Invagination centers within the *Drosophila* stomatogastric nervous system anlage are positioned by *Notch*-mediated signaling which is spatially controlled through *wingless*

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SUMMARY

The gut-innervating stomatogastric nervous system of *Drosophila*, unlike the central and the peripheral nervous system, derives from a compact, single layered epithelial anlage. Here we report how this anlage is initially defined during embryogenesis by the expression of proneural genes of the *achaete-scute complex* in response to the maternal terminal pattern forming system. Within the stomatogastric nervous system anlage, the *wingless*-dependent intercellular communication system adjusts the cellular range of *Notch*-dependent lateral inhibition to single-out three *achaete*-expressing cells. Those cells define distinct invagination centers which orchestrate the behavior of neighboring cells to form epithelial infoldings, each headed by an *achaete*-expressing tip cell. Our results suggest that the *wingless* pathway acts not as an instructive signal, but as a permissive factor which coordinates the spatial activity of morphoregulatory signals within the stomatogastric nervous system anlage.

Key words: cell signaling, cell selection, *Drosophila* neurogenesis, stomatogastric nervous system, tip cell

INTRODUCTION

Neurons are generated by the proliferation of progenitor cells that develop from undifferentiated and pluripotent ectodermal cells at an early stage of embryogenesis. In insects, recent work has focused on the origin of the central (CNS) and the peripheral nervous system (PNS) (reviewed by Campos-Ortega, 1993; Jan and Jan, 1993). Cell ablation experiments in the grasshopper embryo highlighted two important features of neural precursor formation in insects (Doe and Goodman, 1985). First, distinct groups of ectodermal epithelial cells display the potential to form the precursor cells, and second, cell interactions result in the selection of a single cell within each cell group that can potentially form a neural precursor. Mutagenesis screenings in *Drosophila* identified two classes of genes that regulate each of these processes. The 'proneural genes' (Romani et al., 1987; Ghysen and Dambly-Chaudiere, 1989) control competence of ectodermal cells to become neural precursor cells, and the activities of the 'neurogenic genes' (Poulson, 1937; Lehmann et al., 1981) prevent more than one among the competent group of cells from adopting a neural fate. Once identified, the neural precursors, termed neuroblasts (CNS) or sensory organ mother cells (PNS), delaminate from the ectodermal epithelium and give rise to the characteristic and stereotyped patterns of neurons and axonal connections observed during late stages of embryogenesis (reviewed by Campos-Ortega, 1993; Goodman and Doe, 1993; Jan and Jan, 1993).

Proneural genes include the three transcription units of the *achaete-scute* complex (*AS-C*) (Garcia-Bellido, 1979; Romani et al., 1987). The *AS-C* genes encode HLH-type transcription factors (Villares and Cabrera, 1987) which are thought to control the expression of neural-specific developmental genes. In mutants that fail to express the *AS-C* genes, neuroectodermal cells lose their ability to follow a neural fate and contribute instead to the epidermis (Garcia-Bellido, 1979; Jiménez and Campos-Ortega, 1979; Dambly-Chaudiere and Ghysen, 1987; Jiménez and Campos-Ortega, 1990). The *AS-C* genes are initially expressed in small clusters of ectodermal cells (Cubas et al., 1991; Skeath and Carroll, 1992), the proneural cell clusters. From this cell cluster, the neural precursor cell is singled-out and continues *AS-C* gene expression (Cabrera et al., 1987). The singling-out of neural precursor cells within the proneural cell clusters is caused by a process of cell-cell communication referred to as lateral inhibition (Simpson, 1990) and results in a concomitant loss of *AS-C* gene expression in the other cells of the proneural cluster, which thereby restores an epidermal cell fate.

Singling-out by lateral inhibition is mediated by the activity of the neurogenic genes which are integrated into the *Notch*-mediated signaling pathway (Lehmann et al., 1981; Artavanis-Tsakonas et al., 1991; Fortini and Artavanis-Tsakonas, 1993). Within this intercellular communication system, the gene products of *Delta* (*Dl*) and *Notch* (*N*) seem to act as ligand and receptor, respectively (Heitzler and Simpson, 1991). Their activities are mediated by downstream components such as *Enhancer of split* (*E(spl)*) or *mastermind* (*mam*; reviewed by Campos-

Ortega, 1993). Loss-of-function mutants in any one of these genes fail to single-out *AS-C* gene-expressing cells and thereby show an overproduction of neural precursor cells both in the central and the peripheral nervous system (Brand and Campos-Ortega, 1988; Goriely et al., 1991).

Here we describe the early development of the *Drosophila* stomatogastric nervous system (SNS), a system which coordinates ingestion, swallowing and peristaltic movements of the gut (reviewed by Penzlin, 1985). The SNS is composed of four ganglia which derive from a compact epithelial anlage within the dorsal roof of the stomodeal invagination. Once the SNS anlage is formed in response to positional information provided by the maternal terminal system (Perrimon, 1993), three distinct *AS-C*-expressing cells are singled-out under the control of the *N*-mediated signaling pathway. The range of *N*-mediated signaling depends on *wingless* (*wg*) activity. Our results suggest that *wg* acts as a permissive rather than instructive component within the SNS anlage.

MATERIALS AND METHODS

Drosophila strains and mutant embryos

Drosophila strains were kept under standard conditions. Mutant alleles are described in Lindsley and Zimm (1992). Embryos were collected from stocks balanced with *FM7* (*Notch^{55e}* and *Df(1)B57,ac⁻ sc⁻ l'sc⁻*), *CyO* (*mastermind^{IB99}* and *wingless^{IG23}*) or *TM3, Sb* (*Delta^{9P}, Enhancer of split^{x1}, forkhead^{XT6}, huckebein^{A32}* and *Df(3)tailless^G*). The balancer chromosomes carried a *lacZ* reporter gene containing the *fushi tarazu* (*FM7*) or the *hunchback* promoter (*CyO* and *TM3*) which allow homozygous mutant embryos to be unambiguously identified on the basis of the lack of *hunchback* or *fushi tarazu* staining patterns.

tor^{PM} embryos derived from homozygous *tor^{PM}* females. *Df(1)B57; Kr^l* double mutant embryos were distinguished on the basis of the lack of external sense organs in the dorsal epidermis of the trunk and on the basis of the segmentation phenotype of *Kr* (Gaul and Jäckle, 1987). *dsh^{v26}*, *arm^{XM19}* and *sgg^{M11-1}* mutant embryos derived from females bearing homozygous mutant germ line clones. Germ line clones were generated by the 'FLP-DFS' technique as described by Siegfried et al. (1994). These females were mated to *FM7, ftz-lacZ* males. This allows one to distinguish the respective mutant embryos that have received neither the maternal nor the zygotic wild-type product by the absence of *ftz-lacZ* reporter gene expression.

Staining and embedding procedures

Antibody stainings were performed as previously described (Macdonald and Struhl, 1986). Antibodies were diluted at the following concentrations: 1:50 mAb22C10 (Fujita et al., 1982), 1:50 anti-fasciclin II (Grenningloh et al., 1991), 1:50 anti-fkh (Weigel et al., 1989b), 1:500 anti-Kr (Gaul and Jäckle, 1987), 1:3 anti-ac (Skeath and Carroll, 1992), 1:20 anti-crb (Tepass and Knust, 1993), 1:1000 rabbit anti-β-galactosidase (Cappel) and 1:200 mouse anti-β-galactosidase (Promega). Whole-mount in situ hybridizations using digoxigenin-labeled probes for *l'sc*, *sc* and *wg* cDNAs were performed according to the method of Tautz and Pfeifle (1989). After antibody staining or whole-mount in situ hybridization, embryos were embedded in glass capillaries to allow rotation under the compound microscope (Prokop and Technau, 1993).

