Genetic basis of the formation and identity of type I and type II neurons in
Drosophila embryos

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SUMMARY

The embryonic peripheral nervous system of Drosophila contains two main types of sensory neurons: type I neurons, which innervate external sense organs and chordotonal organs, and type II multidendritic neurons. Here, we analyse the origin of the difference between type I and type II in the case of the neurons that depend on the proneural genes of the achaete-scute complex (ASC). We show that, in Notch− embryos, the type I neurons are missing while type II neurons are produced in excess, indicating that the type I/type II choice relies on Notch-mediated cell communication. In contrast, both type I and type II neurons are absent in numb− embryos and after ubiquitous expression of tramtrack, indicating that the activity of numb and the absence of tramtrack are required to produce both external sense organ and multidendritic neural fates. The analysis of string− embryos reveals that when the precursors are unable to divide they differentiate mostly into type II neurons, indicating that the type II is the default neuronal fate. We also report a new mutant phenotype where the ASC-dependent neurons are converted into type II neurons, providing evidence for the existence of one or more genes required for maintaining the alternative (type I) fate. Our results suggest that the same mechanism of type I/type II specification may operate at a late step of the ASC-dependent lineages, when multidendritic neurons arise as siblings of the external sense organ neurons and, at an early step, when other multidendritic neurons precursors arise as siblings of external sense organ precursors.

Key words: Drosophila, sense organ, neuronal identity, cell fate determination

INTRODUCTION

The sensory neurons of arthropods belong to either of two main types, called type I and type II (Zacharuk, 1985). Type I neurons are characterized by their single dendrite whose distal part is a modified cilium and are associated with accessory cells which form the non-neuronal part of the sense organs. In contrast, type II neurons possess several dendrites lacking ciliated structures, and are not associated with accessory cells. This division into type I and type II neurons extends to other phyla, and could represent an ancient feature of sensory systems (Bullock and Horridge, 1965; Lentz, 1968; Blackshaw, 1981).

Type I and II neurons are easily distinguished in the Drosophila embryo, where the sensory neurons are arranged in a highly stereotyped pattern (Fig. 1A; Campos-Ortega and Hartenstein, 1985; Ghysen et al, 1986; Bodmer and Jan, 1987; Hartenstein, 1988). Type I neurons innervate two main types of sense organs: the external sense organs (es organs) and the chordotonal organs (ch organs; Fig. 1A; Ghysen et al, 1986; Hartenstein, 1988), and are called es and ch neurons, respectively. Type II neurons have been named multidendritic neurons (md neurons; Fig.1; Bodmer and Jan, 1987), as most of them have profuse dendritic arborizations which end freely under the epidermis (Bodmer and Jan, 1987). A large body of cellular, genetic and molecular analyses has produced a wealth of information about the development of the ch and es organs and their neurons (Jan and Jan, 1993). In contrast, little is known about the origin and development of the md neurons.

All type I neurons derive from precursor cells (Bate, 1978) through a fixed lineage that also produces the accessory cells of the sense organs (Bate, 1978; Bodmer et al., 1989; Brewster and Bodmer, 1995). By analogy, it has been suggested that md neurons also originate from precursor cells, likely to be born around the same time as es and ch precursors (Bodmer et al., 1989). Recently, the origin of md neurons has been directly assessed using FLP/FRT-induced lacZ clones (Struhl and Basler, 1993). The results (Fig. 1B; Brewster and Bodmer, 1995) suggest that some md neurons are produced by precursors that also generate ch or es cells. In that case, the md neuron appears to be the sister cell of the es or ch neuron. Other md neurons appear to have precursors which do not produce es or
Characterization of the X1 deficiency

ch neurons (‘solo’ md neurons). Finally, some es and ch lineages do not produce md neurons (‘solo’ es and ‘solo’ ch lineages).

The competence to become a precursor cell depends on the expression of one or more ‘proneural genes’ (Ghysen and Dambly-Chaudière, 1989). Among the known proneural genes, those of the *achaete-scute* complex (ASC) are required for the formation of the es organs (Garcia-Bellido and Santamaría, 1978; Garcia-Bellido, 1979; Garcia-Alonso and Garcia-Bellido, 1986). In the embryo, ASC genes are required not only for the formation of all es organs, but also for most md neurons (Dambly-Chaudière and Ghysen, 1987). Another gene, *atonal* (ato), is responsible for the formation of the ch organs and of a small subset of md neurons (Jarman et al., 1993).

In this paper, we investigate the origin of the difference between es and md identities among the ASC-dependent lineages. Our results indicate that this specification uses the same mechanism that assigns the different cell fates within the es and ch lineages (for review, see Posakony, 1994, Jan and Jan, 1995; Vervoort et al., 1997), and suggest that the default state of the ASC-dependent lineages is the md fate. This default state requires the activity of *numb* (Umura et al., 1989) and the silence of *tramtrack* (ttk; Read and Manley, 1992). The alternative fate (es) requires Notch-mediated cell communication (Fortini and Artavanis-Tsakonas, 1993), as well as a new factor that we named *XI*.

