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The *iroquois* complex controls the somatotopy of *Drosophila* notum mechanosensory projections

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SUMMARY

Sensory neurons can establish topologically ordered projections in the central nervous system, thereby building an internal representation of the external world. We analyze how this ordering is genetically controlled in *Drosophila*, using as a model system the neurons that innervate the mechanosensory bristles on the back of the fly (the notum). Sensory neurons innervating the medially located bristles send an axonal branch that crosses the central nervous system midline, defining a 'medial' identity, while the ones that innervate the lateral bristles send no such branch, defining a 'lateral' identity. We analyze the role of the proneural genes *achaete* and *scute*, which are involved in the formation of the medial and lateral bristles, and we show that they have no effect on the 'medial' and 'lateral' identities of the neurons. We also analyze the role of the prepattern genes *araucan* and *caupolican*, two members of the *iroquois* gene complex which are required for the expression of *achaete* and *scute* in the lateral region of the notum, and we show that their expression is responsible for the 'lateral' identity of the projection.

Key words: Sensory neurons, Central projections, Somatotopy, *achaete-scute* complex (ASC), *iroquois* gene complex (IRO-C), *Drosophila*

INTRODUCTION

Sensory neurons form central projections that are often correlated to the position of the sense organ on the body surface. Although the development of topologically ordered projections is nearly universal, the mechanisms underlying their generation are poorly understood. Previous results have shown that, in *Drosophila*, the projections of larval and adult sensory neurons are organized according to the position of the sense organ they innervate (Ghysen, 1980; Murphey et al., 1989; Merritt and Whitington, 1995). Such is the case for the projection of the mechanosensory bristles that cover the back of the adult, also called notum.

The notum of *Drosophila* bears 11 pairs of precisely located large bristles, the macrochaetes, and about 200 smaller bristles arranged in rows, the microchaetes (Fig. 1A). Each bristle is innervated by a single bipolar neuron, which extends its dendrite towards the base of the bristle shaft, and its axon towards and into the central nervous system (CNS). The axons of all neurons innervating the notum bristles enter the thoracic ganglion through the same root and follow a common pathway that extends anteriorly and posteriorly along the pro- and mesothoracic leg neuromeres (Fig. 2). The details of the central projection depend, however, on the position of the bristle (Ghysen, 1980). In the case of macrochaetes, the relative extension of the anterior and posterior branches reflects the position of the bristle along the anteroposterior axis, while the existence and importance of a contralateral projection depends on the laterality of the bristle. The presence of contralateral branches is characteristic of the neurons innervating the medially located macrochaetes (dorsocentrals: DCs, scutellars: SCs, posterior post-alar: pPA, Fig. 3A) and the vast majority of microchaetes (Fig. 4B). On the contrary, the neurons innervating the lateral macrochaetes (notopleurals: NPs, supra-alars: SAs, presutural: PS, anterior post-alar: aPA) and the most lateral microchaetes have a projection confined to the ipsilateral half of the CNS (Figs 2, 3C). Mosaic analysis has shown that neurons project according to the position of the bristle they innervate even when many or most of the bristles are absent, suggesting that the distribution of the axonal terminals in the CNS does not depend on interactions between growing axons nor on competition for target sites (Ghysen, 1980). These results led to the conclusion that local or even intrinsic determinants provide some sort of positional information to the differentiating sensory neurons.

In insects, the sensory neurons are part of the same lineage that produces the other components of the sense organ (Bate, 1978; Jan and Jan, 1993). Thus all cells of one organ derive from the same precursor cell, which is singled out from the undifferentiated ectodermal sheet at the position where the final organ will differentiate. It might be therefore that the neuron takes advantage of whatever positional information was

