Lateral inhibition and cell fate during neurogenesis in Drosophila: the interactions between scute, Notch and Delta

CARLOS V. CABRERA*

MRC, Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

*Present address: Marie Curie Research Institute, The Chart, Oxted, Surrey RH8 0TL, UK

Summary

A comparison of the patterns of expression of AS-C (T3) RNA and protein suggests that an important level of regulation occurs post-transcriptionally. First, when the RNA is abundant in the early embryo the protein is barely detectable. Later, the protein starts to accumulate in only a subset of the nuclei of those cells expressing the RNA. Only the cells in the subsets become the neuroblasts. This post-transcriptional regulation is suppressed in embryos mutant for the genes Notch and Delta; where all cells expressing RNA accumulate protein. These findings suggest that deployment of T3 protein expression is one of the causal factors that assigns specific fates to the neuroblasts and, in consequence, a basis for the mechanism of lateral inhibition is proposed.

Key words: Drosophila, Notch, scute, Delta RNA, neurogenesis, cell fate, lateral inhibition.

Introduction

A fundamental problem in neurogenesis is to understand how highly diversified nerve cells are generated and consolidated into ganglia. It has been proposed that an important component of this process is the formation of a stereotyped pattern of precursor neuroblasts, suggesting a spatial relationship with the underlying neuroectoderm (Bate, 1976, 1982). Indeed, it has been shown that the position in the neuroectoderm where each neuroblast segregates correlates with fate, a fate expressed in the family of neurons that each neuroblast generates through an invariant cell lineage (Taghert et al. 1984; Doe and Goodman, 1985). Both in vivo and in vitro studies suggest that this lineage is cell autonomous, implying that the neuroblasts are born with a precise programme that determines their fate (Doe and Goodman, 1985; Doe et al. 1988; Huff et al. 1989). It is therefore pertinent to ask what this programme is and how it is implemented.

We have proposed that the assignment of a particular developmental fate to each neuroblast is specified by a group of genes of which the achaete–scute complex (AS-C) is a subset (Cabrera et al. 1987). This proposal was based on genetic analysis of the AS-C (Jiménez and Campos-Ortega, 1979; García-Alonso and García-Bellido, 1986), the marked correlation of the patterns of expression of these genes with neurogenesis as well as the apparent dependence of these patterns on positional information on the ectoderm (Cabrera et al. 1987). The finding that the AS-C encodes four homologous proteins provided a molecular basis for the genetic analysis. The set of qualitatively similar functions found by genetic analysis (Jiménez and Campos-Ortega, 1979), including the AS-C and adjacent proximal regions on the X-chromosome, when linked to the homologies, led to the view of a gene family responsible for supplying the complex requirements for the assignment of the wide variety of developmental fates generated during neurogenesis (Villas and Cabrera, 1987; Alonso and Cabrera, 1988). Henceforth I shall refer to the 'scute family' to denote this group of analogous genetic functions of which the AS-C is the best known subset.

In addition to the assignment of each neuroblast to a particular fate, all neuroblasts undergo a more general process of segregation from the neuroectoderm. This seems to be a stochastic event by which interactions between equivalent ectodermal cells assigns one of them to form a neuroblast (Taghert et al. 1984; Doe and Goodman, 1985). Genes of the neurogenic class (Lehmann et al. 1983) have been proposed to mediate this process (Doe and Goodman, 1985; Cabrera et al. 1987; Artavanis-Tsakonas, 1988; Campos-Ortega, 1988; GhySEN and Dambly-Chaudière, 1988; Simpson and Carteret, 1989; Bourouis et al. 1989).

Here I am concerned with neuroblast determination and segregation. My aim is to clarify the functions of the AS-C and neurogenic genes in this early phase of neurogenesis. The recessive amorphic alleles of these two classes of genes elicit complementary phenotypes in the embryo. In AS-C mutants, development of the
nervous system is inhibited (Jiménez and Campos-Ortega, 1979; White, 1980; Dambly-Chaudière and Ghy sen, 1987; Cabrera et al. 1987), whereas in the neurogenics, hypertrophy results (Poulson, 1937; Lehm ann et al. 1983). At the cellular level, these terminal phenotypes appear to be due to failure of some neuroblasts to segregate and deranged appearance of the map of neuroblasts (Cabrera et al. 1987), as well as death of neural precursors in AS-C mutants (Jiménez and Campos-Ortega, 1979); ectopic segregation of neuroblasts seemed the rule in the neurogenics (Poulson, 1937; Lehmann et al. 1983). This suggests that interactions between the two groups of genes might take place. I have tested this hypothesis by studying the expression of one of the AS-C proteins both in the wild type and in backgrounds mutant for two neurogenic genes, Notch and Delta.