**MATERIALS AND METHODS**

*Drosophila* strains

Oregon R was used as wild-type strain. We used the following mutant stocks: w* N55c11 / FM7C and Df(1)N8 / In(1)dl49, y Hw m* g f for the analysis of N phenotypes; numb* / CyO (Umura et al., 1989) for the analysis of *numb*; ttk* c11 / TM3 Sb Ser (Xiong and Montell, 1993) and hs-ttk2069 (Read and Manley, 1992) for *tramtrack*; st e sty2869 / TM3 Sb Ser (Jurgens et al., 1984) for string; phb70* / TM3 Sb Ser and phb11D / TM3 Sb Ser (Lehner, 1992) for *pebble*. *XI* was used as a y *X1 f / FM7C* stock.

As markers, we also used the following enhancer trap lines: E7-2nd-36, in which *lacZ* is expressed exclusively in the md neurons, A1-2nd-29, in which *lacZ* is expressed specifically in the outer accessory cells of the es organs (Bier et al., 1989), and A37, in which *lacZ* is expressed in all sense organ precursors and in their progeny (Ghysen and O’Kane, 1989). We assessed the effects of various mutations on these lines in the progeny of flies doubly heterozygous for the mutation and for the enhancer trap insertion.

**Characterization of the *X1* deficiency**

The stock containing the *X1* deficiency was originally sent to us as Df(1)sol20b, a deficiency which deletes the 13F-14B region of the X-chromosome. We found, however, by using mutations and rearrangements of this region that our stock was not sol20b but harboured another X-linked lethal mutation and renamed it *X1*. We localized the lethality by recombining *X1* with a y w ct m f chromosome. The lethality could not be separated from the w* allele among 612 males progeny. We confirmed the linkage between w* and the lethality and the *X1* phenotypes by testing individually 25 w y w ct m / y w ct m f females, all of which failed to produce w* males and gave one fourth of the embryos with the cuticular (see below) and neuronal *X1* phenotype. We used rearrangements of the w region (position 3C) to localize *X1* more precisely. The lethality due to *X1* is rescued by the duplications Dp(1;1)w2820 (3A4 to 3E8-F1) and Dp(1;1)w36-42 (3C1-2 to 3D6-E1), localizing the locus to the 3C1-2 to 3D6-E1 interval. We observed that *X1* does not complement the deletions Df(1)N71b (3C4 to 3D5) and Df(1)N8 (3C2-3 to 3E3-4), but does complement Df(1)X69b (3C6 to 3D1-4) and Df(1)h34 (3A4-6 to 3C5-6). Taken together, these results suggest that the *X1* mutation maps between 3D1-4 to 3D5 or, if it were a deletion, that it is comprised between 3D1-4 and 3D6-E1.

We characterized the phenotypes of *X1* at the level of the es organs using additional markers to those described in the results section. First, we observed on cuticular preparations (Dambly-Chaudière and Ghysen, 1986), that the external structures of the es organs are absent, while all other cuticular derivatives are present. We confirmed that this absence is related to the absence of the es outer cells by using the A1-2nd-29 enhancer trap line (Bier et al., 1989) and anti-Cut labelling (Blochlinger et al., 1990). We also observed that the es sheath cell markers anti-BarH1 (Higashima et al., 1992) and anti-Propero (Vaessen et al., 1991) fail to label any cell in *X1* embryos.

**Immunolabelling**

Immunolabellings were made as described (Ghysen et al., 1986) using PBS as buffer. Double-labellings were made following Higashima et al. (1992) but using both Nickel and Cobalt ions. All antibodies were used at the published concentrations. We used a rabbit polyclonal anti-

**Heat-shock treatments**

5-6 hours embryos containing two copies of the *hsp70-p69* cDNA construct were placed for 1 hour in a water bath at 37°C and then transferred to 18°C until the end of embryogenesis.

**Dil labelling**

Single sensory neurons of *X1* embryos at stage 17 were stained by in vivo injection with the carbocyanine dye, 1,1’-dioctadecyl-3,3,3’,3’-tetramethyl indocarbocyanine perchlorate (DiI; Molecular Probes) according to Merritt and Whittington (1995). Briefly, the tips of microelectrodes were filled with an ethanolic solution of DiI, brought into contact with a sensory neuron cell body and depolarizing current applied. After fixation, preparations were photoconverted in the presence of 0.2% diamino benzine to give a permanent dark reaction product and mounted in 100% glycerol.