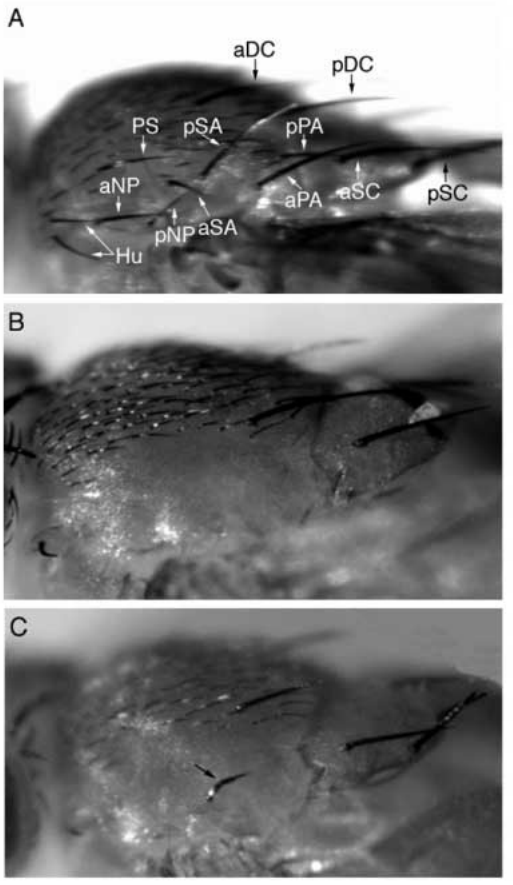

Fig. 1. Lateral view of the thorax in the wild-type and in mutants.(A) wild-type. The macrochaetes are, from anterior (left) to posterior (right): Hu, humerals, aNP, anterior notopleural, PS, presutural, pNP, posterior notopleural, aSA and pSA, anterior and posterior supraalars, aPA and pPA, anterior and posterior postalars, aDC and pDC, anterior and posterior dorsocentrals, aSC and pSC, anterior and posterior scutellars. (B) iro^1/iro^2. The lateral region of the notum lacks both macrochaetes and microchaetes. Note that the humeral bristles are not affected by the mutations.
(C) $Hw^{49c};iro^1/iro^2$. The *Hw* gain-of-function mutation induces the formation of a bristle (arrow) in the *iro*-sensitive lateral region of the thorax.

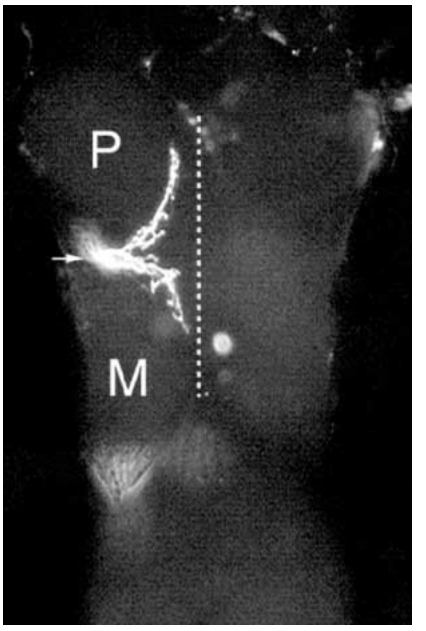

Fig. 2. Central projection of a lateral bristle. The axon enters the thoracic ganglion through the posterior dorsal mesothoracic nerve root (arrow), extends ventrally toward the midline (dashed line) and bifurcates in an anterior and a posterior branch which follow respectively the prothoracic (P) and mesothoracic (M) leg neuromeres. The appearance of several fibers at the tip of the arrow is an artefact due to the fiber being out of focus at this point. Anterior is up.

