suppression (Jarman and Ahmed, 1998). The presence of *sc* RNA in *amos*-expressing cells in the wild-type antenna is consistent with this. The involvement of protein interactions is to be suspected. An interesting parallel is found in vertebrates, where neurogenin1 promotes neurogenesis and inhibits astrocyte differentiation (Nieto et al., 2001). The glial inhibitory effect could be separated from the neurogenesis promoting effect: whereas neurogenesis promotion depends on DNA binding and activation of downstream target genes, astrocyte differentiation was inhibited through a DNA-independent protein-protein interaction with CBP/p300 (Sun et al., 2001; Vetter, 2001). In the case of *amos*, an interesting possibility is that inhibition of bristle formation may involve the sequestering of Sc protein by Amos protein. As discussed above, such a mechanism would have to be sensitive to the level or pattern of *amos*, as general misexpression does not mimic this activity.

Comparison of *amos* and *ato* as olfactory proneural genes

Apart from giving rise to separate classes of olfactory precursor, there are interesting differences in the way that *ato* and *amos* are deployed in the antenna. We characterised three waves of olfactory precursor formation (Fig. 4G). The first and second waves are well defined, giving rise to well-patterned

sensilla coeloconica of the sacculus and the antennal surface, respectively. These precursors express and require *ato*. The third wave of precursors is much more extensive and has little obvious pattern; it gives rise to the much more numerous sensilla basiconica and trichodea. This wave expresses and requires *amos*. For the early waves, *ato* is expressed according to the established paradigm: it is expressed in small PNCs, each cluster giving rise to an individual precursor (Gupta and Rodrigues, 1997). The pattern of the PNCs is very precise and prefigures the characteristic pattern of precursors. *amos* expression is dramatically different. It is expressed in large ectodermal domains for an extended period of time. Densely packed precursors arise from this domain continuously without affecting the domain expression. This shows that singling out does not necessarily require shut down of proneural expression, and therefore has implications for how singling out occurs. In current models, it is assumed that PNC expression must be shut down to allow an SOP to assume its fate. The *amos* pattern better supports the idea that a mechanism of escaping from or becoming immune to lateral inhibition is more likely to be important generally. One prediction would be that *amos* and *ato* (and *ac/sc*) differ in their sensitivities to Notch-mediated lateral inhibition, a situation that has been noted for mammalian homologues (Lo et al., 2002).

Why are the proneural genes deployed so differently? One possibility is simply that there are very many more sensilla basiconica and trichodea than coeloconica. All the coeloconica precursors can be formed by *ato* action in a precise pattern in two defined waves. This would not be possible for the large number of basiconica and trichodea precursors, and so precursor selection has been modified for *amos*. Indeed, *amos* appears to be a particularly 'powerful' proneural gene when misexpressed (Lai, 2003; Villa Cuesta et al., 2003). This may make *amos* a useful model of other neural systems in which large numbers of precursors must also be selected.

For most insects, the antenna is the major organ of sensory input. It is not only the site of olfaction, but also of thermoreception, hygroreception, vibration detection and proprioception, as well as of touch. Patterning the sensilla is therefore complex and three types of proneural gene are heavily involved to give different SOPs (Fig. 7G). It is clear that the study of antennal sensilla will provide a useful model for exploring the fate determining contribution of intrinsic bHLH protein specificity and extrinsic competence factors.

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