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Proneural clusters: equivalence groups in the epithelium of *Drosophila*

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Summary

The segregation of neural precursors from epidermal cells during development of the nervous system of *Drosophila* relies on interactions between cells that are thought to be initially equivalent. During development of the adult peripheral nervous system, failure of the cellular interactions leads to the differentiation of a tuft of sensory bristles at the site where usually only one develops. It is thus thought that a group of cells at that site (a proneural cluster) has the potential to make a bristle but that in normal development only one cell will do so. The question addressed here is do these cells constitute an equivalence group (Kimble, J., Sulston, J. and White, J. (1979). In *Cell Lineage, Stem Cells and Cell Determination* (ed. N. Le Douarin). Inserm Symposium No. 10 pp. 59–68, Elsevier, Amsterdam)? Within clusters mutant for *shaggy*, where several cells of a cluster follow the neural fate and differentiate bristles, it is shown that these display identical neuronal specificity: stimulation of the bristles evoke the same leg cleaning response and backfilling of single neurons reveal similar axonal projections in the central nervous system. This provides direct experimental evidence that the cells of a proneural cluster are developmentally equivalent.

Key words: *Drosophila*, proneural cluster, equivalence group, epithelium, neural precursor, *shaggy*, neuron, sensory bristle.

Introduction

The central and peripheral nervous systems of *Drosophila* develop from single neural precursor cells that segregate individually from over a large area of ectoderm (Hartenstein and Campos-Ortega, 1984; Hartenstein and Posakony, 1989). Each neuroblast or sensory mother cell therefore adopts a developmental fate different from that of neighbouring epidermal cells. Evidence has accumulated, however, that the decision to make a bristle mother cell is initially taken by a small group of cells that are collectively determined. The genes *achaete* and *scute* govern the positions of bristles through the precise spatial distribution of small clusters of cells expressing their transcripts (Romani *et al.* 1989). It has been postulated that such proneural clusters are equivalence groups by analogy to a similar mode of determination in the nematode (Kimble, 1981; Sulston and White, 1980; Palka, 1986; Cabrera *et al.* 1987; Simpson, 1990). Subsequent cell interactions occurring between the equivalent cells lead to the singling out of only one cell that adopts the dominant, neural fate. This dominant cell then inhibits the other members of the group from realising their neural potential by means of a signalling mechanism known as lateral inhibition and they then adopt the secondary epidermal fate (Wigglesworth, 1940; Richelle and Ghysen, 1979; Held and Bryant, 1984; Simpson and Carteret, 1989; Simpson, 1990). The gene *shaggy* and the genes of the neurogenic class mediate this process (Bourouis *et al.* 1989; Lehmann *et al.* 1983; Campos-Ortega, 1985; 1988). When the tissue is mutant for one of these genes, this signalling process fails and many or all of the cells within a cluster adopt the neural fate. This leads to the differentiation of a tuft of bristles at the position of each extant one (Dietrich and Campos-Ortega, 1984; Simpson and Carteret, 1989; Simpson, 1990). From the average number of bristles per tuft, it has been estimated that a proneural cluster is composed of six to seven cells (Simpson, 1990).

Here we have tested the postulate that the cells of a proneural cluster are equivalent. It has been shown that, in a wild-type fly, each large bristle or macrochaete on the thorax, which occupies a unique position, makes a specific neuronal connection in the central nervous system. This is seen both in the specificity of the behavioural response elicited from the fly upon stimulation of the bristle, and in the axonal projection pattern of the bristle neurons in the thoracic ganglion (Vandervorst and Ghysen, 1980; Ghysen, 1980). We have analysed clusters of bristles caused by mutation at the *shaggy* locus that result from a failure of lateral inhibition, and have found that all the bristles of a cluster have identical properties, showing that they are equivalent.

Materials and methods

Flies were raised on standard medium and maintained at 25°C.

Clones mutant for sgg^{D127} (Simpson *et al.* 1988; Bourouis *et al.* 1990) were produced by X-ray-induced mitotic recombination. 24 h egg collections were made and flies of the genotype sgg^{D127} *w s*/+ were irradiated between 48 and 72 h AEL with 1000 R of X-rays (100 kV, 4 mA given for 3 min. 18 s, 1.5 mm aluminium filter, Philips MG102 constant potential X-ray system, beryllium window). The bristles were therefore not marked but in some cases clones were produced with an accompanying labelled twin clone in sgg^{D127} *w s*/*y w* f^{36a} flies.