responsible for the determination of this precursor cell. We have investigated this possibility in the case of the notal bristles. The formation of bristle precursor cells is dependent on the expression of proneural genes, all of which encode highly similar transcription factors of the basic region-helix-loop-helix (bHLH) type. Ectopic expression and rescue experiments have shown that the four bHLH genes of the *achaete-scute* complex (ASC) can substitute for each other to produce morphologically indistinguishable bristles (Rodriguez et al., 1990; Brand et al., 1993; Dominguez and Campuzano, 1993; Martin-Bermudo et al., 1993; Hinz et al., 1994; Giebel et al., 1997). Of the four ASC genes, two are essential for the normal development of the notal bristles: *achaete* (*ac*) and *scute* (*sc*). No bristle ever forms when *ac* and *sc* are both deleted, while the deletion of either gene results in the absence of two nearly complementary subsets of bristles (Raffel and Muller, 1940; Garcia-Bellido, 1979). *ac* and *sc* are co-expressed in clusters of cells in the developing notum, making them competent to become precursor cells (Romani et al., 1990). This co-expression was originally thought to result from cross-activation of each gene by its counterpart (Martinez and Modolell, 1991), but more recent evidence suggests that it may be due to the simultaneous activation of both transcriptional units by common enhancers (Gomez-Skarmeta et al., 1995). Thus it has been concluded that the Ac and Sc proteins are functionally identical, and that the differences in phenotype between *ac* and *sc* mutations are due to the deletion or inactivation of different sets of enhancers.

It should be noted, however, that in the CNS a third member of the complex, *lethal-of-scute*, cannot fully substitute for either *ac* or *sc*, even though it can do so as far as the formation of bristles is concerned (Parras et al., 1996; Skeath and Doe, 1996). Thus not all is redundancy in the AS-C (Campos-Ortega, 1998), and it may be that, even though the ability to promote bristle formation is shared by all members of the complex, some of them have their own specificity at the level of neuronal differentiation. In particular, the closer association of the *ac* and *sc* transcripts with different enhancers might lead to differences in the amount or time of expression of the two genes in different regions of the notum, and thereby play a role in the specification of the type of sensory projection.

Another potential source of positional information to distinguish lateral from medial bristles are the ‘prepattern’ genes. Mosaic analyses have suggested that the competence to form a bristle precursor cell depends on a ‘prepattern’ present

in the undifferentiated ectoderm (Stern, 1954). This prepattern was assumed to control the localized expression of the *sc* (and *ac*) gene, which defines the positions where bristle precursors will form (Richelle and Ghysen, 1979). The prepattern was envisioned as combinations of activating and silencing factors, the products of the prepattern genes, present in different regions of the tissue (Ghysen and Dambly-Chaudière, 1989). One such prepattern factor is *iroquois* (*iro*) (Dambly-Chaudière and Leyns, 1992; Leyns et al., 1996). The molecular analysis revealed that *iro* is a gene complex comprising two very closely related homeobox genes, *araucan* (*ara*) and *caupolican* (*caup*), which are both expressed in the lateral region of the notum where they activate the expression of *ac* and *sc* (Gomez-Skarmeta et al., 1996). The activity of the genes *ara* and *caup* is largely reduced in the viable iro^1 mutation, resulting in a lack of expression of *ac* and *sc* genes in the lateral region of the notum, and in the absence of all lateral bristles (Leyns et al., 1996). Thus *iro* might also be involved in specifying the central projection of the lateral vs the medial bristles.

In this paper we analyze the potential involvement of *ac* and *sc*, and of *ara* and *caup*, in specifying the positional identity of the sensory neurons on the notum. We concentrate on the presence of contralateral branches, a feature that is typical of the neurons innervating medial bristles. We show that altering the expression of *ac* or *sc* has no effect on the projections. On the contrary, loss- and gain-of-function experiments reveal that the expression of either *iro* gene confers a 'lateral' identity to the neurons.

MATERIALS AND METHODS

Fly strains

The $ln(1)y^{3PL}sc^{8R}$ (ac^-), sc^2pn (ase^-), $YDpsc^8$ (ac^+), $sc^{10.1}$ (ac^- sc^-), iro^1, iro^2, Hw^{49c} alleles and *hs-sc* transgenic line are described in FlyBase. The *sca*-GAL4 transgenic line was provided by Yuh-Nung Jan. UAS-ara^{36} is described by Gomez-Skarmeta et al. (1996). The UAS-*caup* transgenic line was provided by Ruth del Corral, José-Luis Skarmeta and Juan Modolell and will be described elsewhere. All flies were raised at 25°C except when mentioned.

