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Runt determines cell fates in the *Drosophila* embryonic CNS

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SUMMARY

The segmentation gene, *runt*, is expressed by a subset of the 30 neuroblasts that give rise to each neuromere of the *Drosophila* embryo. Runt activity in the neuroblasts is necessary for expression of *even-skipped* in the EL neurons. *runt* is therefore a good candidate for a gene specifying neuroblast identities. We have ectopically expressed Runt in restricted subsets of neuroblasts and show that Runt is sufficient to activate *even-skipped* expression in the progeny of specific neuroblasts. Using the marker Tau-green fluorescent protein to highlight the axons, we have found that the extra Even-skipped-expressing neurons project axons along the same pathway as the EL neurons. We find that Runt is expressed in neuroblast 3-3, supporting an autonomous role for *runt* during neuroblast specification.

Key words: *runt*, Neuroblast, Neurogenesis, Cell fate, *Drosophila*, CNS

INTRODUCTION

The *Drosophila* ventral nervous system develops from 30 neuroblasts per neuromere (Bossing et al., 1996; Schmidt et al., 1997). These neuroblasts give rise, through a series of stem cell divisions, to a series of ganglion mother cells (GMCs), which divide once to form a pair of postmitotic neurons or glial cells (reviewed by Goodman and Doe, 1993). Each neuroblast can be identified by its time of delamination, position, lineage and the genes that it expresses. To date, at least 14 neuroblast-identity genes have been identified. These are expressed by different, but overlapping, subsets of neuroblasts (Doe, 1992; Doe and Technau, 1993; Broadus et al., 1995).

The genes expressed by each neuroblast are determined partly by the region of the neuroectoderm from which it delaminates. Neuroblasts express different segmentation genes depending on their anteroposterior position (Patel et al., 1989). Some of the segmentation genes affect neuroblast cell fates autonomously, such as *engrailed (en)* (Bhat and Schedl, 1997) and *gooseberry (gsb)* (Buenzow and Holmgren, 1995; Skeath et al., 1995; Duman-Scheel et al., 1997), and some non-autonomously, such as *wingless (wg)* (Chu-LaGraff and Doe, 1993; Bhat, 1996) and *hedgehog* (*hh*) (Bhat and Schedl, 1997). The pair-rule gene, *runt*, is also expressed in a subset of neuroblasts, ganglion mother cells and neurons and its activity has previously been shown to be necessary for the formation of a subset of *even-skipped (eve)*-expressing lateral neurons, the EL neurons (Kania et al., 1990; Duffy et al., 1991).

There are 8-10 EL neurons per abdominal hemisegment (Frasch et al., 1987; Patel, 1994), which originate from neuroblast 3-3 (Schmidt et al., 1997). The EL neurons are interneurons that express the zinc-finger transcription factor encoded by *eagle*. Using flies carrying the transgene *eagle-kinesin lacZ*, it has been shown that the EL neurons project axons through the anterior commissure across the midline, then turn anteriorly into the longitudinal fascicles (Higashijima et al., 1996). Inactivation of *runt* during neuroblast delamination, using a temperature-sensitive allele of *runt*, leads to a loss of *eve* expression in the EL neurons (Duffy et al., 1991). Eve expression in the EL neurons is not affected when Runt is inactivated after the neuroblasts have delaminated, suggesting that Runt activity is necessary only at the time of neuroblast delamination for the development of the EL neurons.

Interestingly, Runt has not been reported to be expressed in the neuroblast that gives rise to the EL neurons, neuroblast 3-3 (Doe and Technau, 1993; Schmidt et al., 1997), suggesting that Runt might act non-cell-autonomously to induce EL neuron formation. Although detected in the nucleus, the predicted Runt protein sequence encodes a potential N-terminal signal sequence for secretion (Kania et al., 1990). Furthermore, it has been reported that Runt can be secreted in vivo, suggesting that Runt might act as an intercellular signalling molecule (Kania et al., 1990).

Runt is known to be necessary for Eve expression in the EL neurons, but is Runt sufficient to activate Eve expression? We have ectopically expressed Runt in specific neuroblasts and assayed the formation of extra EL neurons. The expression of Eve is just one characteristic of the EL neurons. We have also examined the morphology of the Eve-expressing neurons. A protein fusion of β-galactosidase to the microtubule-binding protein, Tau, marks the cell body and is transported into the axons (Butner and Kirschner, 1991; Callahan and Thomas, 1994; Hidalgo et al., 1995). We have used *UAS-Tau-GFP* (Brand, 1995) to label the axons of those cells in which we mis-expressed *UAS-runt*, in order to examine the axon projections of the neurons.