Materials and methods

Drosophila strains

Notch embryos were obtained from w^a N^St ll / Binsn and Delta embryos from Df(3R)F^y/TM3; Sb, Ser. All stocks were outcrossed to wild type for embryo collection. In the case of Delta, heterozygous individuals from this outcross were subsequently inbred for collection of embryos. In all outcrosses, the expected frequency of hemi or homozygous progeny is one quarter. The attainment of this frequency was assessed while studying abnormal expression of T3 protein. In addition, the association of this frequency with the phenotypes elicited by mutant combinations, neural hypotrophy with AS-C and hypertrophy with Notch and Delta, was shown by staining embryos for a common neural antigen with the antibody 22C10 (Zipursky et al. 1984).

Antibody production

For antibody production, the peptide DDEELLDYISSWQE, corresponding to the C-terminus of the translated sequence of the T3 gene (Alonso and Cabrera, 1988), was kindly synthesised by Dr R. Sheppard at the LMB. 10 mg of this peptide were coupled in a 40:1 ratio to KLH (Sigma) in 50 mm triethanolamine pH8.0-0.2% SDS-0.1% glutaraldehyde by stirring for 16 h at room temperature in the dark. The reaction was stopped by addition of ammonium acetate to 100 mm and stirred for an additional 30 min. The complex was precipitated with 4.5 vol. of acetone, recovered by centrifugation and resuspended in PBS at 1 mg ml^-1. This suspension was dispersed by Dounce homogenisation and used to immunise three Lou rats. The primary injection was carried out subcutaneously with 200 μg of the coupled peptide per animal in incomplete Freund's adjuvant. Booster subcutaneous injections were given every five weeks with half the concentration of the immunogen in incomplete Freund's. Antibody production was monitored by immunoblotting, using the T3 protein translated in vitro. The T3 cDNA cloned in Bluescribe (Vector Labs) was transcribed with T7 RNA polymerase in the presence of m7GpppG as described (Melton et al. 1984). In vitro translation using a rabbit reticulocyte lysate (purchased from the Department of Biochemistry, University of Cambridge) was done according to manufacturer's instructions (Jackson and Hunt, 1983). Lysates from reactions with and without RNA were subjected to SDS–PAGE (Laemmlii, 1970) and electroblotted to nitrocellulose membranes (Wilcox, 1986). Whole sera diluted 1:500 were used to probe these blots and the reaction was visualised by subsequent incubations with a rabbit anti-mouse secondary antibody (Jackson), 125I-protein A (Amersham) and autoradiography. One animal, found positive by this method at the seventh booster injection, was bled out and the 4 ml of serum obtained affinity purified on a column made of peptide coupled to Affigel-15 (Biorad). The antibodies were eluted from the column with five bed vols of 4 M MgCl2, dialysed against PBS-0.1% TritonX-100 and concentrated under vacuum to 0.7 ml. Before use this purified serum was preadsorbed on wild-type embryos in PBT, titered and subsequently used at a 1:25 dilution.

Other procedures

Whole-mount hybridisation followed the protocol of Tautz and Pfeifle (1989). Immunochemistry was performed as described before (Cabrera et al. 1987). Chemical intensification of the immunochromical reaction was undertaken, wherever indicated, as described (Liposits et al. 1984).

Results

Antiserum was raised against a synthetic peptide from the C-terminal conserved domain of the AS-C T3 protein (Alonso and Cabrera, 1988). The closest known homologue of this domain is that of the T4 protein, which differs from T3 by a single amino acid. It was therefore important to show that cross-reaction of the antiserum with other proteins did not occur. This was tested by outcrossing a stock carrying a deletion of the T3 gene and showing that one fourth of the embryos did not stain (Fig. 1).

Wild-type pattern of expression

The T3 protein accumulates in the nuclei, as shown in

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**Fig. 1. Specificity of the antiserum.** Embryos derived from an outcross carrying a deletion for the T3 gene [Df(1)sc4Lsc9R/FM6] were stained with the antiserum. 
(A) Individual showing normal expression representative of three quarters of the cases scored between stages 7 and 11. 
(B) Sibling individual lacking staining representative of a quarter of the cases scored. Embryos are displayed in the standard orientation, anterior to the left, dorsal upwards, unless otherwise indicated.
Fig. 4. Although the protein shows the same regional localisation as the RNA, there are enough differences to warrant reviewing the patterns of RNA expression first (see Cabrera et al. 1987; references to the fate map and embryo staging follow Wieschaus and Nüsslein-Volhard, 1986; Campos-Ortega and Hartenstein, 1985).