**RESULTS**

**Loss of es neurons and overproduction of md neurons in *N*-embryos**

Fig. 1A summarizes the pattern of sensory neurons in the abdominal segments of the embryo, and the proneural genes on which these neurons depend. 27 neurons (open profiles in Fig. 1A) depend on one or more genes of the *achaete-scute* complex (ASC genes), 11 neurons (stippled profile) depend on *atonal* and 3 neurons (dotted profile) depend on neither ASC nor *ato*. We have focused our analysis on the ASC-dependent sensory neurons because most larval md neurons fall in this category. ASC precursors display three major types of lineages, as illustrated in Fig. 1B: some of them produce both es and md cells (es/md precursors) while others produce only one of the
following lineages I and II are related to each other, as shown in IV. Bodmer (1995). We propose (see discussion) that precursors II and III represent three types of lineages described by Brewster and cell (thecogen); to, tormogen (socket cell); tr, trichogen (shaft cell). I, are second-order precursors; pIII is a third-order precursor; sh, sheath protoprecursor; pI es/md, ‘mixed’ es/md precursor; pIIa, pIIb and pII tr to trichogen (shaft cell). I, II and III represent three types of lineages described by Brewster and Bodmer (1995). We propose (see discussion) that precursors following lineages I and II are related to each other, as shown in IV.

two types (‘solo’ es and ‘solo’ md precursors; Brewster and Bodmer, 1995).

The assignment of different cell fates in the es lineages requires cell interactions mediated by the product of the gene Notch (N; Posakony, 1994; Jan and Jan, 1995, Vervoort et al., 1997). In the absence of a functional N gene, sensory neurons are overproduced at the expense of the accessory cells (Hartenstein and Campos-Ortega, 1986; Hartenstein and Posakony, 1990b; Fig. 2A,B). We wondered whether N could also be involved in the assignment of the es and md fates. We analysed the identity of the neurons in N− embryos, using specific markers of es or md neurons. The anti-BarH1 antibody labels es neurons (as well as their associated sheath cells) but none of the md neurons (Higashima et al., 1992). The enhancer-trap line E7-2nd-36 expresses lacZ in most md neurons but not in es ones (Bier et al., 1989).

We observed in N−, E7-2nd-36 embryos large numbers of cells expressing lacZ (Fig. 2C,D) while no BarH1-expressing cells are present (Fig. 2E,F). In the dorsal cluster of neurons, which comprises only es and md neurons in the wild type, all neurons are of the md type in N− conditions. To confirm that the supernumerary md neurons seen in N− embryos are derived from ASC-expressing precursors, we used the anti-Cut antibody, which labels all ASC precursors and their progeny, but not the ASC-independent precursors (Blochlinger et al., 1990). We observed large numbers of cut-expressing cells in N− embryos (Fig. 2G,H), suggesting that most supernumerary md neurons derive from ASC precursors.

We conclude from these experiments that N+ is required to specify the es fate, since in N− conditions only md neurons are produced. This is true not only in the ‘mixed’ es/md lineages, but also in the lineages that normally generate only es neurons (the ‘solo’ es lineages). This result suggests that all ASC-dependent precursors have the potentiality to produce md neurons and that the N+ function is required in all types of ASC-dependent lineages to specify the es alternative.

**Effects of numb on the formation of md neurons**

The progeny of es precursors comprises three types of accessory cells and one or more neurons (Bate, 1978; Bodmer et al., 1989; Brewster and Bodmer, 1995). It is thought that the attribution of different identities to the daughter cells relies both on cellular communications, mediated by N, and on the unequal distribution of an intrinsic determinant, numb (reviewed in Posakony, 1994; Jan and Jan, 1995; Vervoort et al., 1997). In the former section, we showed that N is involved in the specification of es versus md fates among the neurons. We examined whether numb could also be involved in this specification.

Of the two daughters of es precursors, the first to divide (pIIa) produces two accessory cells, while the second one (pIIb) generates the neuron(s) and the associated sheath cell. In the absence of numb, the two daughters usually adopt the same fate, pIIa (Uemura et al., 1989). In some cases, the pIIb cell forms but its two daughters differentiate as sheath cells (Brewster and Bodmer, 1995). Both defects result in the loss of the es and md neurons, which belong to the es/md lineages (Brewster and Bodmer, 1995; our own observations). We observed that, in addition, most or all of the ‘solo’ md are also missing (Fig. 3A; see also Brewster and Bodmer, 1995). For example, in the abdominal ventral and dorsal clusters, the only remaining md neurons are those that do not depend on ASC. In order to determine whether the loss of ‘solo’ md neurons is due to their conversion into es accessory cells, we labelled numb− embryos with anti-Cut. The es sheath cells and neurons are converted into accessory cells as judged by their increased expression of cut. We observed few or no other cell expressing cut (Fig. 3B), suggesting that the md cells do not form, or loose their ability to express cut, or die. We conclude that numb is required to produce all es and md neurons including the ‘solo’ md ones.

**Effects of tramtrack on the formation of md neurons**

Based on the epistatic relationship between numb and tramtrack (ttk), it has been suggested that the main function of numb is to repress the expression of ttk (Guo et al., 1995). Embryos mutant for ttk have a phenotype opposite to that of numb− embryos: the
neurons and sheath cells of the es and ch organs are duplicated, at the expense of the support cells (Salzberg et al., 1994; Guo et al., 1995). We examined whether ttk could be involved in the formation of md neurons by extending the analysis of the ttk phenotypes to the md neurons. We used the ttk/E11 mutation (Xiong and Montell, 1993) which behaves as a strong hypomorphic mutation (Guo et al., 1995), as no null allele is available. We observed a doubling of both md and es neurons of the es/md lineages (by using anti-BarH1 and 22C10 labelling; not shown). This amplification is the expected result of the transformation of pIIa into pIIb. In contrast, we did not detect any modification of the 'solo' md neurons. This is most clearly seen in the case of the ventral abdominal md neurons (arrowed in Fig. 1) which are well separated from all other neurons and hence easily distinguishable. These neurons are present in normal number and pattern in ttk embryos (not shown).