RESULTS

Morphology and origin of the *Drosophila* SNS

The SNS is located in the head region close to the embryonic

Fig. 1. The SNS of late *Drosophila* embryos. (A) Diagram illustrating the components of the SNS (red) and their relative positions with respect to morphological landmarks such as different portions of the gut and brain as seen by mAb22C10 or anti-fasciclin II antibody stainings (B-H); anterior is left. (B) Dorsal view of the SNS (mAb22C10 staining). The frontal ganglion (FG) is formed by two groups of cells which are interconnected by the frontal commisure (fcm). From a mid-dorsal position in the fcm, the frontal nerve (fn) projects anteriorly and innervates the two palisades of dorsal pharyngeal muscles. The recurrent nerve (rn) projects posteriorly and passes below the supraesophageal commisure (sec) connecting the brain hemispheres. At the posterior wall of the pharynx, the rn splits into two short branches connecting, to the right, with the esophageal ganglion 1 (EG1) and, to the left, with the esophageal ganglion 2 (EG2). (C) Lateral view of the embryo shown in B. The EG1 is formed by a cluster of 10 neurons in the posterior-most part of the pharynx (in focus in B) which extends into a row of seven pairs of neurons along the esophagus and a pair of neurons slightly separated from the others. (D) Different lateral focal plane showing that the rn splits to target the EG1 and EG2 at the posterior wall of the pharynx. EG2 connects to the proventricular ganglion (PG) via the proventricular nerve (pn). The PG is located on top of the proventriculus at a position where it connects to the esophagus (not shown). (E) Lateral view of an earlier stage 16 embryo (anti-fasciclin II antibody staining) showing that each cell group of the FG connects ipsilaterally to the subesophageal ganglion via the frontal connective (fcn). In the ventral cord of the CNS, a total of six fascicles within each longitudinal connective can be observed (Grenningloh et al., 1991; not shown). A dorsal fascicle and the most ventral one fasciculate together (arrowhead) at the subesophageal ganglion; they split again anteriorly to contribute to the brain neuropile (bn) and the fcn, respectively. (F) Different lateral focal plane showing that from a mid-dorsal position in the fcn, the rn and two posterior frontal connectives (pfc) project posteriorly. Each pfc runs on top of the pharynx and turns ventrally after passing below the sec to connect ipsilaterally to the subesophageal ganglion in a position near the meeting point of the fcn with the longitudinal connective at the subesophageal ganglion (SEG). In a dorsal position, neural elements of the ring gland (RG) can also be seen with anti-fasciclin II antibody staining. (G) Close-up of the proventriculus of a late stage 17 embryo (mAb22C10 staining). Two groups of nerves project from the PG. The first group is composed of three internal nerves (in) innervating the proventriculus. The proventriculus is made up of three layers as a consequence of the refolding of the gut during development: an external midgut layer (m) and two internal, recurrent (r) and esophageal (e), layers (Skaer, 1993). The three internal nerves run internally between r and e. The second group of nerves projecting from the PG is formed by three midgut nerves (mn) that are apposed externally to the proventriculus. Magnification is 4-fold higher as compared to the specimen shown in B-F. (H) Close-up of the midgut epithelium in a late stage 17 embryo (mAb22C10 staining). The mn projects on top of the midgut epithelium where they branch. Varicosities in the mn suggest the existence of synapses that coincide with the position of mAb22C10 stained cells (as well as other neural markers; not shown) integrated in the midgut epithelium (nc). Magnification is 4-fold higher as compared to the specimen shown in B-F. Orientation of embryos is anterior to the left; in C-F, dorsal side is up.

brain. It consists of four prominent interconnected ganglia (Fig. 1A; overview), i.e. the frontal ganglion (FG), the esophageal ganglia (EG1 and EG2) and the proventricular ganglion (PG) (González-Gaitán et al., 1994). They can be visualized by mAb22C10 (Fujita et al., 1982) (Fig. 1B-D,G,H) or by anti-fasciclin II (Grenningloh et al., 1991) antibody staining (Fig. 1E,F) at late stages of embryogenesis (González-Gaitán et al., 1994; Hartenstein et al., 1994).
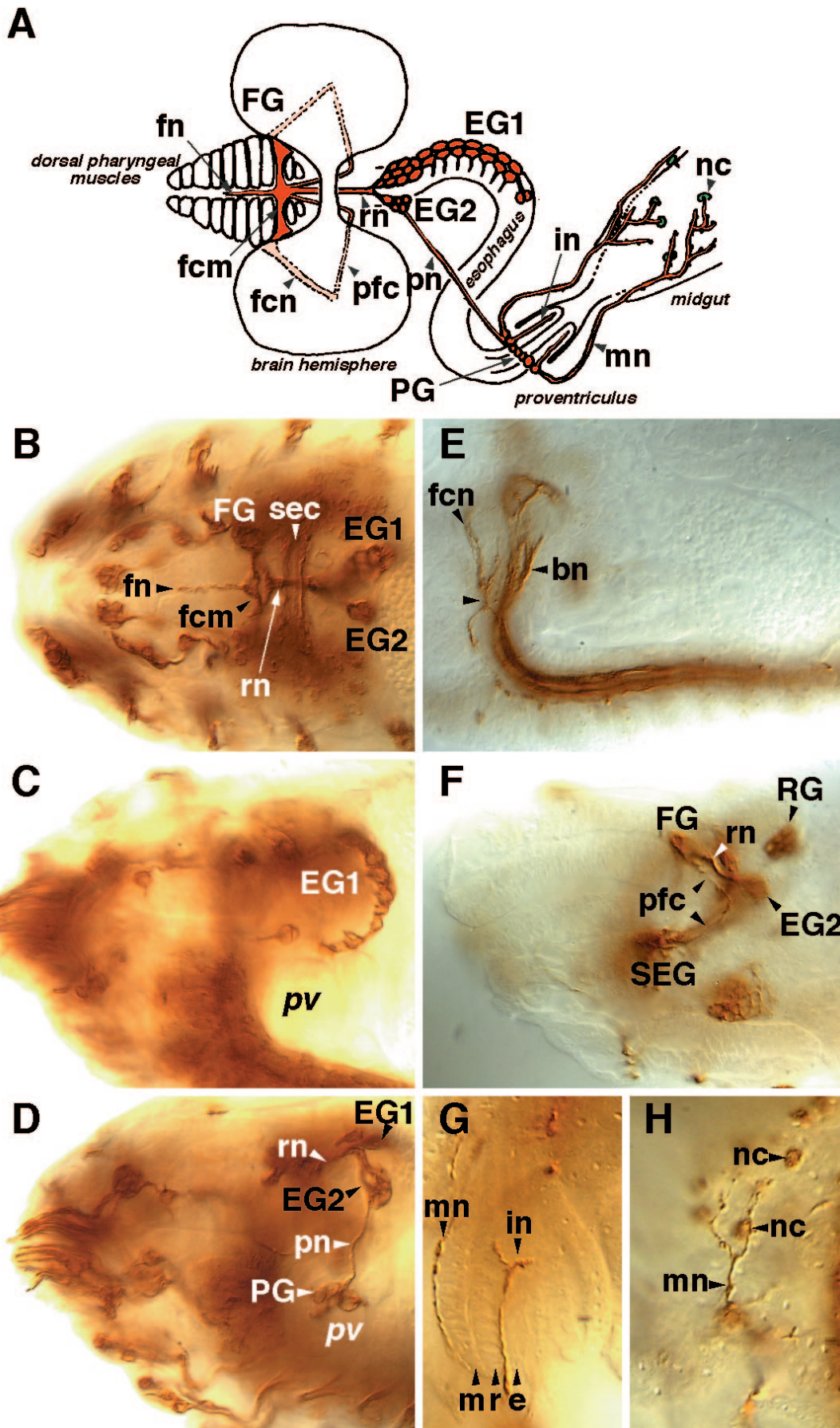
The FG is composed of two groups of neurons, one on either side of the pharynx (Fig. 1A,B). They are connected by axon bundles contributing to the frontal commisure (Fig. 1B). They also connect to the subesophageal ganglion of the CNS via the frontal connectives (Fig. 1E,F). EG1 and EG2 are associated with the esophagus (Fig. 1A,D,F). EG1 extends as a row of seven pairs of neurons apposed to the esophagus (Fig. 1C); EG2 is composed of about 10 tightly clustered neurons (Fig. 1B,D). Both ganglia are connected to the FG via the recurrent nerve (Fig. 1B,D,F). The PG is formed by a cluster of about 10 neurons which are apposed to the proventriculus (Fig. 1A,D); Skaer, 1993). The PG is connected to the EG2 via the proventricular nerve (Fig. 1D). Axons projecting from different SNS ganglia innervate distinct portions of the gut, e.g. the FG innervates the dorsal pharyngeal muscles via the frontal nerve (Fig. 1B), the EG1 innervates the esophagus (Fig. 1C), and the PG innervates both the proventriculus and the midgut (Fig. 1G,H).

The developmental origin of the SNS ganglia can be traced back to a compact, single-layered epithelial anlage at the dorsal roof of the stomodeal invagination (Fig. 2A; Poulson, 1937; Schoeller, 1964; Campos-Ortega and Harten-stein, 1985). During stage 10 (stages according to Campos-Ortega and Hartenstein, 1985), the stomodeal invagination is characterized by the expression of *forkhead* (*fkh*; Fig. 2B), a gene involved in gut development (Weigel et al., 1989a,b). At this stage, the SNS anlage is spatially defined by the localized expression of the *AS-C* proneural genes *achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*) (Romani et al., 1987), and by the expression of the gap gene *Krüppel* (*Kr*; Gaul and Jäckle, 1987) (Fig. 2C-G).

SNS development becomes mor-phologically distinct by the appear-ance of three evenly spaced dorsal invaginations (Schoeller, 1964; Campos-Ortega and Hartenstein, 1985; Hartenstein et al., 1994; Fig. 3A), which can be visualized by anti-crumbs (crb) antibody staining (Tepass and Knust, 1993), by *fkh* and by *Kr* expression (Fig. 3C-F). At stage 14, the invaginations detach from the developing esophagus, and each forms a vesicular structure (Fig. 3B; see also Hartenstein et al., 1994) which continues to express *fkh* (Fig. 3C-F) until the stereotyped pattern of the SNS ganglia emerges (cf. Fig 1B,C and Fig. 3G,H; see González-Gaitán et al., 1994; Hartenstein et al., 1994).