Zygotically expressed T3 is first detected at the cellular blastoderm stage. The RNA pattern is somewhat complex; within the region that will form the metameric germ band there are twelve dorsolateral stripes, separated by a narrow lateral non-expressing strip, a broader non-expressing ventral region encompassing the presumptive mesoderm and a narrow dorsal non-expressing strip of prospective amnioserosa (Fig. 2A). The stripes exhibit alternating (pair rule) levels of intensity. During gastrulation (Fig. 2B), the weak RNA stripes increase in intensity and the spacing becomes more regular so that these bands reflect the three thoracic and nine abdominal metameres. I note that T3 RNA is expressed in cephalic and gnathal regions during the periods being considered here, and that T3 expression also continues beyond stage 9 in the mesoderm, central and peripheral nervous system anlagen. Since these aspects of the expression pattern are not germane to the issues addressed here, they are not discussed further (see Cabrera et al. 1987 for further details).

Concomitantly with the onset of germ band extension, the number of stripes doubles (Fig. 2C); this doubling appears to occur earlier in the more anterior metameres (the far-right bands in Fig. 2C appear single) and seems to involve cell rearrangements due to germ band extension. First, the stripes compress dorsoventrally and their width increases (Fig. 3). This process apparently reshapes the distribution of T3-RNA-expressing cells to produce a semicircle from each component (dorsal and ventral) of the stripes (Fig. 3B). These semicircles appear to be displaced as the germ band extends so that they meet in a ventrolateral position forming the 'circle pattern' (Figs 3B, 4A). Subsequently, the ventral and dorsal ends of the circle break giving rise to the 'double stripe' pattern (Figs 2D and 4C).

For all the studied stages of development, the steady-state levels of T3 RNA appear constant. By contrast the protein levels, as detected by the antibody, vary substantially. Prior to germ band extension, the T3 protein remains barely detectable, although it is made from the blastoderm stage onwards (Fig. 2E, F). Protein accumulation occurs from mid-stage 8 onwards, thus when the underlying pattern of RNA has reached the circle stage (Fig. 2G, H). Once the protein starts to accumulate it is clear that only a subset of the cells that express the RNA also stain for the protein (Fig. 4A, B). This latter differential expression of RNA and protein is, however, transient and occurs concomitantly to the initiation of neuroblast segregation. During the transition to the double-stripe pattern the first wave of neuroblast segregation takes place and this difference disappears (Fig. 4C, D).

Accumulation of T3 protein shows an excellent correlation with the process of neuroblast segmentation. This was established by comparing three aspects of T3 stained cells with morphological features of the neuroblasts. (1) All cells expressing T3 protein segregate from the ectoderm, as neuroblasts do. Indeed, accumulation of T3 antigen occurs first in cells lying on the ectoderm by mid stage 8. All these cells are seen to delaminate to occupy a position just beneath this layer during the transition to the double stripe arrangement. (2) The number of T3 stained cells is similar to the number of neuroblasts. This was undertaken by a detailed study on dissected, flat-mounted embryos to allow counting of stained cells and comparing these estimates with the number of neuroblasts. Counts of stained cells involved five metameres, starting with the second after the cephalic furrow, of at least two embryos of each the circle and double stripe pattern. Chemically intensified specimens (see Materials and methods) were used to improve the resolution of the counts (see Fig. 4B, D). These counts yielded 16 to 19 strongly and 6 weakly labeled cells per hemimetamere. The total number of neuroblasts also ranges from 16 to 25 (Hartenstein and Campos-Ortega, 1984; Hartenstein et al. 1987; Doe et al. 1988). And (3) the distribution of stained cells and the pattern rearrangements discussed above, mimic the descriptions about the neuroblasts. During the first wave of segmentation neuroblasts are found mainly in two rows, medial and lateral to the midline, an organisation very similar to the circle pattern for T3 protein expression (Fig. 4B). This organisation is soon lost due to intermingling of the two populations (Hartenstein et al. 1987). Similarly, the transition from circle to double stripe patterns described above most likely involves cell movements, for it occurs rapidly and the number of cells involved is maintained constant. In summary, all aspects of neuroblast segregation are recapitulated by the expression of T3 protein. I therefore conclude that these two populations of cells are the same.

