Expression and function of the *Drosophila* gene *runt* in early stages of neural development

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Summary

The *Drosophila* gene *runt* was initially identified on the basis of its role during segmentation. Recent molecular and genetic studies have demonstrated that the *runt* gene encodes a novel nuclear protein whose developmental importance is not exclusive to segmentation. This report addresses the functional relevance of *runt* expression in the developmental pathway of neurogenesis. Antibodies against the *runt* protein reveal that it is expressed in a subset of neuroblasts, ganglion-mother cells and neurons. A subset of these neurons also co-express the segmentation gene *even-skipped* (*eve*). Using *eve* as a marker, we show that *runt* is required for the normal development of these neurons. A *runt* P-transposon that lacks neural cis-regulatory elements is used to show that these neurons require *runt* activity independent of its activity during segmentation. These results are confirmed using a temperature-sensitive *runt* allele. Further temperature-shift experiments indicate that the requirement for *runt* is during an early stage of neurogenesis. Based on its pattern of expression and its temporal requirements, *runt* is distinguished as one of the earliest acting genes involved in the generation of diverse cell fates in the developing *Drosophila* nervous system.

Key words: *Drosophila*, segmentation gene, *runt*, *even-skipped*, neural development, neuroblast, cell fate specification.

Introduction

One strategy for generating cellular diversity during development is the expression of key transcription factors in different spatial and temporal contexts. Different combinations of such regulatory products could control unique classes of subordinate genes to produce diverse cell fates. Thus, transcription factors playing a primary role in cell fate specification in one developmental pathway would be excellent candidates for controlling cell fates in other developmental contexts. One such set of regulatory factors are the gap and pair-rule segmentation genes of *Drosophila melanogaster*. These genes act to specify cell fates along the anterior–posterior axis during the blastoderm stage of embryogenesis. Most of these genes encode proteins that appear to be transcriptional regulatory proteins. Different programs of gene expression, or cell fates are established due to the presence of different combinations (and concentrations) of these regulatory proteins in different blastoderm cells. This combinatorial principle is likely to be applicable to the specification of diverse cell fates in many other developmental processes. In particular, the observation that many of the gap and pair-rule genes are re-expressed during *Drosophila* neural development (Carroll and Scott, 1985; Frasch *et al.* 1987; Gaul *et al.* 1987; Schroder *et al.* 1988; Kania *et al.* 1990) strongly suggests these genes participate in generating the incredible diversity of cell types in the nervous system.

The development of the *Drosophila* nervous system starts with the delimiting of a neuro-ectodermal region. All cells in this region have the potential to form neuroblasts (NBs), but only 25% of these cells actually become NBs (for review see Campos-Ortega and Jan, 1991). These cells enlarge and delaminate from the ectoderm in three waves from 4 to 6 h of development. Upon undergoing these changes, the cells can be identified as NBs and distinguished from the underlying ectoderm. NBs then divide asymmetrically to produce a stem cell NB and a chain of smaller ganglion mother cells (GMCs). Each GMC divides once to produce two sibling neurons. A schematic outline of this process as it might be envisioned for a hypothetical neuroblast lineage is shown in Fig. 1.

Several genes are known to be required for the normal development of the *Drosophila* nervous system. The neurogenic genes play a role in restricting the number of NBs that are produced (for review see Campos-Ortega and Jan, 1991). Conversely, the genes...
of the Achaete-scute complex are required for the generation and normal development of most NBs, suggesting an important and general role in determining neural fates (Jimenez and Campos-Ortega, 1990). More specific effects on particular neuronal fates have been documented for several genes, including some of the segmentation genes. There is direct evidence indicating that two of the pair-rule genes are involved in specifying cell fates during the later stages of neurogenesis. The fushi tarazu gene (ftz) is expressed in subsets of GMCs and neurons. Synthetic versions of this gene that lack neural cis-regulatory elements were used to demonstrate that ftz is required for the normal determination of a specific neuron that happens to express another segmentation gene, even-skipped (Doe et al. 1988a). A temperature-sensitive allele of even-skipped (eve) has been used to show that this gene, which is also expressed in subsets of GMCs and neurons, is involved in the determination of particular GMCs (Doe et al. 1988b). The observation that several segmentation genes are expressed in NBs (DiNardo et al. 1985; Baumgartner et al. 1987; Gaul et al. 1987; Schroder et al. 1988) suggests these genes may also play roles in specifying cell fates during earlier stages of neurogenesis. In support of this, the analysis of the nervous system defects in embryos mutant for different segment-polarity segmentation genes provides indirect evidence that gooseberry and patched have roles in specifying NB cell fates that are independent of their roles in segmentation (Patel et al. 1989). However, to date the only gene that has been directly demonstrated to play a role in NB specification is the recently identified prospero (pros) gene (Doe et al. 1991). This gene was not identified based on a role in segmentation, and there is no evidence that pros mutations have any effects on this process. It is interesting to note that pros is expressed in a pair-rule pattern during the blastoderm stage of embryos (Doe et al. 1991).