We have also asked whether Runt acts non-autonomously in the nervous system. We have tested whether the potential signal sequence is required for Runt activity and have expressed Runt-GFP fusion proteins in the developing CNS to determine whether Runt acts autonomously. We show that Runt is sufficient to induce EL neurons and acts cell autonomously.

MATERIALS AND METHODS

Fly strains and genetics

Flies were maintained at 25°C unless otherwise stated. For temperature-shift experiments, eggs were collected for 1 hour at 25°C and aged for 6 hours at the permissive temperature, 18°C. The embryos were then incubated at 29°C for 2 hours so that Runt was inactive at the time of neuroblast delamination. The embryos were allowed to develop for 16 hours at 18°C before fixation.

Antibody generation

His-tagged Runt protein was expressed in bacteria using the pRSET-B vector (Invitrogen). DNA from position 883 to position 1763 of pBED5′, including part of the Runt domain and the C terminus of Runt, was amplified by PCR from pBED5′ (Kania et al., 1990) using the 5′ oligo GCG GGA TCC CCG GTG CAG ATC GCC AGC and a 3′ oligo GCG AAG CTT GTA GGG CCG CCA CAC GG. The PCR product was cloned between the *Bam*HI and *Hin*dIII sites of pRSET-B. Protein was expressed in C41 cells (Miroux and Walker, 1996) and purified over a nickel column (QIAexpress). Rabbits were immunised using standard protocols. The resulting serum was tested at a range of dilutions in embryos for Runt-specific staining. The serum worked best when preadsorbed at 1:10 dilution before use at a final concentration of 1:500.

Generation of *UAS* flies

The *Xho*I site in the polylinker of pUAST (Brand and Perrimon, 1993) was removed by digesting with *Xho*I, filling the 5′ overhanging ends using T4 DNA polymerase and ligating the blunt ends to make plasmid *pUAS-X-T*. To make *UAS-runt* flies, the *runt*-coding sequence was excised from the vector pBED5′ as an *Eco*RI-*Eag*I fragment. This *Eco*RI-*Eag*I fragment was ligated into *pUAS-X-T* using the *Eco*RI and *Not*I sites in the polylinker to make *pUAS-X-runt*. To make *UAS-runt-*ΔSS flies, the 5′ end of the *runt* coding sequence was replaced with a truncated end, which lacked the 93 base pairs encoding the putative signal sequence. The truncated 5′ end was created by amplifying with the 5′ oligo GCG GAA TTC ATG CAG GGT CCT GGG CCG CAG C and the 3′ oligo GCG CTC GAG CTG GTG TTG TTC GTG C. The 5′ end was excised from *pUAS-X-runt* as an *Eco*RI-*Xho*I fragment and replaced with the PCR product.

A Runt-GFP fusion was constructed. The sequence encoding the terminal WRPY of Runt was added to the 3′ end of the GFP-encoding sequence. GFP-WRPY was amplified by PCR from pUAS-GFP$^{65/167}$ (Heim et al., 1995; Heim and Tsien, 1996; A. H. B. and C. Davidson, unpublished) with a 5′ primer containing a *Kpn*I site (GCG GGT ACC AGT AAA GGA GAA GAA CTT TTC) and 3′ primer containing an *Xba*I site (GCG TCT AGA CTA GTA GGG CCG CCA TTT GTA TAG TTC ATC CAT GCC). The 3′ end of *runt* was amplified by PCR from the *Xho*I site at position 492 (GCG GCG CTC GAG CAG CAA CGC CAC CAC CAG TCC GC) to the base pair before the WRPY site sequence with a 3′ primer containing a *Kpn*I site (GCG GGT ACC CAC GGT CTT CTG CTG CAC GGC). The 3′ *runt* PCR product and GFP PCR product were ligated into *pUAS-X-runt* as a three-way ligation between the *Xho*I and *Xba*I sites.

DNA was injected into yw; Sb, P[ry+, Δ2-3]/TM6 Ubx embryos (Robertson et al., 1988) as previously described (Brand and Perrimon, 1993).

Drosophila strains

sca-GAL4 (Klaes et al., 1994) was used for mis-expression throughout the nervous system. Lines expressing GAL4 in subsets of neuroblasts included *en-GAL4* (A. H. B. and K. Yoffe, unpublished) and *eagle-GAL4* (MZ360; Ito et al., 1995). GAL4 lines were characterised by comparing Tau-β-galactosidase expression to the Gsb-d-expressing neuroblasts. For co-expression of *runt* with GFP, flies carrying both *UAS-runt* and *UAS-Tau-GFP*$^{65/167}$ were generated (Brand, 1995).