Labeling of sensory projections

The analysis of the ASC mutants was done with the horseradish peroxidase backfilling technique as described in Ghysen (1980). The analysis of *iro* mutants was achieved using the fluorescent carbocyanine dye, DiI C12 (Molecular Probes, Inc.). Thoraces were fixed overnight at 4°C in 3.7% formaldehyde solution in 0.2 M sodium carbonate buffer (pH 9.5). Single shafts were dislodged from their socket using tweezers. The dye, dissolved in 95% ethanol, was applied to the empty socket. Staining was allowed to proceed for 24 hours in 0.2 M sodium carbonate buffer (pH 9.5) at 22°C. The CNS was then dissected and observed as whole mount in Fluoromount-G medium (Southern Biotechnology Associates, Inc.) under epifluorescence (rhodamine filter).

Thermoinduction of *sc* expression

Late third instar larvae of the $sc^{10.1}$/Y; *hs-sc*/+; *hs-sc*/+ genotype were submitted to a succession of three heat-shocks of one hour at 37°C, separated by intervals of 30 minutes at 25°C.

Ectopic expression of *ara* and *caup*

The *sca*-GAL4;UAS-*caup* and *sca*-GAL4;UAS-ara^{36} flies were maintained at 29°C to maximize the expression and activity of the GAL4 product.

RESULTS

The *ac-sc* gene complex and the specification of bristle projections

In order to analyze whether the *ac* and *sc* genes play a role in the specification of the type of central projection established by the notum mechanosensory neurons, we changed the normal distribution of the Ac and Sc products in various ways, and examined whether these changes have any effect on the presence or absence of contralateral branches.

In the wild-type, the DC projections have contralateral branches (Fig. 3A), while the PS projection remains strictly ipsilateral (Fig. 3C). In an ac^- sc^- double mutant, where no bristle ever forms on the notum, we induced the formation of macrochaetes at the DC and PS positions by inducing the ectopic expression of *sc* in a hs-*sc* line. The resulting bristles display projections that are appropriate to the position of the bristle (Fig. 3B,D), showing that *sc* can promote the formation of both types of projections and that the absence of *ac* has no effect on the type of projection.

We also analyzed the pSA projection in detail. The pSA is the only bristle to depend on both *ac* and *sc*, in that it is often missing when either gene is deleted. We examined the projections of the rare pSA that form in either ac^- or sc^- flies and found them to be identical to the normal pSA projection (Fig. 3E,F), showing that there is no differential effect of *ac* or *sc* on this projection.

Finally, we examined the projections of the microchaetes located in the medial region of the notum in ac^- or sc^- backgrounds and found them to be unchanged relative to the wild type (not shown). Taking in account these various results, we conclude that the proneural genes *ac* and *sc* play no role in specifying the type of sensory projection of the notal bristles.

Loss-of-function analysis of the *iroquois* gene complex

The iro^1 mutation is a null allele of *caup* and a hypomorphic allele of *ara*, while iro^2 is a deletion eliminating both transcripts (Gomez-Skarmeta et al., 1996). The iro^1/iro^2 genotype thus constitutes the strongest viable *iro* combination and is probably close to a complete loss-of-function of the *ara* and *caup* genes. None of the lateral macrochaetes, nor the most lateral of the microchaetes, ever form in this mutant combination (Fig. 1B), due to the lack of activation of the *ac-sc* genes in the lateral regions of the notum (Leyns et al., 1996). In order to examine whether *iro* plays any role in the specification of the lateral projections, we bypassed the *iro* requirement by using a gain-of-function allele of the *ac-sc* complex, *Hairy-wing*^{49c} (Hw^{49c}). In a normal background, Hw^{49c} leads to the formation of supernumerary bristles at various positions of the notum (Balcells et al., 1988). In the iro^1/iro^2 background, Hw^{49c} often leads to the formation of a bristle of variable size in the otherwise naked lateral region of the notum, at a position corresponding to that of the pSA macrochaete (Fig. 1C). Labelling the projection of these bristles revealed the presence of contralateral branches in about 70% of the cases, whether the bristle be microchaete-like (10 out of 15; Fig. 4E) or macrochaete-like (21 out of 29; Fig. 4D). Such a branch was never observed in the projection of the most lateral microchaetes in wild-type flies (n=37) and only once out of 31 cases in the projection of the pSA. Since the genes *ac*

and *sc* have by themselves no effect on the type of projection, we do not expect the Hw^{49c} mutation to be responsible for this effect. Nevertheless we examined the projections of neurons innervating the most lateral bristles in a Hw^{49c}; iro^{+} genetic background, and found that they never display contralateral branches (n=15).