A gain-of-function analysis revealed that the ubiquitous expression of ttk, at stages when the precursors of adult sense organs form, results in a loss of all precursors and consequently in the disappearance of the adult sensory structures (Ramaekers et al., 1997). We examined whether, while not required for the formation of embryonic md neurons, ttk could interfere with it. The ubiquitous expression of ttk at 5-6 hours of development results in an almost complete loss of neurons, including all the md ones (Fig. 3C). At the level of the es organs, we found two phenotypes. Some es organs are completely missing (absence of both 22C10 and anti-Cut labelling; Fig. 3C), probably reflecting the disappearance of the precursors. Other es organs are present and comprise four accessory cells (no 22C10 but strong anti-Cut labelling; Fig. 3C) as in numb- embryos. The extent of these two phenotypes is variable, probably depending on the exact stage at which the embryos were treated. In all cases, we observed a nearly complete loss of both 'solo' md neurons and of the md neurons coming from md/es precursors.

We conclude that the ubiquitous expression of ttk prevents the acquisition of both es and md neural fates, and therefore that ttk is not involved in the choice between es and md identities.

**es and md neurons in the absence of cell divisions**

Taken together, the previous results suggest that the primary fate of ASC precursors is to produce md neurons and that N-mediated cell communication is required to produce the alternative (es) fate. In order to assess directly the primary fate of ASC precursors, we examined the identity of the neurons produced in the absence of precursor divisions. In embryos mutant for the string gene (stg-, Jurgens et al., 1984), which is required for the entry in M phase (Edgar and O’Farell, 1989), all divisions are blocked after the blastoderm stage, when the maternal supply of stg product is exhausted (Edgar and O’Farell, 1989). In such embryos, sense organ precursors do not divide but differentiate into neurons (Hartenstein and Posakony, 1990a; Fig. 4A).

We labelled stg- embryos with es- or md-specific markers (anti-BarH1 and the E7-2nd-36 enhancer trap line). Fig. 4B and C show that both md and es neurons are present. However, the proportion of the two types is dramatically altered. A wild-type abdominal hemisegment comprises 13 es organs and 21 md neurons. In stg- embryos, we observed on the average 6.7±0.9 md cells (n=31) and only 0.7 es cells (0 cells in 60% of the observed segments, 1 in 15%, 2 in 15% and 3 in 10%, n=49). The positions of these cells vary from one embryo to another. The total number of ASC-dependent precursors, as revealed by anti-Cut labelling, was 7.1±1.0 (n=32; Fig. 4D). We conclude that most ASC-dependent precursors, and sometimes all of them, differentiate into md neurons in the absence of a functional stg gene.

stg mutations block not only cell divisions but also the progression through the cell cycle, as Stg protein is required to exit G2 and enter M phase (Edgar and O’Farell, 1989). Recently, this G2 arrest has been shown to be important for cell division control. It has been proposed that Stg protein somehow prevents the acquisition of both es and md neural fates. We labelled stg- embryos with es- or md-specific markers (anti-BarH1 and the E7-2nd-36 enhancer trap line). Fig. 4B and C show that both md and es neurons are present. However, the proportion of the two types is dramatically altered. A wild-type abdominal hemisegment comprises 13 es organs and 21 md neurons. In stg- embryos, we observed on the average 6.7±0.9 md cells (n=31) and only 0.7 es cells (0 cells in 60% of the observed segments, 1 in 15%, 2 in 15% and 3 in 10%, n=49). The positions of these cells vary from one embryo to another. The total number of ASC-dependent precursors, as revealed by anti-Cut labelling, was 7.1±1.0 (n=32; Fig. 4D). We conclude that most ASC-dependent precursors, and sometimes all of them, differentiate into md neurons in the absence of a functional stg gene.

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specification as it prevents the expression of the neuroblast identity gene, *even-skipped*, in some neuroblasts of the embryonic central nervous system (Cui and Doe, 1995; Weigmann and Lehner, 1995). We examined whether the loss of *es* neurons observed in *stg* mutants could be related to the blocking of the precursors in G2 phase, and not to their inability to divide. For that purpose, we analysed *pebble* mutant embryos, in which cytokinesis (and hence cell division) but not cycle progression is prevented (Lehner, 1992; Hime and Saint, 1992). We observed that the precursors differentiate predominantly into *md* neurons (not shown), exactly as in *stg* mutants.

These results indicate that all ASC-dependent precursors have a tendency to form *md* neurons, and that both cell division and cell communication are required to generate *es* neuronal identity. In that view, the occasional appearance of *es* neurons in *stg* embryons could be a direct consequence of cell contacts between adjacent precursors. We tested that possibility by examining *N*; *stg* double mutants where we would expect to observe a complete loss of *es* neurons if the hypothesis is correct. We observed 7.4±1.9 cells expressing the neuronal marker 22C10 (Fig. 4E) and 5.8±1.8 cut-expressing cells (Fig. 4F) per hemisegments in *N*; *stg* embryons, but no *BarH1*-expressing cell was ever detected (not shown).