Embryo collection, fixation and immunostaining

Embryos were collected, fixed and immunostained according to previously published protocols (Patel, 1994). For examination of fixed GFP, embryos were fixed for 30 minutes in 4% formaldehyde (BDH). Primary antibodies were diluted in PBS, 0.1% Triton X-100 and used at the following concentrations: mouse mAb BP102, 1:2 (Patel, 1994; Seeger et al., 1993), rabbit anti-Eagle, 1:500 (Dittrich et al., 1997), mouse mAb 4D9, anti-En, 1:2 (Patel et al., 1989), rabbit anti-Eve, 1:500 (Frasch et al., 1987), mouse mAb 1D4, anti-Fasciclin II, 1:5 (Van Vactor et al., 1993), rabbit anti-GFP, 1:500 (Clontech), mouse anti-Gsb-distal, 1:2 (Zhang et al., 1994), rabbit anti-Odd-skipped, 1:400 (E. Ward, unpublished) and mouse anti-Slit, 1:10 (Rothberg et al., 1988). Secondary antibodies, directly conjugated to horseradish peroxidase (Jackson labs) and diluted 1:300, were detected by using diaminobenzidine (0.3 mg/ml in PBS, 0.1% Triton X-100; Sigma) as a substrate that was intensified with 0.08% NiCl for nuclear stains. Embryos were cleared in 50% glycerol, before mounting in 70% glycerol and examined by DIC optics on a Zeiss Axiophot. Alternatively, secondary antibodies were used directly conjugated to Cy5, FITC or Texas Red (Jackson labs), at a dilution of 1:200. Embryos were mounted in Vectashield (Vector Labs) for examination with a BioRad MRC 1024 confocal microscope. Embryos were staged according to Campos-Ortega and Hartenstein (1985).

RESULTS

Runt is necessary and sufficient to induce *even-skipped* expression in the nervous system

In wild-type embryos, Eve is expressed by 8-10 EL neurons in each abdominal hemisegment and 4-6 EL neurons in each thoracic segment. We have concentrated on the abdominal segments. Eve is also expressed by a subset of medial neurons: the CQ, U, aCC, pCC and RP2 neurons (Fig. 1A). When Runt is inactivated at the time of neuroblast delamination, lateral *eve* expression is lost, although the medial neurons still express *eve* (Fig. 1B) (Duffy et al., 1991). In many segments, only two FasII-expressing longitudinal fascicles are seen on each side of the midline (Fig. 1C), as opposed to three in wild-type embryos (Fig. 1A). Runt is therefore necessary for *eve* expression in the EL neurons and for the correct development of the longitudinal axon tracts. Loss of Runt may either affect the identity of the neurons contributing to the lateral longitudinal fascicle, or affect axon guidance along the lateral longitudinal fascicle.

Runt is necessary for *eve* expression in the EL neurons, but is it also sufficient to induce the formation of EL neurons? If *runt* is sufficient, one might expect to see extra lateral *eve* expression on ectopic expression of *runt*. We used the GAL4 system for targeted gene expression, which can be used to mis-express genes in specific subsets of cells, at specific times during development (Brand and Perrimon, 1993; Brand et al., 1994; Brand and Dormand, 1995). In order to ectopically express *runt* in specific subsets of neuroblasts, we characterised a library of lines expressing GAL4 in distinct, overlapping subsets of neuroblasts.

We generated flies carrying the transgene *UAS-runt* in order to mis-express *runt* in subsets of neuroblasts using the GAL4