SNS anlage formation requires activity of the maternal terminal system

The functional significance of *fkh*, *Kr*

and *AS-C* gene expression for SNS development is apparent from the mutant phenotypes (Figs 4,5). *fkh* mutant embryos lack the SNS (Fig. 4A,B) and fail to express *Kr* and the *AS-C* gene expression in the SNS anlage (Fig. 5A,B). In the absence of *AS-C* gene activities, remnants of SNS structures can be observed (Fig. 5C). In the absence of *Kr*, SNS ganglia are formed (Fig. 5D). Thus, *AS-C* gene expression is required for the normal SNS development, whereas *Kr* activity is not necessary. However, the *AS-C* mutant phenotype is strongly enhanced in embryos lacking both *Kr* and *AS-C* gene activities (Fig. 5E). This observation suggests that *Kr* and *AS-C* genes may function in a common developmental pathway, implying a proneural-like function of *Kr* during an early stage of SNS development.

Since *fkh* expression requires the maternal terminal signal transduction pathway (Gaul and Weigel, 1990; Weigel et al., 1990), we examined the SNS of embryos lacking the activity of *torso* (*tor*). *tor* encodes a membrane-spanning tyrosine kinase receptor activity (Casanova and Struhl, 1989; Sprenger et al., 1989). Upon local activation, *tor* initiates the terminal
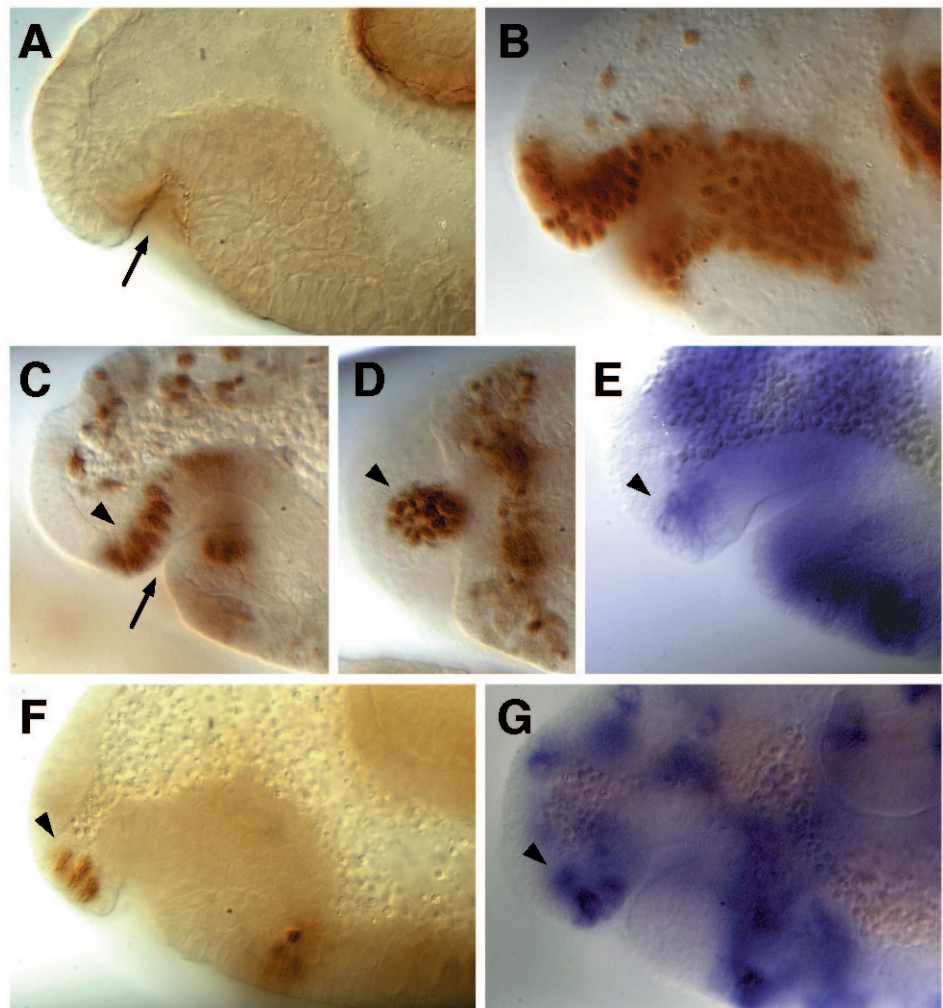
Fig. 2. Gene expression in the SNS neural anlage. Anti-crumbs (A), anti-forkhead (B), anti-Krüppel (C,D) and anti-achaete (F) antibody staining and *lethal of scute* (E) and *scute* (G) in situ hybridization in head regions of wild-type embryos at stage 10. Arrows point to the stomodeal invagination; arrowheads point to the SNS anlage. Except in D (ventral view), lateral views are shown; anterior is left, dorsal up.
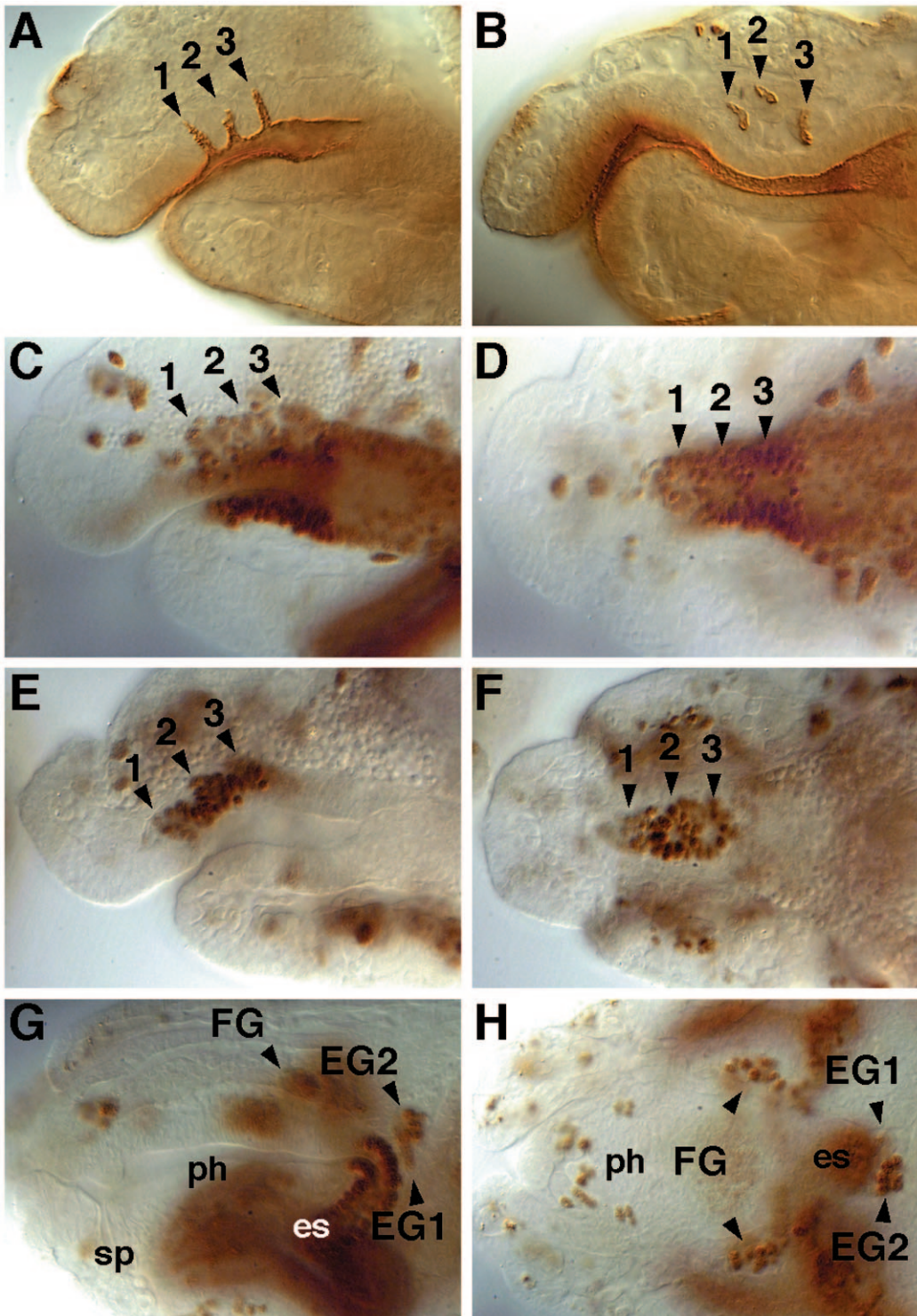
Fig. 3. Invagination and vesiculation of the SNS primordium. (A) Head region of a wild-type embryo stained with anti-crumbs antibody showing three distinct invaginations (1,2,3) in the roof of the stomodeum during stage 11. (B) The invaginations detach from the lumen of the foregut (not shown), and at stage 14, three vesicles containing neural precursors segregate dorsally. (C,D) Head region of a stage 11 wild-type embryo stained with anti-forkhead antibody. (C) Lateral and (D) dorsal view of the same embryo. (E,F) Head region of a stage 11 wild-type embryo stained with anti-Krüppel antibody (E) Lateral and (F) dorsal view of the same embryo. (G,H) Head region of a stage 16 wild-type embryo stained with anti-forkhead antibody. (G) Lateral and (H) dorsal view of the same embryo. Note forkhead expression in the esophagus (es), the proventriculus (out of focus) and the SNS ganglia (FG, EG1 and EG2; PG, out of focus), but not in the neural elements of the ring gland (see Fig. 1F). Orientation of the embryos is anterior to the left; in lateral views, dorsal up. Other abbreviations: ph, pharynx; sp, salivary gland pit.

signal transduction pathway (reviewed by Perrimon, 1993). Fig. 4C shows that embryos fail to develop SNS structures when *tor* activity is absent.