In a previous section, we showed that *numb* is required for the formation of all neurons. We wondered whether this requirement is maintained for the differentiation of precursors as neurons, when cell division is blocked. We analysed *numb*; *stg* embryons and observed that they lack most of the sensory neurons: only occasionally do 1 or 2 cells express neuronal markers (22C10, Fig. 4G; anti-Elav, not shown). In contrast, the number of *cut*-expressing cells is similar to that in *stg*-embryos (7.3±1.2; *n*=24; Fig. 4H). Furthermore, the high level of *cut* expression in these cells is typical of the *es* accessory cells (Blochlinger et al., 1990). We conclude that, in *numb*; *stg* embryons, the ASC precursors differentiate into accessory cells, and therefore that *numb* is required for the acquisition or expression of the neuronal fate even in the absence of cell division.

### X1, a mutation that prevents the acquisition of es fate

During a study of several genomic deletions (M. V., unpublished data), we found a line where the external derivatives of the *es* organs are absent in one fourth of the embryos. This phenotype is due to a previously unreported X-chromosome mutation (see Methods), which we named *X1*. As expected from the cuticular phenotype, we observed a loss of the markers of the *es* accessory cells (not shown; see Methods). Nevertheless, neurons are present in numbers slightly larger than normal (Fig. 5A,B). We assessed the identity of the neurons with the anti-BarH1 antibody. No cells are labelled in the hemizygous embryos (Fig. 5C), indicating that the *es* neuronal identity is lost. On the contrary, many neurons express *lacZ* in *X1*; *E7-2nd-36* mutant embryons (Fig. 5D). In the dorsal abdominal cluster, which comprises only *es* and *md* neurons in the wild-type, all neurons express *lacZ* (Fig. 5E). The conclusion that all or most dorsal neurons are of the *md* type was confirmed by filling dorsal neurons with DiI (Merritt et al., 1993). All the neurons that were filled (*n*=13) show the branched dendritic processes typical of *md* neurons (Fig. 5F). In the lateral regions, besides *md* neurons, several *ch* neurons were also observed (D. J. M., unpublished data).
Some md neurons arise from lineages that are ASC-independent; these neurons are overproduced in X1 embryos (D. J. M. and M.V., unpublished). The excess of md neurons in X1 embryos might therefore be solely due to the overproduction of ASC-independent precursors, and the loss of es cells could be caused by a loss of ASC-dependent precursors. Alternatively, the ASC-dependent precursors might appear normally but form only md neurons. In order to discriminate between these two possibilities, we used the anti-Cut antibody, which labels exclusively cells of the ASC-dependent lineages. We observed the presence of 6-7 md neurons expressing cut in the dorsal cluster of X1 embryos (6.7±1.6 cells, n=29; not shown). These results suggest that ASC-dependent precursors appear normally in X1 embryos and produce md neurons only.

We confirmed that ASC-dependent precursors are formed in X1 embryos in two ways. First, we combined X1 with the A37 enhancer trap line, which allows the detection of all precursors (Ghysen and O’Kane, 1989). At the earliest stage of precursor formation, two pairs of cells are formed within each segment: an anterior pair (A cells), which corresponds to ASC-dependent precursors, and a posterior pair (P cells), which corresponds to ato-dependent precursors (Ghysen and O’Kane, 1989). Soon after the A and P cells are formed, additional precursors appear in more ventral and dorsal positions (V and D cells). At this early stage, we cannot distinguish the hemizygous X1 embryos from their normal siblings. However, we never observed embryos that lack A cells when the first D or V cells start appearing. Furthermore, in some embryos, we observed one A cell and 3-4 P cells before any D and V cells were formed, confirming both the excess of ato-dependent precursors (P cells) and the presence of ASC-dependent precursors (A cells) in mutant embryos.

Second, we used the anti-Poxn antibody which labels specifically some of the es precursors (those that will give rise to poly-innervated sense organs; Dambly-Chaudière et al., 1992) and their progeny. We observed one, or rarely a few labelled cells per segment (not shown), suggesting that the precursors appear and may occasionally divide, but always lose poxn expression much earlier than normal, consistent with the idea that the es fate cannot be maintained in X1 embryos.

We conclude from these experiments that, in X1 embryos, ASC-dependent precursors appear normally but produce only md neurons and no es cells, suggesting that the mutation removes one or more factor(s) required for the acquisition or maintenance of the es fate as an alternative to the md fate within the ASC-dependent lineages. The X1 factor is required for the production of the es identity even when the precursors are unable to divide, as stg−; X1 double mutants lack all es neurons (anti-BarH1 labelling, not shown).