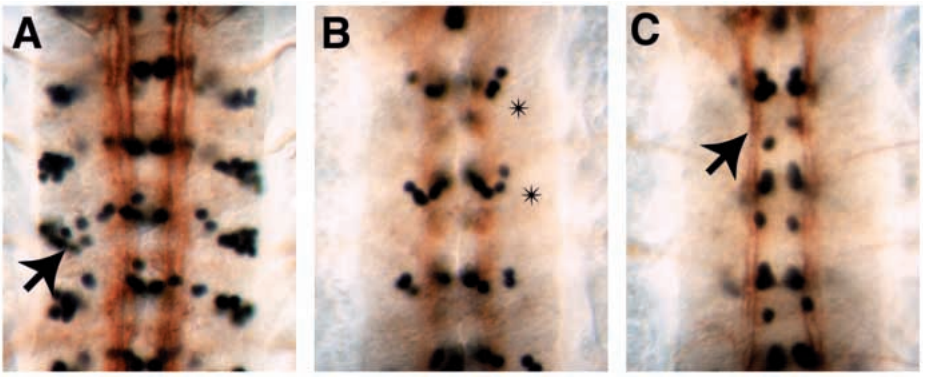

Fig. 1. Inactivation of Runt leads to loss of Eve expression in the EL neurons. The EL neurons are Eve-expressing neurons (black) located laterally to the longitudinal fascicles (brown), detected with mAb 1D4 recognising FasII. The EL neurons are easily identifiable in the wild-type embryo (A; arrow), but are missing when Runt is inactivated at the time of neuroblast delamination in *runt* ^{ts} embryos (B; stars). Inactivation of Runt also leads to disruptions in the longitudinal axon tracts (C; arrow)

system. To check that Runt is translated from *UAS-runt*, we tested whether *en-GAL4* (A. H. B. and K. Yoffe, unpublished) would drive expression of Runt (Fig. 2). *En-GAL4* flies express GAL4 in the *en*-expressing neuroblasts in row 6 and 7 and neuroblast 1-2 (Fig. 2A). Runt is normally expressed in a subset of neuroblasts in rows 2, 3 and 5, but not in the *en*-expressing neuroblasts (Fig. 2B). *En-GAL4* drives *runt* expression in neuroblasts in rows 6 and 7 (Fig. 2C) and so *UAS-runt* can be expressed in specific subsets of neuroblasts.

When *runt* is mis-expressed in all neuroblasts using the pan neural driver, *sca-GAL4* (Mlodzik et al., 1990; Klaes et al., 1994), extra Eve is induced (compare Fig. 3A and B). The average of 9 EL neurons per hemisegment is increased to an average of 16 *eve*-expressing lateral cells per hemisegment (Table 1). Ectopic Runt expression causes a severe disruption of the nerve cord, as shown by the abnormal medial *eve* expression and severe disorganisation of the axons (compare Fig. 3A and B, 3D and E). However, Runt is not sufficient to induce *eve* expression in the progeny of all the neuroblasts. Only a small number of extra *eve*-expressing neurons is seen when Runt is ectopically expressed in all the neuroblasts.

By limiting mis-expression of *runt* to a subset of neuroblasts in each neuromere, we hoped to identify those neuroblasts able to give rise to extra Eve-expressing cells and to identify potential cofactors necessary for EL neuron formation. In order to mis-express *runt* in smaller subsets of neuroblasts, we characterised a library of GAL4 lines expressing GAL4 in overlapping subsets of neuroblasts. *En-GAL4* expresses GAL4 in the *en*-expressing neuroblasts of row 6 and 7 and neuroblast 1-2. Ectopic expression of *runt* in these neuroblasts leads to extra lateral Eve expression resulting in 12 Eve-expressing lateral cells per hemisegment, although medial *eve* expression is relatively normal (Fig. 3C). The posterior commissure is reduced in size or absent (Fig. 3F).

We find a correlation between ectopic expression of *runt* in row 6 neuroblasts and the presence of extra lateral *eve* expression. Extra *eve* expression is induced when mis-expressing *runt* in neuroblasts in rows 4-6, rows 6-7, and rows 1-3 and 6, but not when *runt* is mis-expressed in rows 1-2 or rows 2-4 or row 5 (data not shown). The common row where extra *eve* expression is induced is row 6 excluding neuroblast 6-4, where *runt* mis-expression does not give rise to extra eve expression (*eagle-GAL4*). Thus neuroblast 6-1 and/or neuroblast 6-2 must express another protein that is essential for Runt to activate *eve* expression.

The extra *even-skipped*-expressing cells project EL neuron-like axons

Although the temperature-shift experiments show that Runt is necessary for *eve* expression in the EL neurons, it is not known how Runt affects other characteristics of the EL neurons, such as their axon projections. We co-express the marker Tau-GFP in those cells in which we mis-express *runt*, to examine the axons of the extra Eve-expressing cells and compare them to the axon projections of the wild-type EL neurons. The Tau-GFP fusion protein binds to the microtubules and is transported into axons (Brand, 1995). GFP continues to fluoresce after fixation and antibody staining. We fixed the embryos and then stained them with antibodies to Eve, to show the EL neurons, and FasII, to show the longitudinal tracts.