The *ara* and *caup* genes are weakly expressed in the larval CNS, and only in the ventral part of the brain (Gomez-Skarmeta et al., 1996). Nevertheless one cannot rule out that the formation of contralateral branches by the lateral bristles in Hw^{49c}; iro^{1}/iro^{2} flies could be due to an effect of the *iro* background on the CNS, rather than on the positional identity of the sensory neurons. To examine this possibility we used the prothoracic (humeral) bristles. Their axons enter the CNS in a more anterior position, extend along the same anteroposterior pathway as the mesothoracic (notal) bristles, and do not form contralateral branches (n=17, not shown). Even though the morphology of the humerus is not completely normal in iro^{1}/iro^{2} flies, several humeral bristles are invariably present, and their projections are wild-type. Specifically, a contralateral branch was observed only once out of 17 cases (not shown).

These results suggest that the neurons innervating the lateralmost bristles of the notum lose their lateral identity when they form in the absence of the *iro* products, in as much as they display contralateral branches typical of the neurons innervating medial bristles.

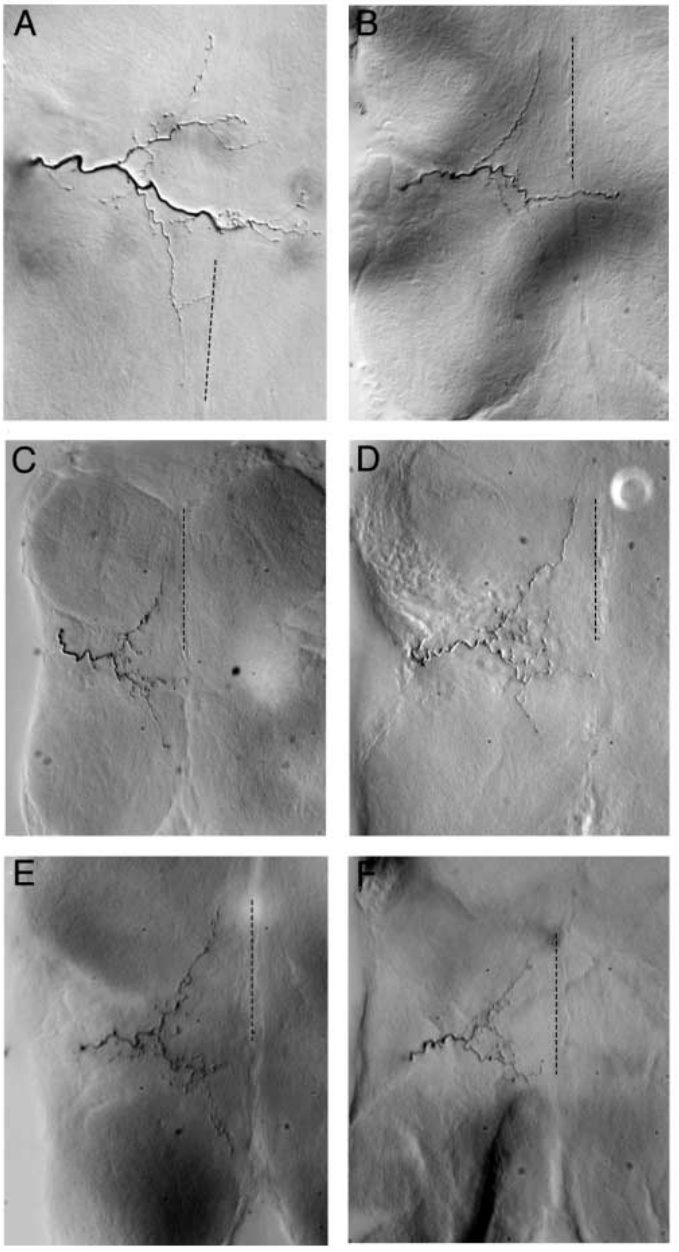

Fig. 3. Central projections in ac^{-} and sc^{-} mutants. (A) Wild-type anterior dorsocentral macrochaete (aDC). (B) Dorsocentral macrochaete induced by *hs-sc* in an ac^{-} sc^{-} genetic background (compare with A), (C) Wild-type presutural macrochaete. (D) Presutural macrochaete induced by *hs-sc* in an ac^{-} sc^{-} genetic background (compare with C). (E) Posterior postalar macrochaete in an ac^{-} genetic background. (F) Posterior postalar macrochaete in a sc^{-} genetic background (compare with E). Anterior is up; the nerve enters in the thoracic ganglion at the left; the dashed line indicates the midline.

Effect of *ara* and *caup* overexpression on the projections of medial bristles