Expression in Notch and Delta

In Notch and Delta embryos, anomalous expression of T3 protein occurs from mid stage 8 onwards, when the protein starts to accumulate in wild type (Fig. 5). At this stage, expression deviates from the normal pattern quantitatively, in that more cells express T3 protein in Notch and Delta embryos than in wild type. This difference has been studied in detail in dissected embryos and is presented in a comparative set of panels in Fig. 6. The most marked increase in the number of cells expressing T3 protein in these embryos occurs in the row of cells proximal to the midline. Cell counts on two mutant embryos, of which these specimens are typical examples, show that, in Delta, the number of cells expressing T3 protein is 1.5- to 2-fold (approx. 26 to 30 cells per hemimetamere) higher than in the wild-type or sibling embryos. Similarly, in Notch this number is 2-fold higher (approx. 32 to 34 cells per hemimetamere). It should, however, be noted that this estimate refers to the earliest time of protein accumulation and
that, given the dynamic pattern of expression, this account may not equally apply to later stages.

Expression of T3 RNA in Notch and Delta is indistinguishable from expression in the wild-type from the blastoderm until stage 9 (Brand and Campos-Ortega, 1989), when the deviations from the normal protein pattern reported here occur. It is, therefore, important to note the resemblance of the pattern of T3 protein in
expression shows, firstly, that there is a substantial delay between the onset of RNA and protein accumulation and, secondly, that once the protein starts to accumulate it appears to do so in only a subset of the cells expressing the RNA. These observations lead to the main conclusion of this paper; namely, that an important level of regulation of T3 activity occurs post-transcriptionally. I further show that this differential behaviour of T3 RNA and protein can be reversed in mutant backgrounds for the genes Notch and Delta, thus indicating the involvement of the neurogenic pathway in the post-transcriptional regulation of T3 expression. In what follows, I elaborate on the significance of these findings for neurogenesis and for the role of the AS-C and neurogenic genes.

Expression of the T3 gene can be divided into two well differentiated periods on the basis of the levels of protein accumulation relatively to the more constant levels of RNA. During the first period the protein remains barely detectable, whereas in the second period it accumulates rapidly. The first period, therefore, corresponds to the expression of the RNA and coincides with the time expected for a phase of pattern building of a factor involved in early neurogenesis, because its synthesis precedes and parallels the appearance of neural precursors. The importance of this period is supported by the correlation of neural fate with position within the ectoderm described in the Introduction (Bate, 1982; Taghert et al. 1984; Doe and Goodman, 1985).

Two mechanisms seem to cooperate in patterning T3 RNA. First, the pair-rule (Cabrera et al. 1987) and dorsoventral modulations of the blastoderm stripes strongly suggest that the pattern of T3 RNA is established in response to positional cues laid down by the segmentation and dorsal groups of genes (Nüsslein-Volhard and Wieschaus, 1980; Anderson and Nüsslein-Volhard, 1986). Indeed, mutations in the pair-rule genes elicit the expected disruptions of the stripes (unpublished observations). And secondly, a poorly understood process of cell rearrangement, triggered by extension of the germ band, reorganises this initial topography, culminating with the circle stage pattern. An interesting derivation of these apparent rearrangements bears on the organisation of the fate map. The dorsoventral extent of the stripes after the reorganisation (circle and double-stripe patterns) coincides with the ventral neurogenic region of the fate map. If, as suggested below, the expression of T3 contributes a component of neural fates, it is most remarkable to find that at the blastoderm stage the anlage for the nervous system extends through what is considered dorsal epidermis (Fig. 2A). If the present interpretation of the pattern rearrangements is correct this would, at least, mean that cell movements induced by extension of the germ band produce a shift in the fate map.

The second period of T3 expression starts with the accumulation of the protein (circle stage). A striking feature of this second period is that the T3 protein appears to accumulate in a subset of those cells expressing the RNA. This feature leads to two conclusions.
First, the neuroblasts (protein-positive cells) occur intermingled with cells that could potentially become neuroblasts (RNA-positive cells). And secondly, it suggests the operation of an active process regulating its differential accumulation, both in time and extent; as supported by the earlier failure to detect reasonable levels of the protein.

It should be borne in mind that the peptide used as immunogen has homology with substrates for protein tyrosine kinase phosphorylation (Villares and Cabrera, 1987). If this type of modification were to occur to the T3 protein, the antiserum is likely to recognize exclusively the unphosphorylated form.