In this paper, we investigate the role of the pair-rule gene runt in neural development. The runt gene encodes a novel type of nuclear regulatory protein (Kania et al. 1990). It is the only characterized gap or pair-rule gene that does not contain a readily identifiable DNA-binding motif. In segmentation, runt plays a key role in regulating the blastoderm expression patterns of other pair-rule genes (Carroll and Scott, 1986; Frasch and Levine, 1987; Ingham and Gergen, 1988). runt function is not restricted to the process of segmentation. We have recently found that runt plays a vital role in the activation of the Sex-lethal gene in female blastoderm stage embryos (Duffy and Gergen, 1991). runt is also expressed and required during later stages of development (Gergen and Butler, 1988; Kania et al. 1990; Butler et al. unpublished data). Here we show that runt is required during the early stages of neurogenesis for the normal development of a specific subset of central nervous system neurons. We also provide evidence indicating that runt is involved in specifying the fates of the NBs that give rise to these neurons.

**Materials and methods**

**Drosophila stocks**

The runt<sup>L.B5</sup> mutation is a small deletion that spans the structural gene (Gergen and Butler, 1988). The runt P-transposon OP1 is described in Gergen and Butler (1988). It consists of a 14.5 kb segment of runt genomic DNA in the transformation vector CaSpR. Hemizygous runt<sup>L.B5</sup> embryos with two copies of the OP1 transposon were generated by crossing runt<sup>L.B5</sup>/FM7z; OP1/+ females to FM7z/Y; OP1/+
Fig. 2. *runt* is expressed in NBs, GMCs and neurons. (A) A lateral view of a wild-type embryo at ~4.5h of development. NBs can be seen expressing *runt* as they delaminate (arrowhead). Note the characteristic morphology of the NBs. Progeny GMCs also expressing *runt* can be seen just above the NBs (arrow). (B) A ventral view of a double-stained embryo at ~9.5h of development (*runt*-expressing cells are brown, *eve*-expressing cells are blue and cells with co-expression are black.) Two clusters of EL cells that are expressing *runt* and *eve* are indicated by arrows. Both embryos are oriented with anterior to the left. The CQ cells that co-express *runt* and *eve* are located more medially (see Fig. 3 for orientation), and are slightly out of focus in this picture.
The expression of runt is observed in neuroblasts, GMCs and neurons. Antibodies and immunocytochemistry have been utilized to investigate runt expression. We previously demonstrated that runt is expressed in a subset of neuronal cells that express eve. This indicates that runt expression is present in different cell types (NBs, GMCs and neurons). Therefore, we conclude that runt is expressed in both the GMCs and neurons, which suggests that runt has multiple roles in neural development.

### Table 1. Effect of reduced runt activity on eve expression in the EL lineage

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age at shift 18°C→30°C</th>
<th>No. of hemisegments with eve expression in N EL cells&lt;sup&gt;2&lt;/sup&gt;</th>
<th>No. of hemisegments (embryos) scored</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N=0</td>
<td>1-3</td>
</tr>
<tr>
<td>wild-type</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LB5; OP1</td>
<td>–</td>
<td>46</td>
<td>6</td>
</tr>
<tr>
<td>YP17&lt;sup&gt;18°C&lt;/sup&gt;</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>YP17</td>
<td>3.5–4.5 h</td>
<td>34</td>
<td>2</td>
</tr>
<tr>
<td>YP17</td>
<td>4–5 h</td>
<td>119</td>
<td>1</td>
</tr>
<tr>
<td>YP17</td>
<td>4.5–5.5 h</td>
<td>92</td>
<td>4</td>
</tr>
<tr>
<td>YP17</td>
<td>5.5–6.5 h</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>YP17</td>
<td>6.5–7.5 h</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>YP17</td>
<td>7.5–8.5 h</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>YP17</td>
<td>4–5 h</td>
<td>124</td>
<td>22</td>
</tr>
<tr>
<td>YP17&lt;sup&gt;(2 h pulse)&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Development at 18°C is at ~ half the rate as development at 25°C. The age at the time of the shift indicates the times as corrected for development at 25°C.