The activated *tor* signaling pathway eventually leads to localized zygotic target gene expression including the terminal gap genes *tailless* (*tll*) and *huckebein* (*hkb*) (Weigel et al., 1990). The activities of both genes are required for *fkh* expression (Gaul and Weigel, 1990; Weigel et al., 1990). *tll* or *hkb* embryos fail to develop normal SNS ganglia, although

remnants can be observed (Fig. 4D,E). This suggests that *hkb* and *tll* act through *fkh* and that the remaining *fkh* activity observed in their absence (Gaul and Weigel, 1990; Weigel et al., 1990) is sufficient to generate some SNS remnants.

Invagination centers are selected by *N*-mediated signaling

ac, *sc* and *l'sc* are initially expressed in all cells of the SNS anlage (Fig. 2E-G). While *l'sc* expression continues during the
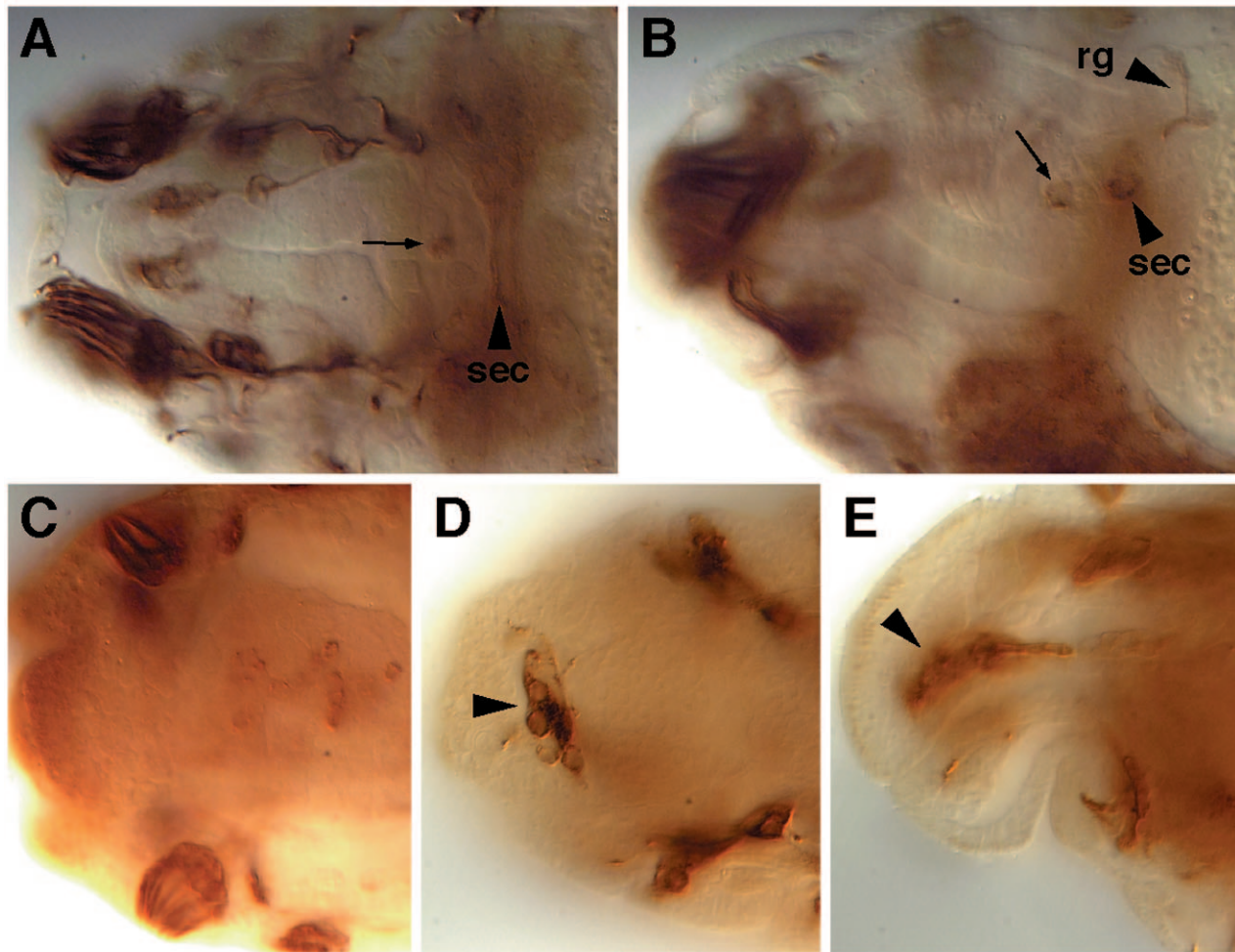
Fig. 4. Maternal and zygotic components of the terminal system are necessary for SNS development. (A,B) fkh^{XT6}, (C) tor^{PM}, (D)hkb^{A32} and (E) tll^{G} mutant embryos stained with mAb22C10 at stage 17. (A) Dorsal and (B) lateral view of the same fkh^{XT6} mutant embryo where SNS ganglia fail to develop. Occasionally a single cell stained with mAb22C10 (arrow) can be detected anterior to the supraesophageal commisure (sec) where the FG is normally located. Dorsally, the ring gland (rg) remains unaffected, confirming its origin outside the stomodeal SNS anlage (cf. Fig. 3G,H). (C) Embryos deriving from homozygous tor^{PM} females fail to develop SNS ganglia, while in hkb^{A32} (D) and tll^{G} (E) homozygous embryos remnants of the SNS (arrowheads) are observed. (C,D) Dorsal view; (E) lateral view. Orientation of the embryos is anterior to the left and dorsal up (lateral views).

subsequent invagination process (Fig. 6A,B), both *ac* and *sc* expression become restricted to three cells (Fig. 6C,E). The singled-out *ac*- and *sc*-expressing cells represent invagination centers in the SNS anlage and they characterize the tip cells of each invagination (Fig. 6G,H). Once the folds are maximally extended, *ac* and *sc* become re-expressed throughout the epithelial folds and consequently all SNS cells undergo a second round of proneural gene expression (Fig. 6B,D,F).

In order to establish the genetic requirement for the singling-out of invagination centers, we used *ac* expression as a marker to examine the selection of invagination centers in neurogenic mutant embryos that lack integral components of the *N*-mediated signaling pathway (Lehmann et al., 1981; Artavanis-Tsakonas et al., 1991; Fortini and Artavanis-Tsakonas, 1993). In *N* mutant embryos, initial *ac* expression covers the SNS anlage as in wild type (not shown). During late stage 10, however, when the three *ac*-expressing cells are normally singled-out, *ac* expression continues throughout the SNS anlage (Fig. 7A). Instead of forming distinct invagination

folds, the *N* mutant SNS anlage invaginates *en masse* (Fig. 7E). In *Dl*, *E(spl)* or *mam* homozygous mutant embryos, *ac* expression is also not restricted (Fig. 7B-D), and the SNS anlage invaginates as observed in the *N* mutants (Fig. 7F-H). These results indicate that the singling-out of *ac*-expressing cells in the wild-type SNS anlage is dependent on *N*-mediated lateral inhibition as observed for early CNS and PNS development. However, instead of delaminating, the *ac*-expressing cells of the SNS anlage define the position of invagination centers.