Flies bearing selected appropriate thoracic clones were anaesthetized, decapitated and left for at least an hour in a moist chamber, following the protocol of Vandervorst and Ghysen (1980). Individual bristles were tickled with a fine hair and the leg cleaning responses recorded.

Backfills of thoracic sensory neurons were achieved following the protocol of Ghysen (1978, 1980). Flies with selected thoracic clones were immobilised in plasticine on a microscope slide and a drop of horseradish peroxidase ($\simeq$75 mg ml^{-1}) placed over the mutant bristles. We obtained greatest success by first breaking off the bristle with tweezers and then scraping off the stump with the rough edge of the side of the tweezers. Flies were left between 16 and 20 h at 18°C. Thoracic ganglions were then dissected, fixed and stained with diaminobenzidine and H_2O_2. The preparations were examined as whole mounts and drawings of the stained axonal projections were made.

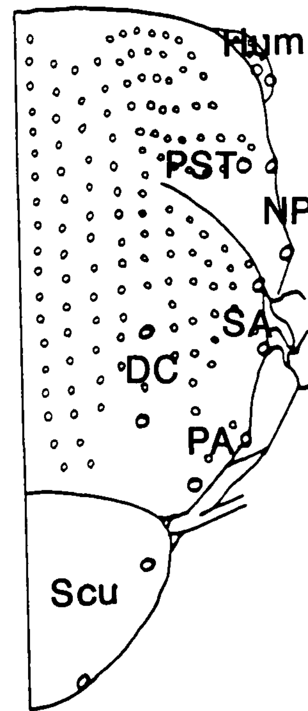

Fig. 1. Standard diagram of the wild-type hemithorax showing the positions of macrochaetae, large circles, and microchaetae, small circles. The macrochaetae are named as follows: DC, dorsocentral; Scu, scutellar; PA, postalar; SA, supraalar; NP, notopleural; PST, presutural; Hum, humeral.

Results

Behavioural response to stimulation of individual bristles within mutant clusters

The positions of the large mechanosensory bristles, the macrochaetae, on the notum of the fly are shown in Fig. 1. Tactile stimulation of single bristles induces, among other responses, a cleaning movement by a leg (Vandervorst and Ghysen, 1980). In some cases, stimulation of specific bristles will evoke a response from a specific leg (ibid). We chose the anterior postalar, presutural, humeral and posterior postalar bristles for our study as stimulation of these bristles leads to a fairly constant response.

Clones of cells mutant for *shaggy* (*sgg*) differentiate a cluster of bristles at the site of each extant one. This phenotype is due to the fact that a greater number of cells adopt the neural fate at the expense of epidermal cells. Complete penetrance for this phenotype is seen in clones mutant for some alleles of *Delta*, and in this case a dense tuft of adjacent bristles forms (Dietrich and Campos-Ortega, 1984; Simpson, 1990). The incomplete penetrance of mutant *sgg* cells for the bristle transformation phenotype means that in all clones some epidermal cells form between the bristle precursors (Simpson and Carteret, 1989). Subsequent division of these epidermal cells leads to dispersion of the macrochaetae (Simpson, 1990), which facilitates the stimulation of individual bristles within the tuft. Nevertheless, in order to be able to stimulate individual bristles, we found it necessary to select clones with small clusters composed of two to three bristles. Here we present the results of the testing of each bristle of a cluster individually. We chose not to label the mutant clones with marker mutations of the cuticle since it has not been shown that these mutations are without effect on the growth and connections of bristle neurons. Clones were recognized because of additional macrochaetae and an increased density of microchaetae. (In wild-type flies additional macrochaetae are extremely rare, Simpson, 1990).

The behavioural responses observed after testing individual bristles within the mutant clusters are presented in Table 1. In 98 % of the cases that responded, individual bristles of the same cluster behaved similarly to one another and elicited a cleaning response from the same leg. Therefore, we conclude that bristles mutant for *sgg* are innervated and that all the bristles of a cluster generally make the same connections.

We then tested whether stimulation of the mutant bristles provoked the same response as in the wild type. As controls the corresponding wild-type bristle on the contralateral hemithorax was tested for each fly bearing a mutant tuft of bristles. The results, presented in Table 2, show that the mutant bristles behave like the wild-type ones and elicited the same behavioural response. The neurons of bristles mutant for *sgg* therefore make the appropriate connection in the central nervous system.