Given that *iro* is necessary for the lateral bristles to display the appropriate ipsilateral projection, we examined whether the expression of either *iro* gene is sufficient to specify this projection. We used the GAL4-UAS system (Brand and Perrimon, 1993) to induce the expression of *caup* or *ara* in all proneural clusters of the developing notum, including the medial clusters that do not normally express the *iro* complex and give rise to contralaterally projecting neurons, and tested the capability of the *iro* products to modify the projections of the medial bristles.

In flies carrying both *sca*-GAL4 and UAS-*ara* or UAS-*caup*, the ectopic expression of the two genes does not affect the projections of the medial macrochaetes, which display contralateral branches as in the wild-type. On the contrary, the neurons innervating the most medial microchaetes lack all contralateral branches (Fig. 4F), i.e., their projection has now become typical of the most lateral microchaetes. The efficiency of the effect depends on the transcript used: about 80% (11 out of 14) show a transformation in the case of *caup*, and about 30% (5 out of 17) for *ara*. The *sca*-GAL4 insertion has by itself no effect on the central projections, which retain a contralateral branch in nearly all cases (18 out of 19). The presence of ARA or CAUP proteins is thus sufficient to change the projection of the neurons innervating the medial microchaetes to one corresponding to the most lateral microchaetes. The fact that we observed no effect on the medial macrochaetes suggests that the transformation of the microchaete projection (disappearance of contralateral branches) is probably not due to an effect on the CNS.

DISCUSSION

The genes of the *iro* complex, but not of the *ac-sc* complex, are required for the lateral specification of neuronal projections

One key feature of sensory projections is their ability to establish a central projection that is correlated to the position of the innervated organ on the body surface. In the case of the