The range of *N*-mediated signaling in the SNS depends on the *wg* pathway

When analysing *wingless* (*wg*) mutant embryos, we noted an SNS phenotype (see below) which suggested that the range of *N*-mediated signaling in the SNS anlage may depend on a second intercellular communication system which depends on *wg* activity (reviewed by Perrimon, 1994). The essential components of the *wg* signaling pathway include *wg* itself, *dishev-*
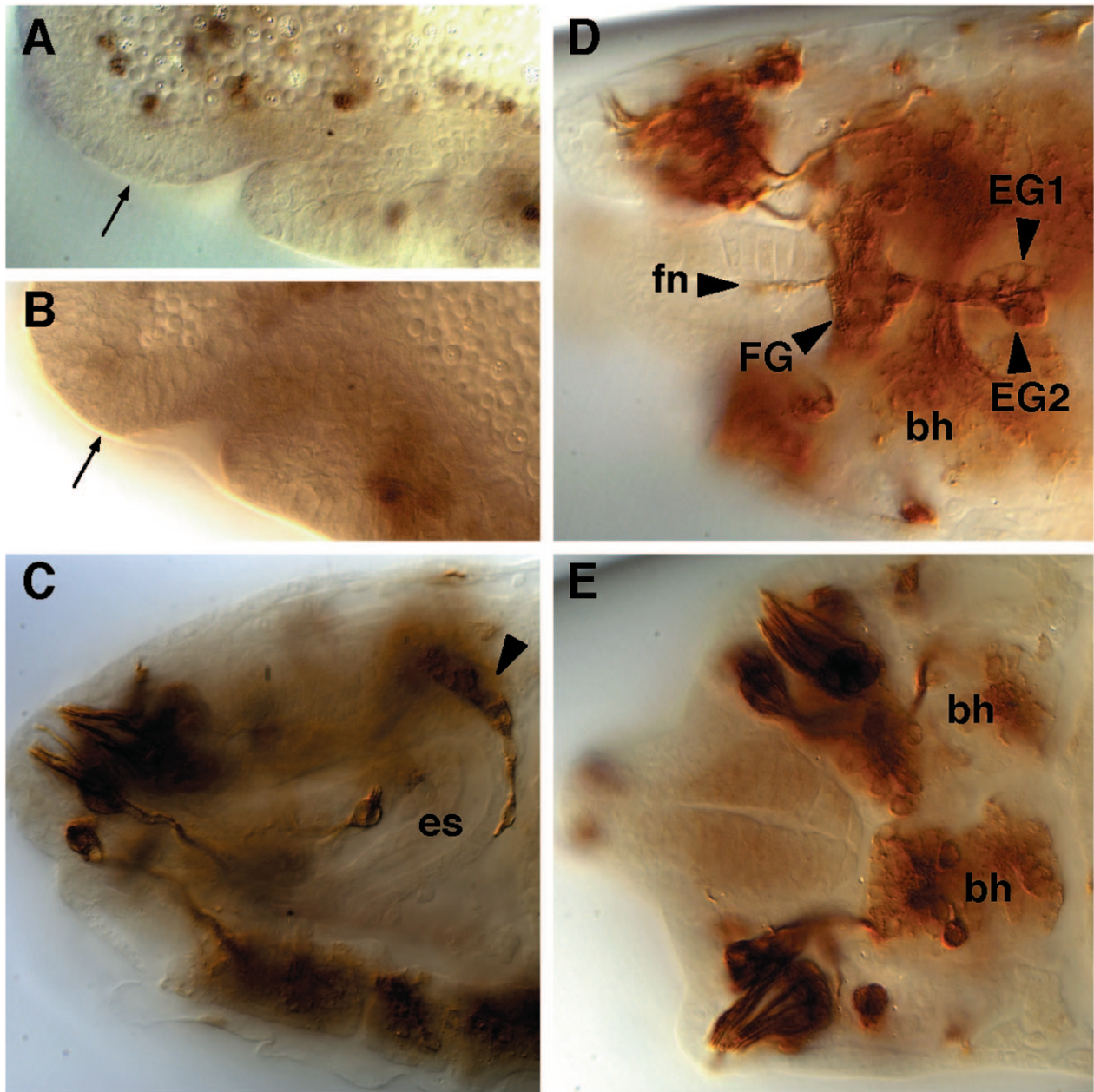
Fig. 5. *Krüppel* and the *achaete-scute* complex genes act synergistically during SNS development. (A) Anti-Kr and (B) anti-ac antibody stainings of *fkh^XT6* mutant embryos at stage 10. No expression of these genes is detected in the roof (arrows) of the stomodeum which is substantially reduced. (A,B) Orientation of the embryos is anterior to the left and dorsal up (lateral views). (C) mAb22C10 staining at stage 17 of *Df(1)B57* mutant embryo, lacking the *AS-C* genes. Remnants of the SNS ganglia can be detected in *Df(1)B57* homozygous mutant embryos. Most frequently the EG1 (arrowhead) is seen in a position apposed to the esophagus (es); remnants of the other SNS ganglia are occasionally detected. Orientation of the embryo is anterior to the left and dorsal up (lateral view). (D) mAb22C10 staining at stage 17 of *Kr²* mutant embryo. Note that the SNS structures are present (FG, EG1, EG2, fn; other nerves and the PG, out of focus). The abnormal appearance of some structures is caused by the *Kr* segmentation defect, which affects the head involution. Orientation of the embryo is anterior to the left (dorsal view). (E) mAb22C10 staining at stage 17 of *Df(1)B57*; *Kr¹* double mutant embryo. Note that all SNS ganglia are lacking when both *Kr* and the *AS-C* genes are absent. Remnants of what appear to be SNS structures were observed infrequently. Orientation of the embryo is anterior to the left (dorsal view). Abbreviation: bh, brain hemisphere.

elled (*dsh*) (Klingensmith et al., 1994), *shaggy/zeste white 3* (*sgg*; Siegfried et al., 1992) and *armadillo* (*arm*; Peifer and Wieschaus, 1990). *dsh*, *sgg* and *arm* are expressed in all *Drosophila* cells, while the expression of the secreted wg protein is spatially and temporally restricted (van den Heuvel et al., 1989; Siegfried et al., 1992; Li and Noll, 1993; Peifer et al., 1993; Klingensmith et al., 1994). Analysis of their epistatic relationships showed that *wg* activates *dsh*-dependent inhibition of *sgg*, which in turn acts negatively on *arm* (Noordermeer et al., 1994; Perrimon, 1994; Siegfried et al., 1994).
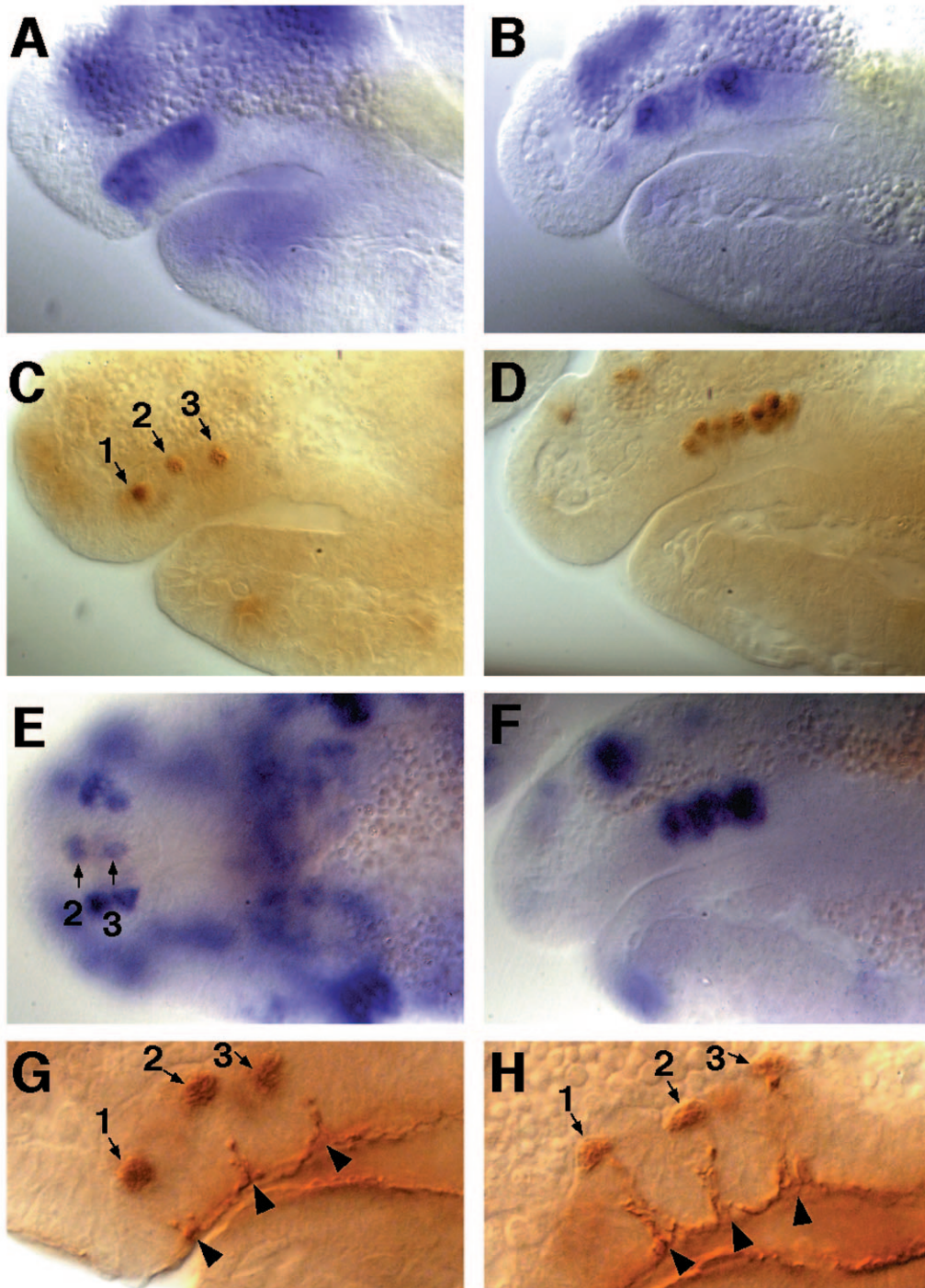
Fig. 6. *ac* and *sc*, but not *l'sc* expression, are restricted to SNS invagination centers and tip cells. (A) *l'sc* expression throughout the SNS primordium of late stage 10 embryos as revealed by whole-mount in situ hybridization. (B) Overall *l'sc* expression throughout the SNS primordium persists during stage 12; later on, *l'sc* expression also continues in the vesicles (not shown). (C) *ac* expression, which covers the SNS anlage at early stage 10 (see Fig. 2F), becomes restricted to three cells (1,2,3) as revealed by anti-achaete antibody staining at late stage 10. *ac* expression in these three cells persists during stage 11 when the invaginations occur (see G,H). (D) At stage 12, *ac* expression is reinitiated in all the cells within the invagination folds. (E) *sc* expression as revealed by whole-mount in situ hybridization. *sc* expression is restricted to three cells (1 is out of focal plane) at late stage 10. (F) *sc* expression is reinitiated at stage 12 in all the cells within the primordium as has been observed with *ac* expression (see D). (G) Anti-achaete and anti-crumbs double antibody staining showing nuclear staining (ac) and apical membrane staining (crb) in wild-type embryos at early stage 11. Note that the expression of ac is restricted to the three tip cells (1,2,3) of the incipient invagination folds (arrowheads). (H) Restricted tip cell expression of ac (1-3) persists until invagination (arrowheads) is completed late during stage 11. Orientation of embryos is anterior to the left; (A-D, F-H) lateral view (dorsal up), (E) dorsal view.