<sup>2</sup>The number of eve-expressing cells at the lateral position of the EL neurons was counted in abdominal segments in embryos of the indicated genotypes. The numbers in these columns indicate the number of hemisegments with eve expression in the indicated no. of cells at the lateral position of the EL neurons. The number of hemi-segments in the column corresponding to the most predominant category in each experiment is highlighted in bold.

Antibodies and immunocytochemistry

The eve antibody was isolated from a polyclonal rabbit antiserum that was elicited by Manfred Frasch. The runt antibody is a cocktail of the purified IgG fractions of the monoclonal antibodies. This cocktail contains 50 μg ml<sup>−1</sup> of each of these antibodies. The β-galactosidase antibody is a mouse polyclonal antibody (Sigma). The double-staining reactions were done as described previously (Kania et al. 1990).

### Results

Runt is expressed in neuroblasts, GMCs and neurons

We have previously shown that runt is expressed throughout embryogenesis, including extensive expression in the developing nervous system (Kania et al. 1990). In order to address the function of runt during neural development, we first set out to determine in what cell types runt is expressed. Neural expression of runt is first detected in delaminating NBs at ~4.5 h of development (Fig. 2A). Based on the morphology and location of these cells, as well as the timing of this expression, there are approximately 5 runt-expressing NBs per hemisegment. Also clear in Fig. 2A is the expression of runt in a daughter GMC of this NB. As development proceeds the number of runt-expressing cells increases and the pattern becomes highly complex.

In order to facilitate the identification of neural cells that express runt at these later stages, we did double-staining experiments with antibody markers for specific neurons. These experiments revealed that runt is expressed in a subset of neuronal cells that express eve (Fig. 2B). At ~12 h of development each hemisegment shows eve expression in the aCC, pCC and RP2 neurons, in a cluster of 5–6 CQ neurons, and in a cluster of ~10 EL (eve-lateral) neurons (Doe et al. 1989). The co-expression of eve and runt is most obvious in the EL neurons (Fig. 2B). The GMCs that give rise to the EL neurons also express eve (Doe et al. 1991). The double-stained embryo shown in Fig. 2B is at a stage where this cluster should be composed of both GMCs and neurons. All cells in this region that express eve also express runt. Therefore we conclude that runt is expressed in both the GMCs and the neurons of the lineages that generate the EL neurons. There is also evidence of eve and runt co-expression in some of the CQ neurons. Other cells of the CQ cluster stain only for eve, and there are cells adjacent to this cluster that stain only for runt. The expression of runt in different neuronal lineages, as well as in different cell types (NBs, GMCs and neurons) within these lineages strongly suggests that runt has multiple roles in neural development.
Runt is required for subsets of eve’s neural expression pattern

A straightforward functional analysis of runt's expression during neurogenesis is complicated by the earlier activity of runt during segmentation. The absence of runt activity at cellular blastoderm (~3 h) causes severe segmentation defects, which result in a grossly malformed nervous system. To circumvent this problem, we used two approaches to provide runt activity during segmentation and to reduce it during neurogenesis. In the first approach, we used a genomic runt transposon (OP1) that provides runt function during segmentation (Gergen and Butler, 1988), but lacks regulatory elements necessary for most of runt's post-blastoderm expression. The details of runt expression from this transposon both during segmentation and neurogenesis will be documented elsewhere (Butler et al. unpublished data). The number of neuroblasts in which runt expression is detected from this transposon is reduced to ~2 per hemisegment and the level of runt expression in these neuroblasts is significantly reduced when compared to wild-type expression. In the second approach, we used the temperature-sensitive allele runtYPn'. To look for changes in neural cell fates, we stained runt~ embryos generated from both approaches with antibody to eve. As shown above, a subset of cells that express runt also express eve.

Embryos with two copies of the runt genomic transposon OP1 and hemizygous for the null allele runt785 (runt is on the X chromosome) have nearly wild-type segmentation (Fig. 3B, also Butler et al. unpublished data). Embryos of this genotype (runt~; OP1) were generated and stained with antibody to eve. The expression of eve in the EL cluster is dramatically affected in all segments of these embryos (Fig. 3B). eve expression in this cluster was completely absent in 46 of 56 hemisegments with wild-type segmental morphology analyzed from 7 different embryos (Table 1). The alteration of eve expression in the EL cluster in regions with wild-type segmental morphology strongly suggests that this defect is not an indirect consequence of runt's role during segmentation, but rather reflects an independent requirement for runt activity in the pathway that gives rise to the EL neurons. There are other defects in eve’s neural expression pattern in these embryos. The most consistent and reproducible defect is a severe reduction in the number of eve-expressing cells in the cephalic region (Fig. 4). This indicates that the normal development of these eve-expressing cells also requires runt.