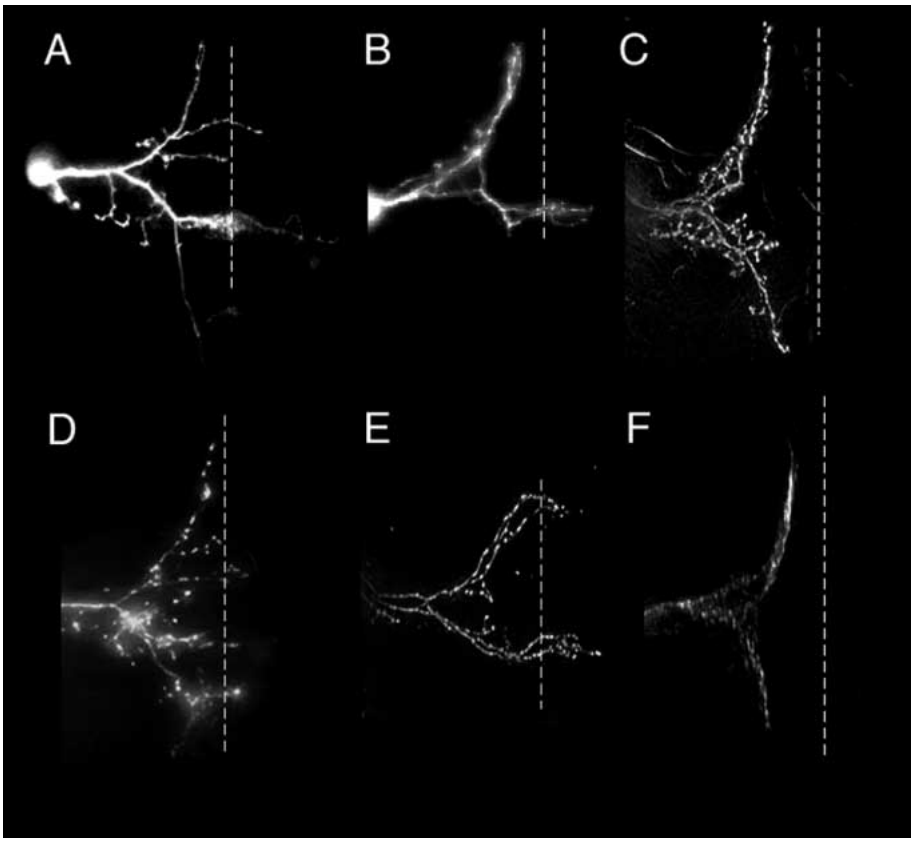

Fig. 4. Central projections in iro loss-of-function and gain-of-function mutants. (A) Wild-type medial macrochaete (aDC). (B) Wild-type medial microchaete. (C) Wild-type lateral macrochaete (pSA). (D) Hw^{49c}; iro^1/iro^2 lateral macrochaete (compare with C). (E) Hw^{49c}; iro^1/iro^2 lateral microchaete. (F) *sca*-GAL4;UAS-*caup* medial microchaete (compare with B). In C, the thin fluorescent lines in the background are not related to the projection. Anterior is up; the nerve enters in the thoracic ganglion at the left; the dashed line indicates the midline.

notal bristles of *Drosophila*, one of the major positional correlates is the absence of contralateral branches in the neurons innervating the lateral organs. Thus the presence of contralateral branches defines a 'medial identity', while their absence corresponds to a 'lateral identity'.

We analyzed whether the proneural genes *ac* and *sc*, which are necessary for the formation of medial and lateral macrochaetes respectively (Raffel and Muller, 1940; Garcia-Bellido, 1979), are also involved in specifying the 'medial' vs 'lateral' identity of the projection. Our results show that the identity of the projection is not correlated to which of the two proneural genes has induced the formation of the bristle.

We have also analyzed the effect of the prepattern genes of the *iroquois* complex, *ara* and *caup*. The products of both *ara* and *caup* are expressed in the lateral region of the notum and are necessary for the activation of the *ac-sc* genes in this region (Gomez-Skarmeta et al., 1996). The absence of lateral bristles in *iro* mutants can occasionally be rescued in the Hw^{49c} mutant, due to the ectopic expression of the *ac-sc* genes. We observed that, in the absence of ARA and CAUP, the lateral bristles that form in Hw^{49c} flies display sensory projections that have contralateral branches. This phenotype was never observed in Hw^{49c}; iro^+ flies, ruling out an effect of Hw^{49c} itself.

The abnormal presence of contralateral branches observed in Hw^{49c}; iro^- flies could possibly be due to an effect on the CNS rather than on the sensory neurons. However we found that the projection of the humeral bristles, which do not depend on *iro* to be formed, remains strictly ipsilateral in iro^1/iro^2 mutants. The results are therefore consistent with the conclusion that the positional identity of the lateral sensory neurons has been transformed to a 'medial' one (presence of contralateral branches) due to the iro^1/iro^2 mutant background.