At stage 10, *wg* expression encompasses the SNS anlage of wild-type embryos (Fig. 8A). In the absence of *wg* activity, initial *ac* expression in the SNS anlage is normal (not shown), but only one *ac*-expressing cell was observed in the center of the SNS anlage of the *wg* mutant embryos (compare Figs 6C, 8D), and only one corresponding central invagination fold was formed (Fig. 8E). The same observations were made in embryos lacking *dsh* or *arm* activity (Fig. 8F-I). In contrast, more than three *ac*-expressing cells (Fig. 8J) and corresponding numbers of invaginations were observed in *sgg* mutant embryos (Fig. 8K-M). The opposite effects seen in *sgg* mutants

as compared to the *wg, dsh* or *arm* mutants reflect a restriction versus an expansion of the range of *N*-mediated signaling. This implies that the *wg* pathway functions to spatially control the range of *N*-mediated signaling in the SNS anlage to allow for the selection of the three invaginations (Fig. 9).

DISCUSSION

We show that the larval SNS originates from a single-layered epithelium in which distinct invagination centers are selected.
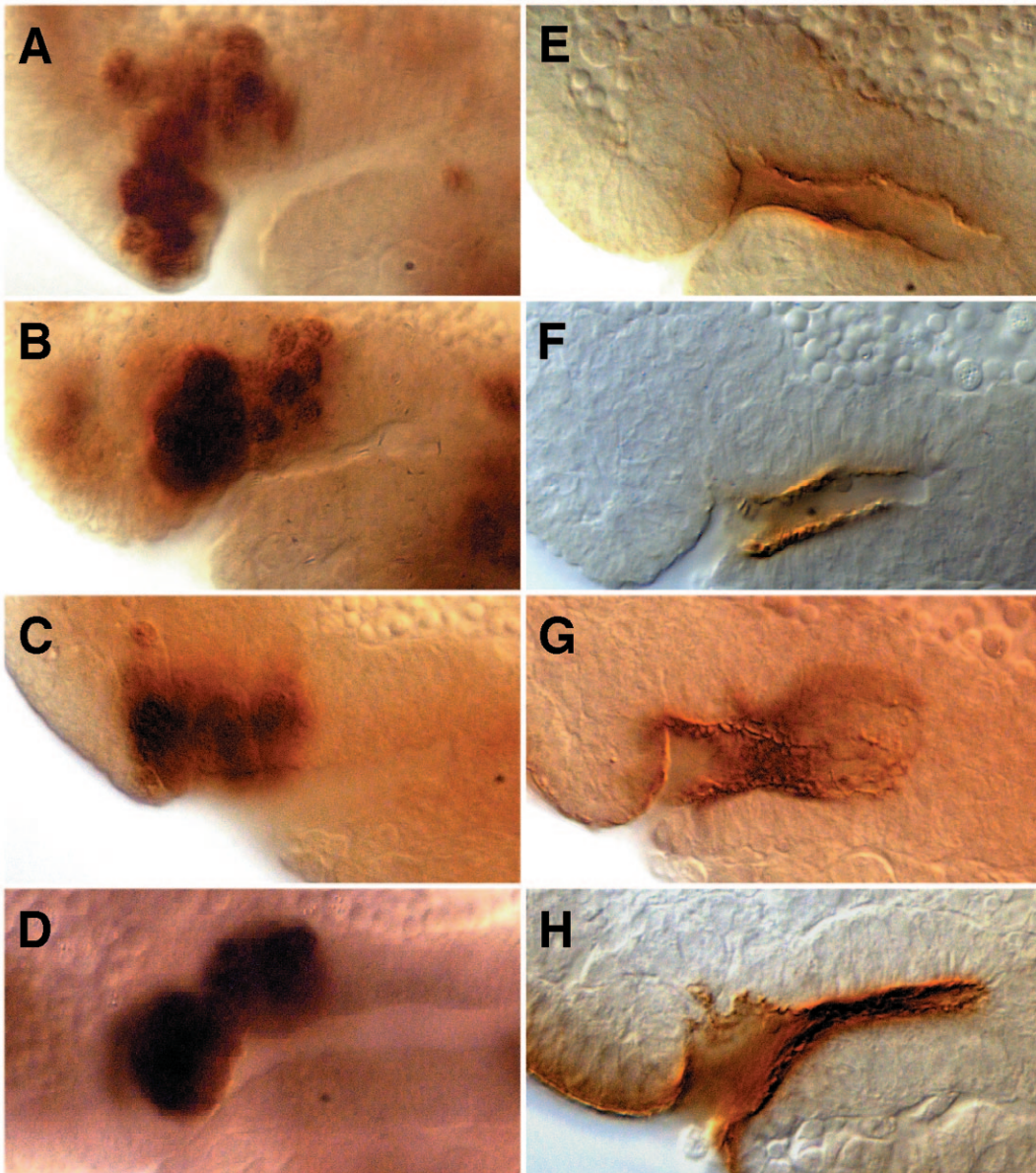
Fig. 7. Singling-out of *ac*-expressing invagination centers depends on *N*-mediated signaling. (A-D) Anti-achaete and (E-H) anti-crumbs antibody stainings of embryos mutant for different components of the *N*-mediated signaling pathway. (A,E) *Notch^{55e}*, (B,F) *Delta^{9P}*, (C,G) *Enhancer of split^{x1}* and (D,H) *mastermind^{IB99}* mutant embryos at stage 11. Note that ac expression persists throughout the SNS anlage (A-D) and that the SNS anlage invaginates *en masse* without forming distinct invagination folds (E-F). Orientation of the embryos is anterior to the left and dorsal up (lateral views).

The selection process depends on lateral inhibition by *wg*-dependent *N*-mediated signaling and results in three singled-out *ac*-expressing cells from which distinct invagination folds are formed, each headed by an *ac*-expressing tip cell. Thus, unlike PNS and CNS development, SNS morphogenesis is not initiated by delamination of single neural precursor cells in a scattered pattern but rather resembles the process of vertebrate neurulation from a compact epithelium (reviewed by Gilbert, 1994).

SNS anlage formation and neural fate

The genetic circuitry that establishes the SNS anlage includes the known maternal and zygotic components necessary to control pattern formation in the anterior terminal region of the embryo (Perrimon, 1993). Of those, *fkh* activity is required for the expression of *Kr* and the genes of the *AS-C*. While the SNS is absent in *fkh* mutant embryos, the lack of the *AS-C* gene activities causes only a reduction of neurons, as observed during CNS development (Jiménez and Campos-Ortega,

1990), and the absence of *Kr* activity has no significant effect on SNS development. However, the absence of both *Kr* and *AS-C* gene activities causes a dramatic reduction of the SNS which exceeds the degree of reduction in the absence of *AS-C* gene activities. This observation suggests that *Kr* provides proneural-like function, which can be compensated for by *AS-C* gene activities. Since remnants of SNS ganglia can still be detected in the absence of *AS-C* and *Kr* gene activities, other components with proneural activity must act in the SNS anlage of these embryos.