The EL neurons express *eagle* (Higashijima et al., 1996). *eagle-GAL4* (Ito et al., 1995; Dittrich et al., 1997) can therefore be used to target expression of Tau-GFP to the EL neurons. The EL axons cross the midline through the anterior commissure (Fig. 4A) and then project anteriorly in the dorsal, medial fascicle of the five longitudinal fascicles detected by mAb 1D4, anti-Fasciclin II (E. L. D. and A. H. B. unpublished).

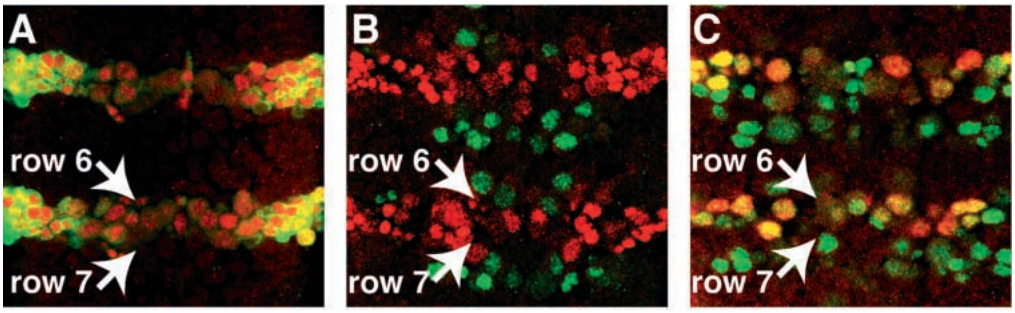

Fig. 2. The GAL4 system can be used to ectopically express Runt in subsets of neuroblasts. (A) *en-GAL4* flies express GAL4 in the En-expressing neuroblasts, as shown by expression of Tau-GFP (green) and En (red) in embryos containing *en-GAL4* and *UAS-Tau-GFP*. (B) In wild-type embryos, Runt (green) is not expressed in the En-expressing cells (red). (C) When *en-GAL4* flies are crossed to *UAS-runt* flies, the progeny express Runt in the En-expressing cells – colocalisation of Runt (green) with En (red) appears yellow.

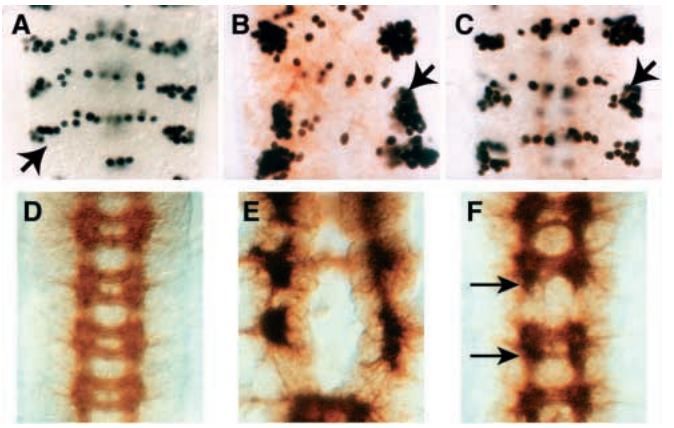

Fig. 3. Ectopic Runt induces extra Eve expression. (A) In wild-type embryos, the clusters of EL neurons each contain 8-10 cells (arrow). (B) When *runt* is mis-expressed in all neuroblasts, using *sca-GAL4*, the EL neuron clusters are significantly enlarged (arrow), however, the medial *eve*-expressing cells are also disrupted. (C) Mis-expressing *runt* in row 6 and 7 neuroblasts, with *en-GAL4*, leads to a more specific induction of extra EL neurons, with normal medial *eve* expression (arrow). (D) The axons of the nerve cord normally form a ladder-like array, as revealed by mAb BP102. (E) The neuropile is severely disrupted when *runt* is mis-expressed in all the neuroblasts. (F) Mis-expression of *runt* in row 6 and 7 neuroblasts leads to breaks in the longitudinal connectives and reduced or missing posterior commissures (arrows).