The genes of the *iro* complex may confer a 'lateral' identity to sensory neurons

The ectopic expression of either ARA or CAUP transforms the projections of the medial microchaetes, which now assume a strictly ipsilateral distribution typical of the most lateral microchaetes. We conclude that, in the microchaetes of the notum, the expression of either *ara* or *caup* is necessary and sufficient to confer a 'lateral' identity to the sensory neurons. Our results show therefore that the *iro* gene complex, besides its role in the activation of the *ac-sc* proneural genes in the lateral region of the notum, is also necessary to specify the positional identity of the sensory neurons innervating both microchaetes and macrochaetes. The gain-of-function transformation is not observed in the case of the medial macrochaetes (DCs and SCs), which retain their contralateral sensory projections. This negative result strongly suggests that the transformation of the microchaete projections is not due to an effect of the ectopic expression of the *iro* genes on the central nervous system.

Our present knowledge of the expression pattern of *ara* and *caup* genes is limited to the late larval stages, corresponding to the determination of macrochaete precursor cells (Gomez-Skarmeta et al., 1996; Leyns et al., 1996). We do not know whether *ara* or *caup* (or both) are still expressed at the time the neuron differentiates. Two possibilities are conceivable: the first is that the positional specification is conferred at the time the precursor cell is determined, and is transmitted through the

lineage to the innervating sensory neurons by perdurance or by an undefined genetic relay; the second possibility is that the specification is conferred to the sensory neuron by a late expression (or re-expression) of the *iro* genes.

We do not know whether the absence of an effect of *iro* on the medial macrochaetes is due to an insufficient level of expression at the critical time, or whether other factors may override the effect of *iro* gain-of-function in the medial macrochaetes. In this context, it is worth noting that the expression of the *sca*-GAL4 line used to drive the *iro* genes seems to be turned down in the macrochaete precursors, but not in the microchaete precursors, before they undergo their first division (N. Gompel and K. Usui, personal communication). Thus the difference between microchaetes and macrochaetes might be due to a sustained expression of GAL4 in the former, consistent with the idea that positional information is conferred early in the lineage, and is simply transmitted to the neuron by perdurance of the *iro* products.

The difference between medial and lateral sensory projections may be determined by the capability of the growth cone to cross the midline boundary. The special importance of midline structures and molecules in the shaping of neural connections has been well documented both in invertebrates and in vertebrates, leading to the identification of signaling molecules which have been conserved through evolution (Stoeckli and Landmesser, 1998; Thomas, 1998). Such molecules could mediate the different behaviours of medial and lateral neurons, and the corresponding genes might be direct targets through which the *iro* genes specify differences in pathway recognition.

Dual function of the prepattern genes

The ‘prepattern’ hypothesis postulated by Stern defined the prepattern as a set of ‘regionalized tissue-properties’ to which *ac*-containing and non-containing cells respond differentially (Stern, 1954). However, Ghysen and Dambly-Chaudière suggested that the prepattern factors could be ‘region-specific expressions of activators and repressors of *ac* and *sc* expression’ (Ghysen and Dambly-Chaudière, 1989). The results that we obtained in the case of the *iro* gene complex suggest that both definitions could hold true, since the ARA and CAUP transcriptional factors are certainly involved in the activation of *ac-sc* gene expression, yet they also specify the positional identity of these ‘ac-containing cells’, i.e. the type of sensory projections established by the derived neuron. The analysis of other prepattern genes such as *pnr* (Ramain et al., 1993; Heitzler et al., 1996) and *wg* (Phillips and Whittle, 1993) in the specification of the positional identity of sensory neurons may reveal whether the dual function of the *iro* genes is a general feature of prepattern genes.

Recent results have shown that the vertebrate *iro* homologues are involved in a ‘prepattern’ function during the *Xenopus* nerve chord development (Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998). It would be interesting to investigate whether the functional conservation holds true for the neuron positional identity as well.

We thank A. García-Bellido for pointing out that *iro* was a prime candidate for providing positional information to the neurons. We thank Ruth del Corral and Juan Modolell for allowing us to use their UAS-*caup* lines prior to publication, Juan Modolell for giving us free access to his lab’s resources and in particular for making the *hs-sc* and the UAS-*ara* line available to us, and Yuh-Nung Jan for the *sca*-GAL4 line.

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