Table 1. *Frequency with which different bristles in a mutant proneural cluster show the same specific response to tactile stimulation*

Bristle*	No. of clusters	Frequency (%) with which individual bristles of each cluster gave				Average clone size
		Same response	Different response	No response	Only some responded	
APA	39	82	3	10	5	2.3±0.1
PST	28	82	0	7	7	2.3±0.1
HUM†	26	73	0	15	11	3.0±0.1
PPA	26	88	0	12	0	2.2±0.15
Total	119	81	2	11	6	2.5±0.1

* See Legend to Fig. 1 for abbreviations.
† It is difficult to distinguish the two clusters of macrochaetae on the humerus and therefore all the bristles here were considered together.

Table 2. *Frequency and specificity of leg cleaning response to stimulation of individual bristles mutant for* shaggy

Bristle	Experimental (%)			No. of bristles	Control (%)			No. of bristles
	L1	L3	No response		L1	L3	No response	
APA	1	86	11	83	0	87	13	39
PST	52	38	10	60	65	24	10	29
HUM*	72	5	23	79	79	2	19	52
PPA	5	84	11	57	0	88	12	26

Controls were the appropriate non-mutant bristle on the contralateral hemithorax.
* see footnote to Table 1. The two humeral macrochaetae were scored together in the controls.
L1, first leg; L3, third leg.

In the case of the wild-type postalar bristle, 100 % of those that responded induced a cleaning action from the metathoracic leg. Similarly, stimulation of the wild-type humeral bristles led to a cleaning action from the prothoracic leg in virtually all those cases where a response was obtained. Clusters of bristles mutant for *sgg* at the postalar bristle sites and the humeral bristle sites similarly gave a cleaning response from the third or the first leg, respectively. The response obtained after stimulation of the wild-type presutural bristle varied however: in some flies, the first leg and, in others, the third leg responded. Interestingly, the clusters of mutant bristles also gave both responses in more or less the same proportion as the wild-type population. Therefore, in one fly the mutant cluster at the presutural site will cause a response from the first leg, but in another, a response from the third leg. Nevertheless, in spite of two possible responses, the mutant bristles within the same cluster always behaved in the same way. Furthermore the choice between first or third leg response is apparently made independently in each hemithorax: of 27 thoraces where a response was obtained from both the mutant cluster and corresponding contralateral wild-type bristle, 67 % gave the same response but 33 % gave different responses. A control experiment was performed in wild type flies where it was found that 81 % of cases gave the same response for the presutural bristles of either side of the thorax, but 19 % gave different responses (n=36).

Central projection of neurons of individual bristles within mutant clusters

The axonal projection pattern of neurons from specific thoracic macrochaetae show considerable individual variation, but some constant reproducible features are also seen (Ghysen, 1980). We focused on the two dorsocentral bristles as they are positioned close to one another on the thorax but nevertheless make distinguishable projections. The axonal pathways followed by their neurons in the wild type are shown in Fig. 2. The posterior dorsocentral axons project further posteriorly and display the metathoracic cross branch. These features are not seen for the anterior dorsocentral bristle.

Clones of cells mutant for *sgg* that differentiated three or four bristles at the site of either the anterior or posterior dorsocentral bristles were selected. Backfilling was attempted for all the bristles of a cluster, although in most cases only a single one was successfully filled. The success rate, however, was no lower than that achieved for the backfilling of single bristles in wild-type animals (not shown). Seventeen cases of anterior dorsocentral bristle clusters were analysed; in four of these, two neuronal axons had stained. Drawings of five projections are shown in Fig. 3. In spite of individual variation all show the characteristic anterior dorsocentral projection seen in the wild type. Twelve backfills from posterior dorsocentral bristle clusters were analysed, one of which stained two