The extra *eve*-expressing cells project axons (Fig. 4B-D) that cross the anterior commissure (Fig. 4C,D), as do the EL neurons. As a control, when *en-GAL4* drives *UAS-Tau-GFP* alone, two ventral fascicles cross the anterior commissure (Fig. 4E). When *runt* is mis-expressed in row 6 and 7 neuroblasts using *en-GAL4*, many more axons cross the anterior commissure, which are not organised into fascicles (Fig. 4F). This is consistent with the extra *eve*-expressing cells projecting axons along the pathway taken by the EL neurons. The extra axons crossing the anterior commissure may be at the expense of the posterior commissure, which is reduced or absent (Fig. 3F)

The N-terminal putative signal sequence is not necessary for Runt to specify neuroblast fates

Previous reports indicated that Runt is not expressed by neuroblast 3-3 and yet Runt is essential for the development of the EL neurons formed by this neuroblast, suggesting that Runt may act non-autonomously. Since *runt* encodes a putative signal sequence for secretion, Runt could act as an intercellular signalling molecule. If the N-terminal signal sequence is necessary for Runt to induce the formation of extra EL neurons, a truncated form of Runt lacking the N terminus would be unable to induce extra EL neurons. When we express Runt lacking the putative secretory signal sequence in those neuroblasts which are able to form extra EL neurons, we find that the truncated form of Runt is still able to induce extra EL neurons (Fig. 5A-C). The number of lateral *eve*-expressing cells is similar when the full-length and truncated forms of Runt are mis-expressed (Table 1). Thus the hydrophobic N terminus of Runt is not necessary to specify the EL neuron fate.

Runt acts cell autonomously to specify neuroblast fates

We assayed whether the extra Eve-expressing cells induced by ectopic Runt are formed by Runt-expressing cells or by neighbouring cells. If Runt acts cell autonomously to induce

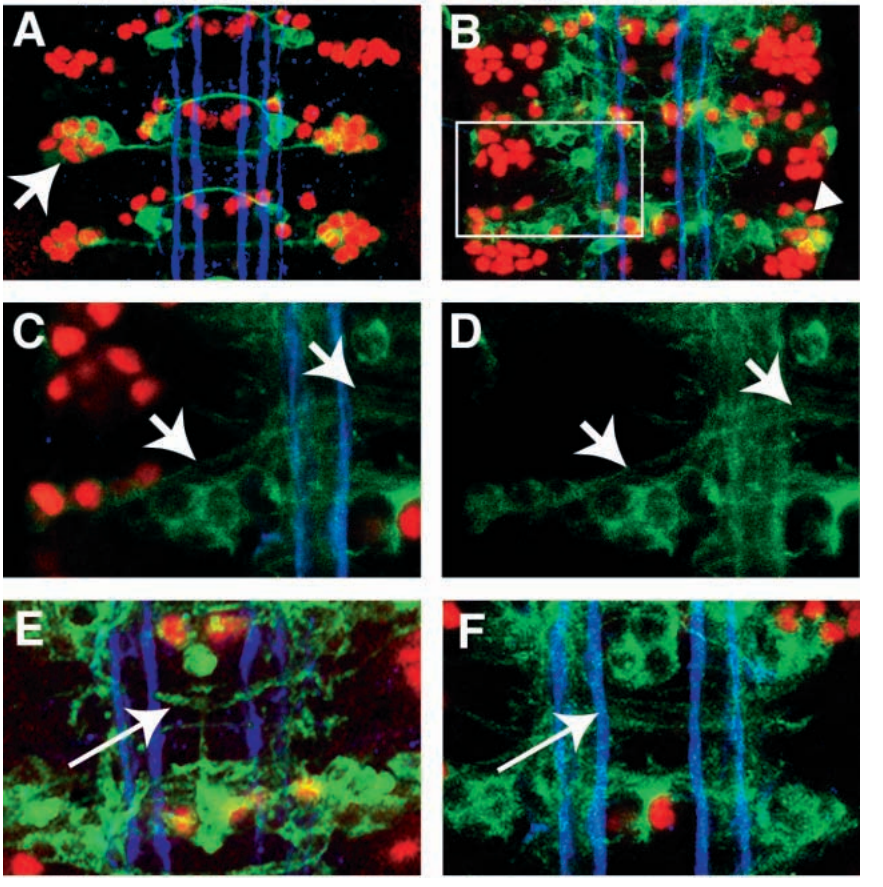

Fig. 4. The extra *eve*-expressing cells project axons with the EL neurons. Axon projections can be followed by GAL4-mediated expression of Tau-GFP (green). Fixed embryos were labelled with antibodies against Eve (red) and FasII (blue). (A) The wild-type EL neurons, examined in embryos containing *eagle-GAL4* and *UAS*-Tau-GFP (arrow), project axons across the anterior commissure. (B) On co-mis-expression of *runt* and Tau-GFP in row 6 and 7 neuroblasts, using *en-GAL4*, the extra *eve*-expressing cells also express Tau-GFP (arrowhead). (C,D) In the enlarged section (of the boxed area of B, the extra *eve*-expressing cells can be seen sending out axons towards the anterior commissure (arrows). (E) In the anterior commissure of normal embryos expressing Tau-GFP in the *en-GAL4* pattern, there are two fascicles. (F) When *runt* is mis-expressed using *en-GAL4*, there appear to be more axons crossing in the anterior commissure, which are defasciculated.