**DISCUSSION**

**Asymmetric cell divisions and cell fates**

Sense organ precursors produce a set of different cell types through fixed lineages. For example, in the case of ‘solo’ es organs, four different cells are generated: two accessory cells, maintenance of the es fate as an alternative to the md fate within the ASC-dependent lineages. The X1 factor is required for the production of the es identity even when the precursors are unable to divide, as stg−; X1 double mutants lack all es neurons (anti-BarH1 labelling, not shown).

**Fig. 4.** Differentiation of the precursors in the absence of cell divisions. (A) In stg− embryos, precursors differentiate into neurons as shown by 22C10 labelling. (B,C) Both md and es neurons can be observed, but while several md neurons are present (B; anti-β-Gal labelling of E7-2-36, stg− embryos), few es neurons can be observed (C; anti-BarH1 labelling of stg− embryos). The embryo in C is an extreme case: only one es cell (arrow) is present (see text for numbers). (D) Anti-Cut labelling of stg− embryos showing the presence of ASC-dependent sensory cells. (E) In N−; stg− double mutants, precursors differentiate into neurons (labelled by 22C10; arrows). (F) Several of these neurons come from ASC− dependent precursors, as they are labelled by anti-Cut labelling (arrows). (G) In numb−; stg− embryos, ASC-dependent precursors usually differentiate into es accessory cells: very few neurons are labelled with 22C10 (arrows in G) while many cells express strongly cut (H).
a sheath cell and a type I neuron (Bate, 1978; Bodmer et al., 1989, Brewster and Bodmer, 1995; Fig. 1). It is thought that the specification of these different identities is made progressively, through a succession of asymmetric cell divisions (Posakony, 1994; Jan and Jan, 1995; Vervoort et al., 1997). In a first step, the pl precursor produces two equipotent pII cells whose potentiality is subsequently restricted such that one (pIIa) will generate the two accessory cells while the other (pIIb) will generate the inner cells. In a second step, each pII cell divides to produce two equipotent cells which subsequently adopt two different fates: the daughters of pIIa differentiate as tormogen and trichogen cells, while the daughters of pIIb differentiate as sheath cell and neuron.

Several genes are involved in the allocation of the different cell types constituting the sense organ (Posakony, 1994; Jan and Jan, 1995; Vervoort et al., 1997). Mutations of these genes lead to the formation of abnormal sense organs with one or a few cell types overproduced at the expense of the others. For example, in the absence of N, two pIIb cells and no pIIa are produced, and the pIIb cells give rise to neurons only, and not to sheath cells (Hartenstein and Posakony, 1990b). The same set of genes functions at the different steps of the lineage, acting as ‘symmetry breaker’ (Garcia-Bellido, 1996) at each step.

The specification of different fates would then be due to the reiteration of the same developmental operation and the lineage itself can be viewed as a concatenation of asymmetric divisions (Horvitz and Herskovitz, 1992). This concatenation would ensure that at each step one daughter cell will adopt the default state corresponding to this step while its sister will necessarily adopt the alternative fate, and therefore that the entire complement of cells necessary for the proper functioning of the organ will be unerringly generated.

In this paper, we asked whether and how the type II (md) neuronal fate fits in this fate allocation system. We show that when the asymmetry-generating system is inactivated, in N− embryos, ASC-dependent precursors generate md neurons at the expense of the other cell types. This result suggests that the specification of the md fate is made through the same system used to specify the other cell fates and that the md neuronal fate is the primary or default state in these lineages.

‘mixed’ and ‘solo’ lineages

We will consider the case of the ‘mixed’ md/es lineages first. In this case, one of the daughters of pIIb behaves as a third order precursor, pIIb, which further divides to produce a md neuron and one (or more) es neuron(s) (Brewster and Bodmer, 1995). Since es neurons are absent and only md neurons are produced in N− embryos, we conclude that N+ is required to allow one of the daughters of pIIb to become an es neuron. The easiest interpretation of this effect is that the specification of es and md identities involves the same type of asymmetric division that acts in the previous steps, and that the pIIb precursor cell gives rise to two equipotent daughter cells which then adopt two different fates, one of them becoming a md neuron, the other one an es neuron.

We have to consider, however, that not all ASC-dependent

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**Fig. 5.** The X1 mutation induces the loss of es neurons and the overproduction of md ones. (A,B) Anti-Elav labellings of wild-type and X1/Y embryos. Anti-Elav labels all the neurons. A significant increase in the number of cells labelled by anti-Elav is observed in X1/Y embryos. Arrows point to the dorsal clusters of sensory neurons of two abdominal segments. (C) X1/Y lacks most of the es neurons and sheath cells (anti-BarH1 labelling; compare with Fig. 2C). Arrowheads highlight epidermal staining. The arrow points to a strongly labelled cell that belongs most probably to an es organ, highlighting the incomplete expressivity of the X1 phenotype (see text). (D) md neurons are overproduced in X1/Y; E7-2-26 as seen by anti-β-Gal labelling (arrows point to two dorsal abdominal clusters; compare with Fig. 2C). (E) The dorsal and lateral clusters of three successive abdominal segments of a X1/Y; E7-2-26 embryo double-labelled with 22C10 (membrane labelling) and anti-β-Gal (nuclear staining). The arrowheads point to the dorsal clusters where all neurons express lacZ and are probably of the md type. In the lateral clusters (arrows), many cells do not express lacZ, and probably belong to the ch type. (F) Randomly selected sensory neurons from three adjacent segments of an X1/Y embryo were stained with DiI. All of them are md neurons, as evidenced by the presence of finely branched dendrites (arrows). Axonal misrouting is frequently seen in these embryos: the lower arrow indicates a bifurcating axon with one branch running ventrally and the other dorsally.
md neurons arise from ‘mixed’ md/es lineages: some precursors produce only es cells and others only md neurons (Fig. 1; Brewster and Bodmer, 1995). We have observed that es neurons coming from ‘solo’ es precursors are also lost in N+ mutants.