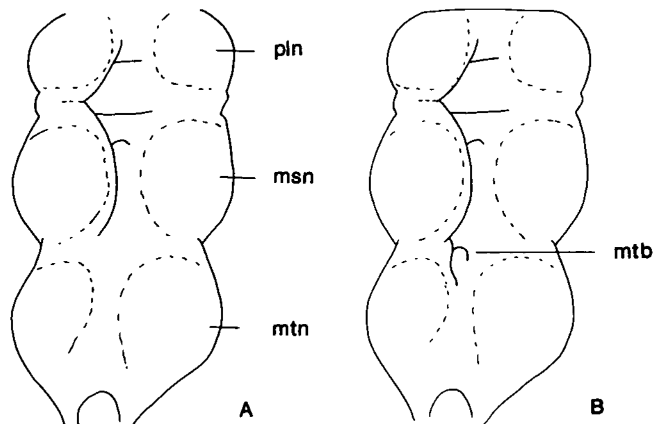

Fig. 2. Ventral half of the thoracic ganglion: pln, prothoracic leg neuromere; msn, mesothoracic leg neuromere; mtn, metathoracic leg neuromere.
The pathways followed by the sensory neurons of the anterior dorsocentral bristle (A) and the posterior dorsocentral bristle (B). The neurons of these two thoracic bristles enter the ganglion through the posterior dorsal mesothoracic nerve and project both anteriorly and posteriorly. Both neurons display a number of cross branches but the posterior dorsocentral bristle neuron extends much further posteriorly and unlike the anterior dorsocentral bristle neuron also projects along the metathoracic cross branch (mtb). Adapted from Ghysen (1980).

neurons and another three neurons. Five of these are also shown in Fig. 3. All stainings revealed the characteristic posterior dorsocentral bristle pattern, recognizably different from the anterior dorsocentral pattern by the presence of the metathoracic cross branch.

Discussion

Within epithelia mutant for *sgg*, a tuft of several macrochaetae differentiates at each site where, in the wild type, a single one develops. This is thought to be the result of a failure of lateral inhibition between a group of equipotential cells (a proneural cluster) all of which have the potential to form a bristle. This observation reveals that the cells of a cluster are developmentally equivalent in that they all follow the neural fate and produce sensory bristles rather than epidermis. It has therefore been suggested that these groups of cells constitute equivalence groups (Simpson, 1990). Kimble *et al.* (1979) defined an equivalence group as a group of cells that share a common developmental potential but that subsequently follow different fates as a result of cell interactions. Similar developmental phenomena have been observed in the leech (Weisblat and Blair, 1984; Shankland and Weisblat, 1984) and are thought to operate in insect embryos during the separation of epidermal and neural lineages (Kuwada and Goodman, 1985; Doe and Goodman, 1985; Technau and Campos-Ortega, 1986).

If the cells of a proneural cluster are equivalent then not only could they each develop a sensory bristle but the bristles should display identical neuronal specificity. We first used a physiological assay to test whether the individual bristles in a mutant tuft are functionally equivalent. Our results show that the stimulation of individual mutant bristles elicits the same behavioural response as that of the corresponding wild-type one. Mutant bristle neurons therefore make the appropriate connections in the central nervous system. Stimulation of some macrochaetae, such as the presutural bristle, can elicit a cleaning response from either the first or the third leg in wild-type flies. Each presutural bristle tested in the wild type will provoke either one or the other response but not both, suggesting that at this site there may be a choice of target neurons in the central nervous system. This choice is apparently not a random event since more bristles lead to a first leg response in both the wild type and the mutant. Interestingly, in the case of a mutant cluster of bristles, all the bristles gave either a first or third leg response. Thus the individual bristles of a cluster apparently make the same connections. While the physiological basis underlying this result is not clear, it reinforces the conclusion that all cells within a given cluster are identical. Therefore all of the bristles within a cluster mutant for *sgg* exhibit the same neuronal specificity. This suggests that all cells of a proneural cluster can potentially produce a bristle with the same neuronal identity, and that they therefore constitute an equivalence group.

A second test of similarity between the bristles of a given cluster involved the study of the axonal projections of neurons of mutant bristles. There is evidence that the detail of axonal projections is a function of the position in the epithelium at which the neuron (or sensory precursor cell) was born (Ghysen, 1980; Walthall and Murphey, 1984; Taghert *et al.* 1984; Doe and Goodman, 1985; Patel *et al.* 1989; Doe *et al.* 1988*a,b*). In the thoracic imaginal disc the bristle precursors are dispersed over a wide area. The specific positional identity that will lead to a specific neuronal identity in only a single cell is likely to be a property of a small area of the epithelium at the site where each macrochaete will form. Therefore if a small group of cells at that position collectively adopt a neural fate then their positional identities may be the same and consequently their projection patterns would be identical. We found, in fact, that the individual projections of bristles of a cluster are the same. We conclude that the equivalence groups that are established at specific positions are composed of a sufficiently small number of cells that have received the same positional specification. Subsequently if more than one cell of the group adopt the neural fate they will display the same neuronal specificity.

These results also reinforce our earlier conclusions on the role of the gene *sgg*. *shaggy* is required for the selection of a single cell from each proneural cluster. It does not play a role in determining which bristle types differentiate where (Simpson *et al.* 1988; Simpson and Carteret, 1989).