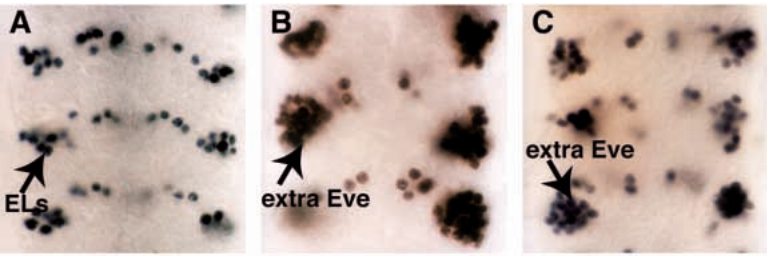

Fig. 5. The putative signal sequence is not necessary for Runt to induce extra EL neurons. (A) The normal clusters of 8-10 EL neurons (arrow) are enlarged on mis-expression of both (B) full-length Runt and (C) a truncated form of Runt, which lacks the putative signal sequence.

the formation of extra EL neurons, the extra cells will be formed by the those neuroblasts expressing GAL4. We constructed a transgene to express a Runt-GFP fusion protein. *UAS-runt-GFP* was driven by *en-GAL4* and the presence of *runt* protein monitored by its fluorescent tag. *en-GAL4* is not expressed in the EL neurons (Fig. 6A). Expression of Runt-GFP in the *en*-expressing cells is able to induce extra EL neurons and the extra cells coexpress Runt-GFP, suggesting that the fusion protein acts cell-autonomously (Fig. 6B).

However, if Runt-GFP is secreted, it would not be an autonomous marker of Runt expression. We therefore compared the expression of an independent, autonomous marker (Tau-GFP), with the induction of Eve expression by Runt. By expression of *UAS-runt* and *UAS-Tau-GFP*, it can be seen that the extra EL neurons also express Tau-GFP (Fig. 6C). Therefore, the EL neurons are formed by the *runt*-expressing cells. Both Runt-GFP and the Runt antibody are localised in the nucleus, consistent with Runt acting as a transcription factor. We conclude that Runt acts cell autonomously to induce the formation of extra EL neurons.

Runt is expressed in the neuroblast that gives rise to the EL neurons

Although Runt expression has not been reported in neuroblast 3-3, which gives rise to the EL neurons, ectopic *runt* induces EL neuron formation autonomously. Therefore, it is possible either that Runt expression was missed in neuroblast 3-3, or that Runt acts autonomously when ectopically expressed but non-autonomously in wild-type embryos. Since previously published antibodies to Runt are no longer available, we generated new anti-Runt antibodies (Fig. 7). Runt is expressed in seven stripes of cells in the cellular blastoderm embryo, which are just posterior to and overlapping with the *eve* stripes (Fig. 7A), consistent with previously published expression patterns (Kania et al., 1990). To determine which neuroblasts express Runt, we triple labelled embryos with anti-Runt, anti-En and anti-Gsb-d (Fig. 7B,C). En is expressed by neuroblasts in row 6 and 7 and neuroblast 1-2; Gsb-d is expressed by neuroblasts in row 5 and 6 and neuroblast 7-1. Contrary to the previously published expression pattern, which showed expression of Runt in the neuroblasts only up to stage 10, we find that Runt is expressed in neuroblasts throughout neurogenesis.

Table 1. Average and range of numbers of EL neurons per abdominal hemisegment

Genotype	Number of EL neurons per hemisegment	Standard deviation	Range	Number of hemisegments examined
yw	9	0.80	7-10	109
sca-GAL4/+	8.9	0.73	8-10	120
en-GAL4/+	8.9	0.74	8-10	120
UAS-runt/+	9.0	0.78	8-10	120
UAS- runt-ΔSS/+	9.1	0.79	8-10	120
sca-GAL4/*UAS-runt*	15.5	4.06	9-25	120
sca-GAL4 × *UAS- runt*-ΔSS	16.4	3.94	10-28	120
en-GAL4/*UAS-runt*	12.7	2.92	8-20	120
en-GAL4 × *UAS- runt*-ΔSS	13.5	3.81	9-27	120