One possible explanation would be that all ASC-dependent precursors follow a lineage of the ‘mixed’ type and that, in some of them, the md neuron fails to differentiate (‘solo’ es lineages) while, in others, the es neuron and support cells fail to differentiate (‘solo’ md lineages). In this case, however, one would expect that, in N- embryos, where the two daughters of pIIIn take on the md fate, both should fail to differentiate in the ‘solo es’ lineages. This does not happen, since we observed an excess of md neurons. For this and other reasons listed below, we favour an alternative explanation.

We propose that these ‘solo’ es precursors (pI-es) are in fact sisters to the ‘solo’ md precursors (pI-md), and arise from the division of a protoprecursor cell (p0) which has both es and md potentiality (Fig. 1B). In the absence of N, the two daughters of the protoprecursors will adopt the same fate, the md precursor fate. The specification of es and md fates in these lineages would hence be similar to that proposed for the ‘mixed’ es/md precursors lineages and rely on an asymmetric cell division of the protoprecursor cell.

The existence of protoprecursors is supported by three lines of evidence. The first line is based on the fact that partial deletions of ASC lead to the absence of a subset of es organs, including ‘solo’ es organs (Dambly-Chaudière and Ghysen, 1987), and of a subset of md neurons, including ‘solo’ md neurons (M. V., unpublished observations). For example, in Df(1)sc34, the three ventralmost es and four of the five ventral ‘solo’ md neurons are usually absent. A segment-by-segment analysis reveals that the two phenotypes are correlated, as the four md neurons are never present when the three es neurons are absent and are always present when the three es neurons are formed (M. V., unpublished observations).

Second, the analysis of the embryonic expression of achaete, a member of the ASC, reveals the existence of 11 proneural clusters (and precursors) for ASC-dependent sense organs (Ruiz-Gomez and Ghysen, 1993). This is much too low to account for the formation of the 13 es organs and the 7 ‘solo’ md neurons (inferred from Brewster and Bodmer, 1995) if those originated from independent precursors, and suggests that some precursors give rise to both es and ‘solo’ md lineages.

Third, Brewster and Bodmer (1995) often observed FLP/FRT-induced lacZ-positive clones containing both ‘solo’ es organs cells and ‘solo’ md neurons, indicating that the corresponding precursors may derive from the same cell.

It may seem surprising that the decision to choose between es and md identity might, in the case of the ‘solo’ lineages, precede the choice between pIIa and pIIb identities, for it seems a departure from the rule that cells make progressive choices in a specific order. Yet the capability of the precursors to differentiate as neurons in stg and pebble mutants suggests that the precursor and neuronal fates may be closely related. Furthermore, precursors can differentiate as either md or es neurons, indicating that they can opt between es and md identities. The reason why this choice would operate at the level of the precursors in some lineages, and at the level of the neurons in others, remains however mysterious.

**md neurons as default fate**

The product of the gene Notch is part of a cellular communication system used to specify the fate of cells among equipotent cells (Fortini and Artavanis-Tsakonas, 1993; Artavanis-Tsakonas et al., 1995). Its implication in the sensory lineages suggests that, after each division, the two daughter cells form a small equivalence group where fates are determined through cellular communications. In such a group, the fate taken by the cells in the absence of signals is called the ‘default state’ (Greenwald and Rubin, 1992). Our results suggest that, in the lineages produced by ASC-dependent (proto)precursors, the default state is the md neuron and that the es identity is the alternative state.

We confirmed the idea that the md neuron is the ‘default state’ by looking at the situation where the lineage is reduced to a single cell. This is possible thanks to mutations (string and pebble) which block the division of the (proto)precursors and let them differentiate in the absence of any influence from equivalent (sister) cells. We found that the (proto)precursors predominantly differentiate into md neurons, confirming the md as the default state.

We have however to explain that es neurons are occasionally produced, though at very low frequency, in the absence of precursor divisions (see Results). Our interpretation is based on the idea that whenever two ASC (proto)precursors are in direct contact the md fate is inhibited and the es identity is acquired in one of them. In wild-type conditions, this happens between the daughters of pIIIn or between the daughters of p0, leading to the determination of an es and a md fate. We propose that this interaction can also happen in stg+ between different (proto)precursors, so that one of them will differentiate as md neuron, while the other as es one. These interactions between (proto)precursors are made more likely by their close proximity, due to the reduced number of ectodermal cells in stg embryos. We observed that the number of es neurons is low and variable in stg− embryos (as monitored by anti-BarH1 labelling), indicating that the interactions between precursors happen randomly and at a rather low frequency. The idea that the occasional presence of es neurons is due to cell communication is supported by the observation that only md neurons form in stg− embryos where cell communication is prevented (in N+; stg+ embryos).