We used the temperature-sensitive allele runtYP17 (YP17) to confirm the requirement for runt in the development of these different eve-expressing lineages. Hemizygous YP17 embryos maintained at the permissive temperature (18°C) have weak or no segmentation defects, whereas YP17 embryos maintained at the restrictive temperature (30°C) have a strong segmentation phenotype (Gergen and Wieschaus, 1986). Control YP17 embryos (maintained at 18°C) showed a normal pattern of eve expression (Table 1 and Fig. 5A). YP17 embryos shifted to 30°C after cellular blastoderm (>3.5 h at 25°C) had wild-type segmentation (Fig. 5B, C), but showed a strong reduction in the number of hemisegments with eve EL-expression.

Fig. 3. runt is required for the development of the EL neurons. Pattern of eve expression in the trunk of wild-type (A) and runt~; OP1 (B) embryos at ~10 h of development. Each panel is a ventral view with anterior oriented up. The following symbols indicate eve-expressing neurons: RP2 (thin arrow); aCC, pCC, and CQ cluster (arrowhead); EL cluster (thick arrow); absence of EL cluster (hollow arrow). (A) In this wild-type embryo note the presence and position of the EL cluster in each hemisegment. (B) In this runt~; OP1 embryo note the reduction in the number of hemisegments with eve expression at the lateral position of the EL neurons.
A role for runt in neural development

Fig. 4. Expression of eve in the cephalic region requires runt. Pattern of eve expression in the cephalic region of wild-type (A) and runt \( \text{OP1} \); embryo at \( \sim 10 \text{ h} \) of development. Each panel is a ventral view with anterior oriented up.

(Table 1 and Fig. 5B). Such a shifted embryo with wild-type segmentation and lacking eve expression in the EL cluster is shown in Fig. 5B. eve expression in the GMC that gives rise to the aCC and pCC neurons, as well as the pattern of eve expression in the aCC, pCC, RP2 and most of the CQ neurons, appears unaffected in these shifted embryos (Fig. 5B). Expression of eve in the cephalic region was also affected in these embryos (data not shown). Based on these results, we conclude that runt activity is required for the normal development of a subset of the neuronal cells that express eve, confirming the results obtained with the OP1 transposon.

The following lines of evidence strongly suggest that this activity is independent of runt's activity during segmentation. runt is a member of the pair-rule class of genes, but defects in the expression of eve in the EL

Fig. 5. eve expression is affected in YPI7 embryos. Each panel is a ventral view with anterior oriented up. The symbols are the same as in Fig. 2. (A) YPI7 embryo maintained at 18°C (control). eve expression in the EL cluster is present in each hemisegment. (B) YPI7 embryo shifted to 30°C at 4.5–5.5 h of development. No eve expression in the EL cluster is evident in any hemisegment. (C) YPI7 embryo shifted to 30°C at 5.5–6.5 h of development. eve expression in the EL cluster is present in each hemisegment.
neurons are seen in every segment. These defects were scored in segments with wild-type morphology and in some cases in embryos with wild-type segmentation. Although the EL cluster is affected in the YP17 embryos, the remainder of the eve neural pattern appears unaffected. Finally, YP17 embryos maintained at the restrictive temperature during segmentation and shifted to the permissive temperature after the onset of gastrulation have grossly abnormal segmentation, but show clear evidence of eve-expressing cells in the lateral regions of the EL neurons (data not shown). Therefore, we conclude that the requirement for runt activity in the development of the EL neurons is independent of its activity during segmentation.

There is residual EL-expression of eve in both the OP1 and YP17 mutant embryos. Based simply on the number of eve-expressing cells that we scored in the region of the EL neurons, the YP17 phenotype appears to be stronger. The YP17 embryos that were shifted at 4-5 h of development yielded only 1 eve-expressing EL cell in the 120 hemisegments that we scored (Table 1). The EL cells that remain in YP17 embryos could well be due to residual runt activity. Although this allele has a strong segmentation phenotype at the restrictive temperature, it is not equivalent to a null allele. This suggests that there is an absolute requirement for runt in the normal development of the EL lineages.