Intriguing parallels between CNS/PNS and SNS development are not only apparent with respect to the initial expression patterns of the proneural genes, but also with respect to the singling-out of *ac*-expressing cells in response to neurogenic gene activities. However, while the selected cells already represent the CNS and PNS neural precursors (reviewed by Campos-Ortega, 1993; Goodman and Doe, 1993; Jan and Jan, 1993), the corresponding three cells of the SNS anlage define the invagination centers from which

epithelial folds are generated. Furthermore, *l'sc* expression is not part of the singling-out process in response to *N*-mediated signaling and *ac* and *sc* expressions become reinitiated. Thus, the cells of the developing SNS combine *ac*, *sc* and *l'sc* activities during a second round of *AS-C* gene expression. In view of this second round of *AS-C* gene expression, the first one may not determine neural fate, but rather sets a morphoregulatory process into motion that causes epithelial invaginations. The second round of *AS-C* expression may therefore represent the functional equivalent to the proneural gene expression during CNS and PNS development. At present we do not know how the *N*-dependent singling-out of *l'sc*-expressing cells in the SNS anlage is

circumvented and how the reinitiation of *ac* and *sc* gene expression is controlled.

Fig. 8. The number of *ac*-expressing cells and invagination folds depends on the *wg* pathway. (A) *wg* expression encompasses the SNS anlage of stage 10 wild-type embryos as revealed by whole-mount in situ hybridization; lateral view, anterior to the left, dorsal up. (B) Anti-achaete and (C) anti-crumbs antibody staining of wild-type SNS primordia at stage 11; dorsal view, anterior to the left. (D-M) Anti-achaete (D,F,H,J) and anti-crumbs (E,G,I,K-M) antibody stainings of wg^{IG23} (D,E), dsh^{v26} (F,G), arm^{XM19} (H,I) and sgg^{M11-1} (J-M) mutant embryos at stage 11; (D-I,L,M) lateral view; (J,K) dorsal view. Note a single *ac*-expressing cell (arrows) and the corresponding single invagination fold (arrowhead) in wg^{IG23} (D,E), dsh^{v26} (F,G) and arm^{XM19} (H,I). In contrast, five *ac*-expressing cells (arrows) are found in sgg^{M11-1} (J, compare with the wild-type *ac* pattern in B). Note that the corresponding number of invagination folds appears in sgg^{M11-1} mutants (K-M). (K) A sgg^{M11-1} embryo with four invagination folds (compare with C). (L,M) Different focal planes of a lateral view of the same embryo to demonstrate the four invagination folds. Note that the number of both *ac*-expressing cells and the corresponding invaginations in sgg^{M11-1} embryos is variable; there are more than three and up to five. The dsh^{v26}, arm^{XM19} and sgg^{M11-1} mutant embryos lack the respective gene activity both maternally and zygotically. For a description of the generation of such embryos see Materials and Methods. Note that during late stage 11, when the invagination process is completed, wild-type *wg* expression is restricted to a ring in the inner part of the esophagus that will contribute to the proventriculus development (not shown).

Lateral inhibition   Invagination   signaling control
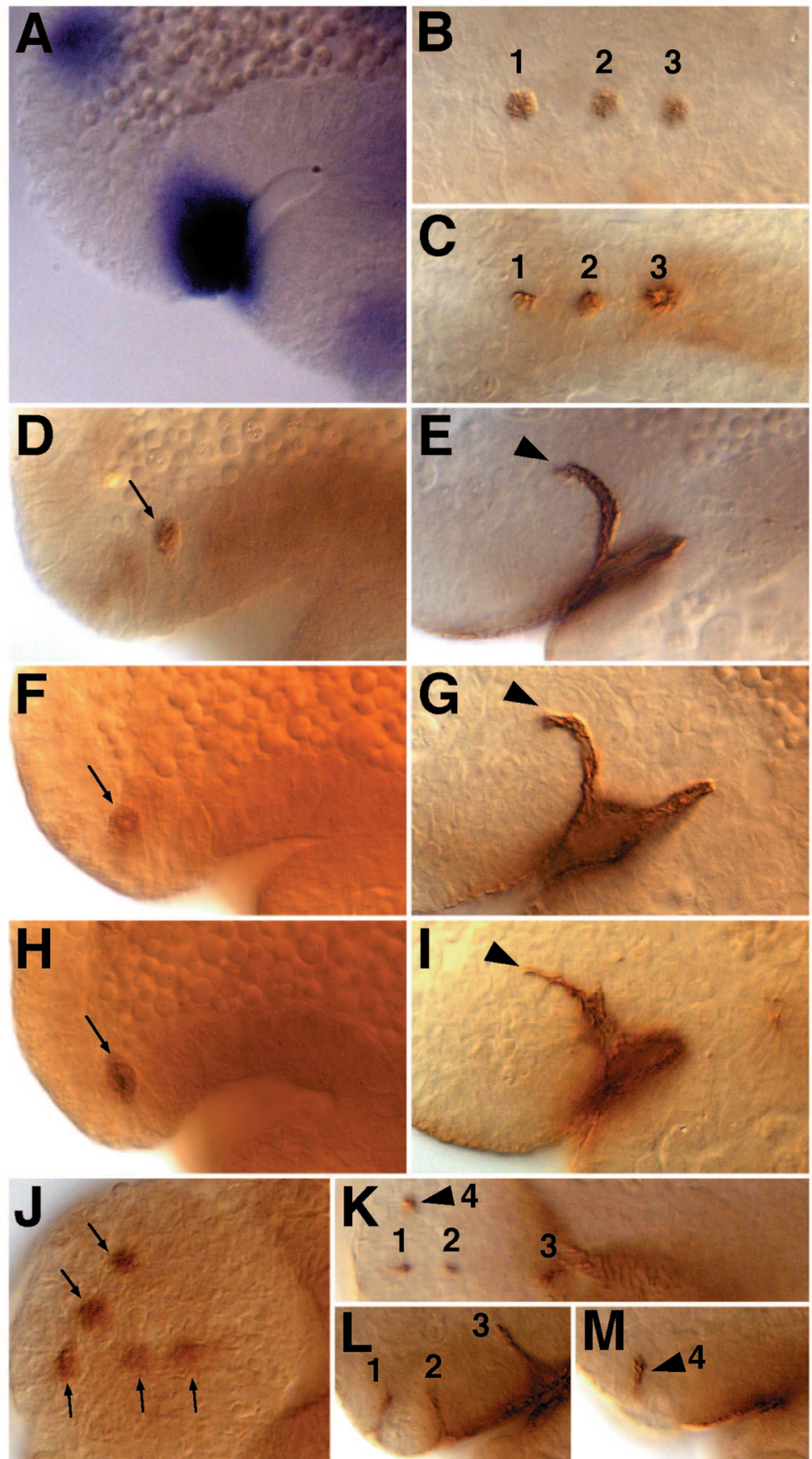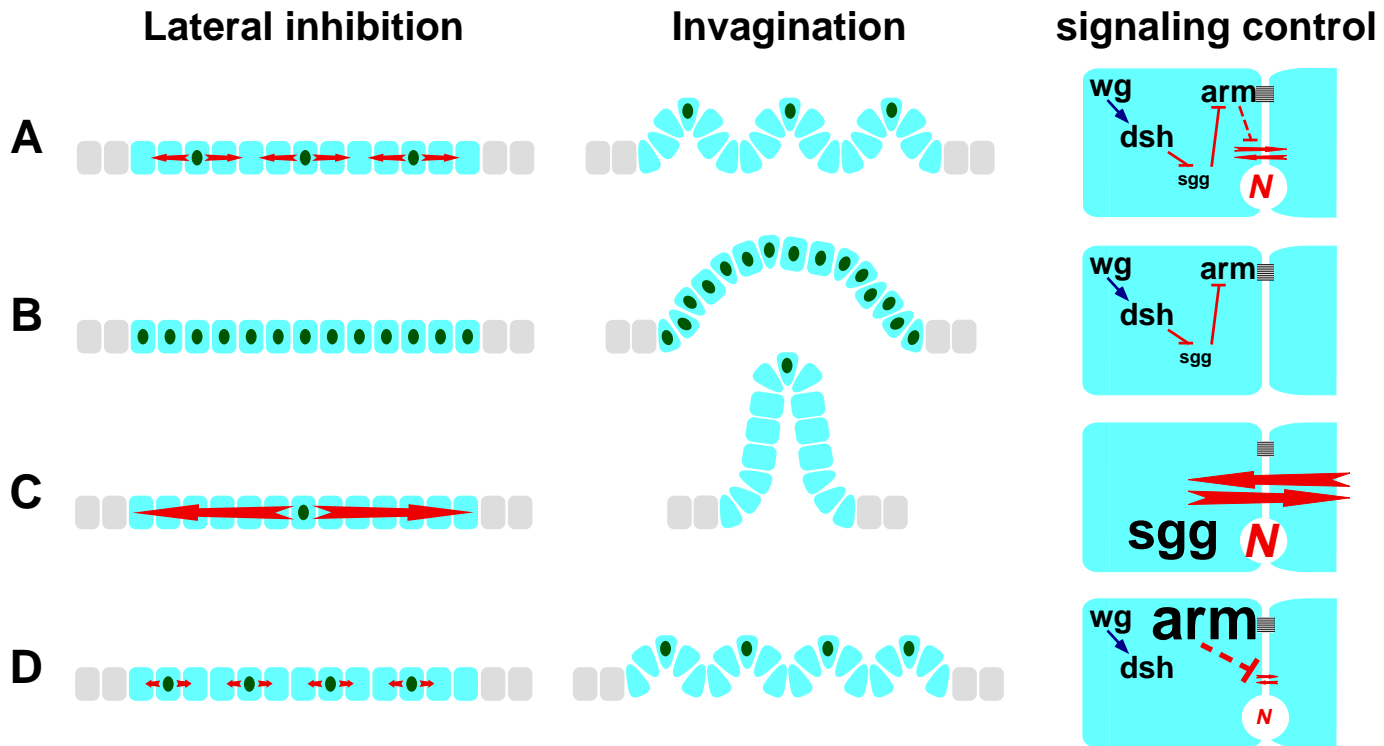
Fig. 9. Formation of invagination centers by a *wg*-dependent range of *N*-mediated signaling in the SNS anlage. (A) The wild-type SNS anlage (blue cells) is outlined within the stomodeum (grey cells). *N*-mediated signaling (red arrows) functions to single-out three *ac/sc*-expressing cells (green nuclei) within the SNS anlage by lateral inhibition (left panel). Three invagination folds are then formed, each headed by an *ac/sc*-expressing cell (central panel). The range of *N*-mediated signaling is controlled by the *wg* pathway (right panel). Active arm protein (associated with cell junctions; square between cells) may decrease the reception and/or emission efficiency of the *Notch*-dependent signal (*N*) and hence shorten the range of *Notch*-dependent lateral inhibition (red arrows). *wg* activity, which is present throughout the SNS anlage (see Fig. 8A), reduces (but does not abolish) the level of *sgg* activity via *dsh* activity, which in turn diminishes *arm* activity. Thus, the range of *N*-mediated signaling is critically dependent upon the levels of *arm* activity and this is determined by *wg*-adjusted *sgg* activity. (B) In the absence of *N*-mediated signaling, the singling-out process does not occur. Thus, *ac* expression persists throughout the SNS anlage (see Fig. 7A-D) and leads to an uncoordinated invagination of all *ac*-expressing cells (see Fig. 7E-H).(C) Absence of *wg* or *dsh* causes high levels of *sgg* activity and therefore low levels of active *arm* (Perrimon, 1994). Thus, *wg*, *dsh* or *arm* mutations lead to a maximal range of *N*-mediated signaling, which then encompasses the SNS anlage and allows the singling-out of only one *ac*-expressing cell (Fig. 8D,F,H) that leads the single invagination in the middle of the anlage (see Fig. 8E,G,I). (D) In *sgg* mutants, *arm* activity is not decreased. This high level of *arm* activity causes a stronger reduction of the efficiency of *N*-mediated signaling than in wild-type which shortens the range of lateral inhibition. As a consequence, more than three *ac*-expressing cells are singled-out (see Fig. 8J) and each supernumerary cell leads to the formation of an extra invagination fold (see Fig. 8K-M).