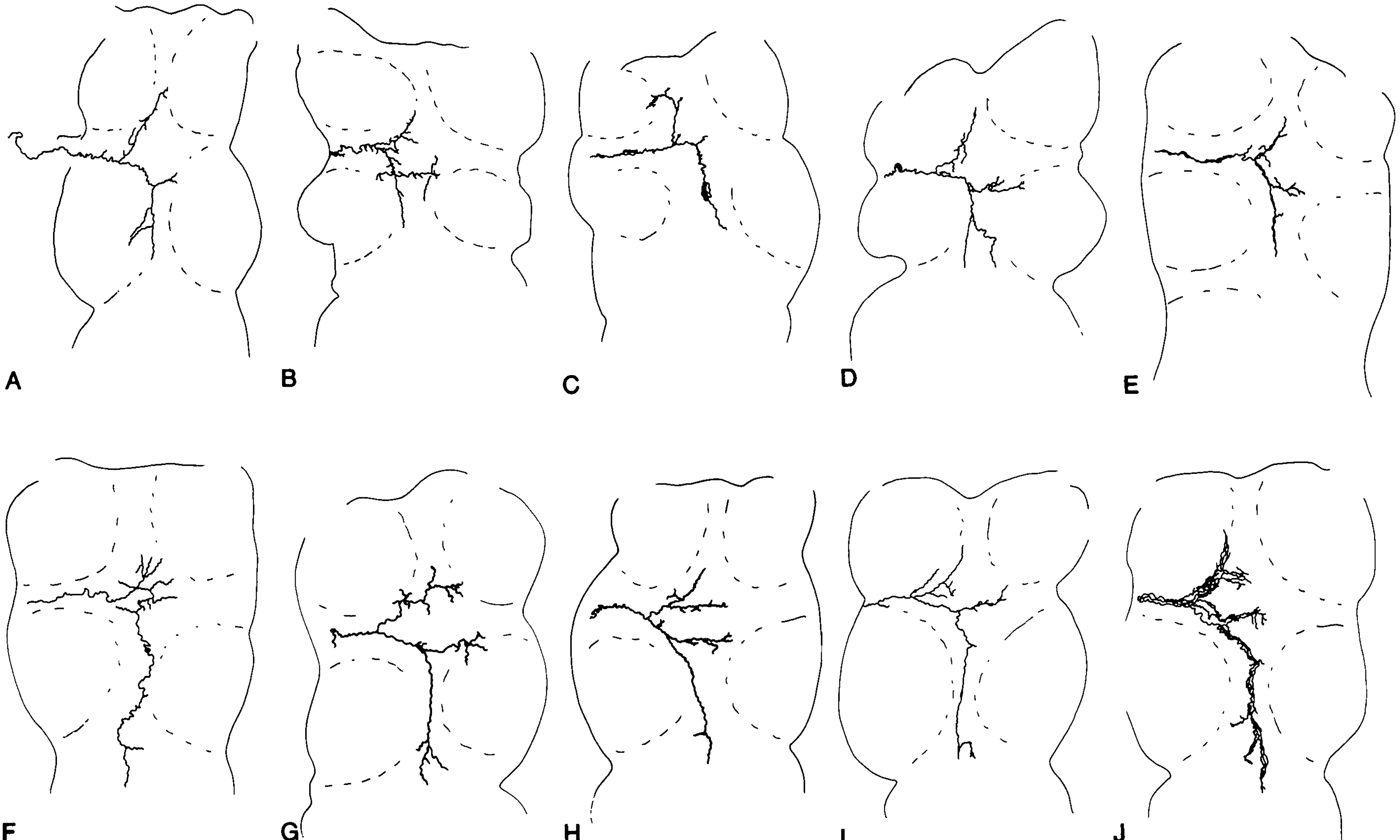

Fig. 3. Projection patterns of the neurons of supernumerary thoracic macrochaetae mutant for *shaggy*. Mutant clones displaying a tuft of bristles at the position of the extant anterior or posterior dorsocentral bristles were selected. Backfill and staining was attempted for all bristles of the cluster but in most cases only one succeeded. (E) The projection of two neurons and (J) the projection of three neurons; (A–E) sensory projection patterns from anterior dorsocentral bristles. These neurons display the characteristic pattern schematised in Fig. 2A. (F–J) Sensory projection patterns from posterior dorsocentral bristles. These neurons display the pattern schematised in Fig. 2B and unlike those of anterior dorsocentral bristles extend posteriorly into the metathoracic leg neuromere and display the metathoracic cross branch.

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