Evidence in support of the regulation of the differential accumulation of T3 protein is provided by the Notch and Delta embryos. It is clear that a major requirement for the products of these two genes occurs in the second period of T3 expression. This requirement concerns the number of cells which, by accumulating the T3 protein, segregate as neuroblasts. The neurogenic genes, therefore, provide a device for pattern refinement (Cabrera et al. 1987), which has been classically known as 'lateral inhibition' (Wigglesworth, 1940). By means of this process, one cell out of a group of equivalent ones segregates into the pathway the group has been assigned to, at the same time actively preventing its neighbours from doing so. Experimental support for this process has been obtained by laser ablation during neurogenesis in the grasshopper (Doe and Goodman, 1985), pattern formation in Anabaena (Wilcox et al. 1973) and for the vulva lineages in C. elegans, where the term 'equivalence groups' was coined to describe the
identical potential fate invested in a set of cells (Sulston and White, 1980; Kimble, 1981; Kimble and White, 1981). A similar situation was also described in the classic mosaic analysis of Stern (1968) with the adult sensory organs of *Drosophila* (see also Simpson and Carteret, 1989). The present findings strongly suggest that equivalence groups correspond to Stern’s prepattern, and that they can be defined by the extent of T3 RNA expression. Further, the lateral inhibition process, by which the final pattern is attained, is recognizable by accumulation of the protein in a subset of the cells expressing the RNA. The data as they stand, however, do not allow the resolution of individual equivalence groups, for T3 is only one of the genes of a larger family required for this process.

The products of the *neurogenic* genes may mediate cell interactions (Wharton *et al.* 1985; Kidd *et al.* 1986; Knust *et al.* 1987; Kopczynski *et al.* 1988) and be part of a process of signal transduction (Hartley *et al.* 1988). The data presented here are consistent with the hypothesis that a nuclear target for this process is the T3 protein and that, consequently, this protein might be either unstable or phosphorylated.

During the second period of expression, accumulation of T3 protein accompanies the first morphological sign of neurogenesis: the segregation of the neuroblasts. This is most remarkable, for every detail described histologically about the behaviour of the neuroblasts is mimicked by the expression of the AS-C (also Cabrera *et al.* 1987). These correlations provide strong support for a role of the *scute family* in determining neuroblast fate. Further, the fact that the T3 protein, as detected with the present antibody, accumulates exclusively in neuroblasts, both in wild type and mutant backgrounds where ectopic neuroblasts occur, suggests that the epitope hereby monitored is carried by the biologically active protein.

We have proposed that the *scute family* could carry out its role by acting as transcriptional activators of other genes downstream on the neuroblast lineage (Alonso and Cabrera, 1988). Recently it has been shown that, indeed, the T3 gene encodes a protein that,
in conjunction with the *daughterless* protein, forms a heterodimer that binds to specific DNA sequences (Murre et al. 1989 and unpublished observations).

In considering this regulatory role, it is important to note that the activity of the *scute family* appears to be partially redundant. This has been discussed before in

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**Fig. 6.** Comparative protein expression in wild-type, Delta and Notch embryos. Embryos shown in Fig. 5A, B and F were dissected and mounted flat. Ventral views of (A) wild type; (B) Delta and (C) Notch. Notice the larger number of T3-protein-expressing cells in the two latter and compare with the expression of RNA in the wild type (Fig. 4A). Stained nuclei occur at different depths and thus they are not fully depicted in the pictures. See text for cell counts. Arrowhead marks the position of the midline. Magnification is identical in the three panels. Anterior is to the top.
relation to the adult sensory organs and a molecular basis for this redundancy was then suggested in terms of the sequence homologies between the AS-C genes (Villares and Cabrera, 1987). Similarly, the partial redundancy found for the requirements for various AS-C genes during the development of the larval sensory organs was interpreted in the same terms (Dambly-Chaudière and Ghysen, 1987). At the level of expression support for this redundancy has been obtained in the case of the mesectodermal anlage, where both the T3 and T4 RNAs co-exist in the same cells and, at a lower level of resolution, in the ventral neurogenic region for the T3 and T5 RNAs (Cabrera et al. 1987 and unpublished observations).

Taken together these data suggest that expression of a member of the scute family may determine whether or not an ectodermal cell becomes a particular neuroblast. If more than one member of the family are expressed by one cell, the absence of one of them might not preclude neuroblast segregation or completion of its lineage; the function of one gene thus becoming substituted by another member of the family. However, it is also plausible that, in the absence of one gene, the lineage might be mis or partially specified; an alternative consistent with the observed death of neural precursors in deficiencies of the AS-C and adjacent chromosomal regions (Jiménez and Campos-Ortega, 1979). It is thus conceivable that the coordinate cooperation of members of the scute family might be the causal factor that assigns specific fates to the neuroblasts.

In summary, it is clear that, in the lack of specific lineage markers, we are just starting to grasp the intricate relationship between the expression and requirements of the scute family and the fate of the neuroblasts. This complexity possibly reflects the magnitude of the task, that is the specification of 25 different lineages per hemimetamere that will generate some 250 neurones.

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