Singling-out of invagination centers

N-mediated signaling is both necessary and sufficient to provide lateral inhibition throughout the SNS anlage in a manner analogous to that seen in the proneural cell clusters initiating both PNS and CNS development (Campos-Ortega, 1993). This argument is supported by the finding that in the absence of *wg* activity, one *ac*-expressing invagination center is selected. The selection of three invagination centers in the wild-type SNS anlage therefore depends upon the functional range of *N*-mediated signaling, which is controlled by *wg* activity (Fig. 9A).

Local *wg* activity causes *dsh*-dependent inhibition of *sgg* which, in turn, acts negatively on *arm*, *i.e.* *wg* activity eventually causes the increase of active *arm* protein at the receiving end of the pathway (Noordermeer et al., 1994; Perrimon, 1994; Siegfried et al., 1994). The finding of only one selected *ac*-expressing invagination center in *wg*, *dsh* and *arm* mutant embryos indicates that a reduction or loss of *arm* activity leads

to an expansion of the range of *N* function in the SNS anlage (Fig. 9C). Conversely, the absence of *sgg* leads to increased *arm* activity which generate supernumerary *ac*-expressing cells and the corresponding number of invaginations (Fig. 9D). This means that critical levels of *sgg* activity must be present at functional levels in the wild-type SNS anlage. *wg* activity, which covers the entire wild-type SNS anlage, does not repress, but rather reduces its level so that the range of lateral inhibition becomes adjusted for the selection of three centers as outlined in the model presented in Fig. 9A.

How *wg* and *N* may interact in the SNS anlage

sgg encodes a cytoplasmic serine/threonine kinase (Bourouis et al., 1990) which had been proposed to act as an integral component of the *wg* pathway as well as of *N*-mediated signaling (Simpson et al., 1993). In the notum, clonal analysis of *sgg* mutant cells suggested the possibility that *sgg* functions downstream of *N* during lateral inhibition (Ruel et al., 1993;

Simpson et al., 1993). However, our results show that the lack of *sgg* activity does not cause a *N*-like SNS phenotype (Fig. 8J). Thus, *sgg* cannot be essential for *N*-mediated signaling in the SNS anlage per se. In our view, the selection of supernumerary invagination centers in *sgg* mutants can be explained by high levels of active arm protein that shortens the range of *N*-mediated lateral inhibition (Fig. 9D). Likewise, the increased levels of sgg kinase activity (in *wg* or *dsh* mutants) should then lower the level of active *arm* so that *N*-mediated signaling functions throughout the SNS anlage, generating a single invagination fold in the center (Fig. 9C).

arm encodes a protein similar to β-catenin and placoglobin (Peifer and Wieschaus, 1990), leading to the suggestion that *arm* regulates cellular junctions to control the reception of signaling molecules (reviewed by Peifer et al., 1993; Martínez Arias, 1994). Since *wg* is expressed throughout the SNS anlage, it may act to affect *arm*-dependent cell junctions throughout the epithelium. Our findings are consistent with *wg* acting autonomously to affect the reception of other molecules by controlling a cell adhesion prerequisite for signaling. Moreover, the control of cell adhesion has the potential to modify or prevent the transmission of signals between adjoining cells by either affecting the emission or reception of the signal. This implies that the *wg* pathway is not necessarily generating an instructive signal in its own right but rather regulates the functional range of other instructive signals (Fig. 9).

Is *wg* action in the SNS anlage a paradigm?

The proposal that the activated *wg* pathway modulates instructive signals appears at variance with the orthodox view that *wg* initiates a signaling cascade to instruct neighboring cell fates nonautonomously (Immerglück et al., 1990; Chu-LaGraff and Doe, 1993; Couso et al., 1993; Li and Noll, 1993; Struhl and Basler, 1993; Couso et al., 1994; Couso and Martínez Arias, 1994; Perrimon, 1994) and with the recent finding that wg may directly interact with the *N*-encoded transmembrane receptor as suggested by allele-specific combinations of *wg* and *N* mutations (Couso and Martínez Arias, 1994). The latter suggestion could be integrated into our model only if one assumes that *wg*-dependent *arm* function ultimately controls the effects generated by the proposed interaction of wg and N.

Our proposal is consistent with the observation of short-range effects of the *wg* activity in different embryonic developmental systems such as epidermal segmentation (reviewed by Perrimon, 1994), gut (Immerglück et al., 1990) and CNS development (Chu-LaGraff and Doe, 1993), and with the recent demonstration that the segment polarity function of *wg* affects *engrailed* target gene expression only in the adjoining epidermal cells (Vincent and Lawrence, 1994). However, it does not explain the long-range effect of *wg* activity observed during imaginal development (Struhl and Basler, 1993). This phenomenon could be due to an initial short-range effect of *wg,* which has been propagated through cell proliferation and by local cascades of cell interactions, as was recently shown for the non-secreted sgg protein (Díaz-Benjumea and Cohen, 1994). Furthermore, the non-diffusible membrane-associated vertebrate homolog of *wg, Wnt-1,* provides both long-range effects in the *Xenopus* axis duplication assay and short-range effects on adjoining cells in cell culture transfection assays (Parkin et al., 1993). These findings are consistent with the view that although *wg* encodes a secreted protein (van den

Heuvel et al., 1989), both long-range diffusion and morphogen function may be irrelevant to its action as described here in the SNS anlage. Thus, wg and wg-like molecules of different species may exclusively function to control morphoregulatory signals using an evolutionarily conserved molecular strategy of *wg* action, i.e. *wg* activity may serve to regulate morphoregulatory signals and thereby act as a permissive component rather than as an instructive signal (Sampedro et al., 1993).

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