The precise nature of the defects that occur during the development of the EL lineage in the absence of runt activity are not known. The expression of eve was previously the only known marker for these cells. However, based on our double-staining experiments runt is also expressed in the EL GMCs and neurons. Thus, the one other marker for the affected cells in the EL cluster is the expression of runt itself. The expression of runt in the EL cluster is also reduced in both OP1 and YP17 runt- embryos (data not shown). Therefore either the fate of the precursor cell that normally gives rise to these co-expressing cells has been altered such that these cells are not generated, or alternatively the cells that should express runt and eve are generated but have altered programs of gene expression. In either case, it is clear that runt plays a role in the development of this neuronal lineage.

Discussion

The following simple model is consistent with all of our results. runt expression is initiated in the EL precursor NB(s) and continues to be expressed in its progeny GMCs and neurons. (refer back to Fig. 1). runt activity is required in this precursor NB(s) to ensure the normal development of this lineage (GMCs and neurons). Based on our temperature-shift experiments, the critical requirement for runt in this lineage is from 4-7 h of development. This is prior to the appearance of eve expression in the GMCs that will ultimately generate the EL neurons. This strongly suggests that runt is acting in the NB that will give rise to these GMCs. In this model, the elimination of runt activity during the critical period would alter the fate of this NB such that it could no longer generate its normal complement of GMCs. Whether runt is actively involved in establishing a program of gene expression and thereby in specifying the fate of this NB, or is passively required for such a process is not known. However, the function of runt in regulating particular programs of gene expression in other developmental pathways suggests that runt plays an active role in specifying the fate of this NB. An alternative to our simple cell-autonomous model is that runt expression in other cells is important for the normal development of the EL lineage. However, the
results of experiments where NBs are isolated and cultured in vitro suggest that cell interactions do not play an important role in the specification of individual NB cell fates (Huff et al. 1989).

Proof of our simple model requires the unambiguous demonstration that runt is expressed and required in the progenitor NBs for the EL neurons. The lineage that gives rise to the EL neurons has not been described in detail, but it is thought that the EL neurons in each hemisegment are all derived from the same NB (C. Doe and N. Patel, personal communications). Unfortunately, in Drosophila the progeny from different NBs can intermingle, making it difficult to identify unambiguously lineages based solely on topology (Hartenstein and Campos-Ortega, 1984). The Drosophila nervous system is homologous to that of the grasshopper (Thomas et al. 1984). Lineages are topologically separated and therefore are more readily defined in this larger insect. It will be very interesting to determine if there is a grasshopper homolog of runt with a similar pattern of expression during neurogenesis. If so then it should be straightforward to determine if runt is expressed in the NBs for the EL lineages.

The function of runt in the development of the EL neurons is not known. The positively acting role that runt plays in regulating eve in this lineage is different from the mutually repressing interactions observed between these genes during segmentation (Ingham and Gergen, 1988). Similar discrepancies were previously observed in the hierarchical relationships between ftz and eve in segmentation versus neurogenesis (Doe et al. 1988a; Doe et al. 1988b). The difference between the time at which runt is required in neural development (~4-7 h) and the time at which eve EL-expression first becomes apparent (~9-10 h) suggests that the effects on eve regulation may not be direct.

It is interesting to contrast the requirements that we have described for runt with the requirements that have been described for pros, the one other gene shown to be involved in specifying cell fates during the early stages of neurogenesis. pros mutations eliminate eve expression in the aCC, pCC and RP2 neurons, and in most of the CQ neurons (Doe et al. 1991). Expression of eve in the EL neurons is not affected. These effects are complementary to those that we describe for runt. Further studies on the roles of the runt and pros genes in these developmental pathways will indicate whether there is any functional significance to this intriguing complementarity.

The runt gene encodes a nuclear protein that is involved in regulating the transcription of other genes during the process of segmentation. Unlike all of the other characterized gap and pair-rule proteins, the runt protein does not contain an obvious DNA-binding motif. Therefore the mechanism by which runt regulates gene expression is unknown. The results described here establish an important role for runt in the pathway of neurogenesis. We have also recently determined that runt plays a key role in regulating the expression of the sex-determining gene Sex-lethal in blastoderm-stage female embryos (Duffy and Gergen, 1991). Taken together, these observations suggest that the apparently novel regulatory function of runt may be widely utilized in developing systems. The runt gene cross-hybridizes to DNA from several vertebrate species, as well as to other fragments of Drosophila DNA (M. Pepling and JPG, unpublished observations). This observation suggests that runt may be the prototype member of a new class of nuclear regulatory proteins. It will be interesting to determine if there are functional homologs of runt that participate in the generation of cell diversity in other developmental contexts.

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