In conclusion, the function of N+ in the sensory lineages would be to force after each division one daughter cell to adopt the ‘alternative state’: in mixed lineages, pIIa versus pIIb, sheath cell versus pIIIn, and es versus md neuron; in protoprecursors lineages, es-pI versus md-pI.

**Function of numb and ttk**

Besides the N-mediated communication system, other genes are also involved in cell fate specification in the sensory lineages (Posakony, 1994; Jan and Jan, 1995, Vervoort et al., 1997). One of them is numb whose product is asymmetrically distributed to one daughter of pI (Rhyu et al., 1994), as well as probably to one daughter of pIIb. Numb determines the fate of the cell in which it is located and promotes pIIb versus pIIa fate, and neuron versus sheath cell fate (Jan and Jan, 1995; Brewster and Bodmer, 1995). Numb appears to repress tramtrack (ttk), a gene that promotes pIIa versus pIIb and sheath cell versus neuron (Guo et al., 1995; Ramaekers et al., 1997).
We have analysed the requirement for these two genes in the formation and specification of the md neurons. We first looked in the ‘mixed’ es/md lineages. In numb- embryos, both md and es neurons from ‘mixed’ lineages are usually missing (Brewster and Bodmer, 1995). Their absence is likely due either to the absence of pIIa cells, which results from the conversion of pIIb into pIIa (Uemura et al., 1989) or to the conversion of pIIa into a sheath cell (Brewster and Bodmer, 1995).

This absence makes it impossible to determine whether numb plays a role in the specification of es versus md neuronal identity. In ttk- embryos, two pIIb cells are produced, both of which generate a sheath cell, an es and a md neuron. This result is consistent with the observation that ttk is never expressed in neuronal cells (Guo et al., 1995; Ramaekers et al., 1997) and indicates that ttk plays no role in the specification of neuronal types.

In the case of the protoprecursors lineages, the inactivation of numb or the ectopic expression of ttk results in the absence of the pI-md cells. We do not know whether this absence is due to cell death, or to transformation into another cell type. One possibility would be that pI-md are transformed into pI-es. Each pI-es resulting from this transformation, should produce four es accessory cells (as in mixed lineage), and we should then observe clusters of eight cut-expressing cells in numb mutants. We never observed such clusters, however, (Fig. 3B), suggesting that md precursors fail to form neurons, rather than being transformed into es precursors.

These results confirm that the presence of Numb and the absence of Ttk are required to produce both es and md neural fate. As in the case of the ‘mixed lineages’, our results neither support nor rule out a possible function of numb in es/md specification, as no neuronal types can be observed in the absence of numb. In ttk- both pI-es and pI-md appear and generate respectively es and md cells. This result confirms that ttk is not involved in es/md specification. We can therefore say that, if numb has any role in the es/md specification, it must be through a pathway that does not include ttk.

In conclusion, we have shown that the presence of Numb and the absence of Ttk are required for the acquisition of both es and md neuronal fates. In the case of numb, this requirement is maintained when the (proto)precursors are unable to divide. This result is consistent with the conclusion that the expression of ttk, which probably depends on N activation (Guo et al., 1996), prevents a cell from adopting a neuronal identity (Guo et al., 1995; Ramaekers et al., 1997). The role of numb would be to prevent ttk from exerting this inhibition, probably by blocking the transcription of ttk (Guo et al., 1996). Our observations suggest, therefore, that the determination of es and md neuronal identity comprises two aspects: the acquisition of a neuronal fate, which involves N, numb and ttk, and the specification of the type I/type II identity, which involves N but not ttk and probably not numb.

X1 defines a new factor required for the alternative es fate

We found a new factor required to produce the es fate from ASC-dependent precursors. This factor has been defined through the analysis of a X chromosome mutant line, X1. In the mutant embryos, the ASC precursors appear normally but only produce md neurons, and all es neurons as well as accessory cells are missing. This is similar to the effect of N inactivation and suggests that the X1 factor may function in the same pathway as N. However, while N mutations lead to the emergence of many ASC-dependent precursors (Brand and Campos-Ortega, 1989; Goriely et al., 1991; Ruiz-Gomez and Ghysen, 1993), no such phenotype is observed in X1 embryos. We do nevertheless observe that X1 embryos present neurogenic phenotypes at the level of the ASC-independent md neurons, and in the central nervous system (M. V. and D. J. M., unpublished). These phenotypes are also present in N-embryos (Hartenstein and Campos-Ortega, 1986). These similarities suggest that the ‘X1 factor’ may be one among several effectors of N-mediated cell communication, and therefore could be involved only in some of the processes in which N acts. Further genetic and molecular analyses will be required to understand the function of the ‘X1 factor’ during sense organ formation.

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REFERENCES


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