Neuroblasts 2-2, 2-5, 3-1, 3-2, 5-2 and 5-3 express Runt from the time of their delamination (Fig. 7B; stage 10). By stage 11, Runt is also expressed in neuroblasts 2-3 and 3-3, and expression is lost from neuroblast 2-5 (Fig. 7C). We verified that Runt is expressed in neuroblast 3-3 by double staining for Eagle, which is expressed by neuroblasts 2-4, 3-3, 6-4 and 7-3 (Fig. 7D). The previously published expression pattern of Runt had not shown Runt expression in neuroblast 3-3 (Doe and Technau, 1993), which gives rise to the EL neurons (Schmidt et al., 1997). Furthermore, it was previously reported that neuroblasts 1-1 and 4-1 also express Runt (Doe and Technau, 1993, neuroblast numbers corrected to Broadus et al., 1995), but this is not seen with our antibody. Therefore, Runt is expressed in five neuroblasts in rows 2 to 3 (neuroblast 2-2, 2-3, 3-1, 3-2 and 3-3) and two neuroblasts in row 5 (neuroblast 5-2 and 5-3). The Runt expression in neuroblast 3-3 further supports our conclusion that Runt acts cell autonomously in the nervous system.

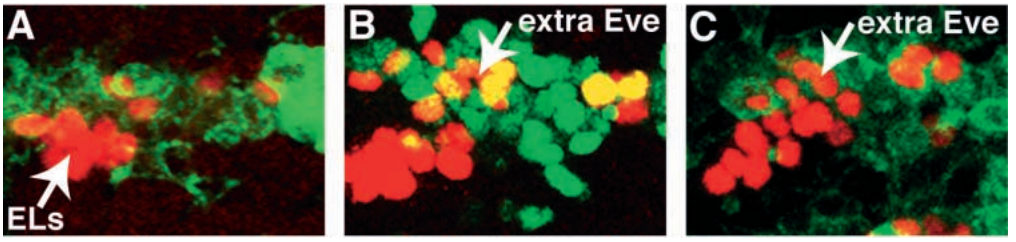

Fig. 6. Runt acts autonomously to induce Eve expression. In the progeny of *en-GAL4* flies crossed to *UAS*-Tau-GFP flies (A), none of the lateral GFP-expressing cells (green) express *eve* (red). (B) Expression of a Runt-GFP fusion protein (green) or (C) co-expression of Tau-GFP (green) and *runt* show that the extra *eve*-expressing cells also express Runt. Single hemisegments are shown. Arrows, EL neurons.

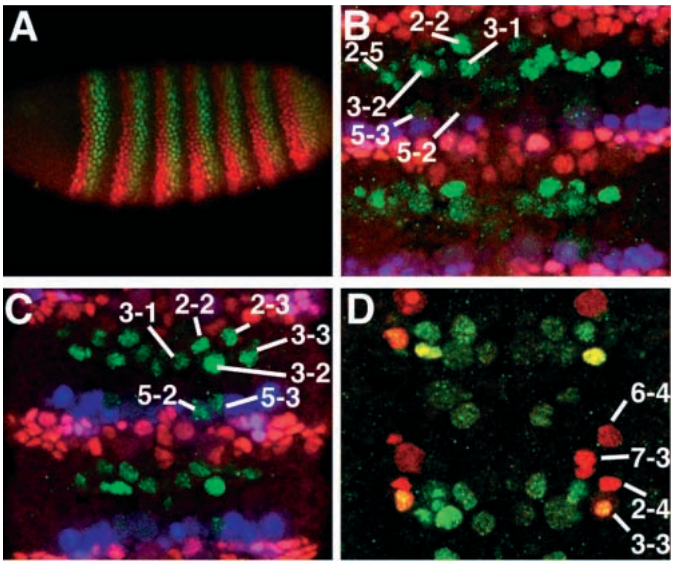

Fig. 7. Runt expression in the ectoderm and neuroblasts. (A) In the blastoderm embryo, Runt (green) is expressed in seven ectodermal stripes posterior to the Eve stripes (red). (B, stage 10; C, stage 11) Runt is expressed by a subset of neuroblasts (Runt, green; En, red; Gsb-d, blue). (D, stage 11) There is coexpression of Runt (green) and Eagle (red) in neuroblast 3-3 (yellow).

Runt is also expressed in a subset of neurons and midline cells

Runt is expressed by a large number of GMCs and neurons including the EL neurons (Fig. 8A-D). Runt is also expressed in a cluster of two to four cells on the midline and in a pair of neurons one on each side of the midline. By double labelling with anti-Odd, which labels MP1 and dMP2, we found that the neurons on each side of the midline are the MP1 neurons (Fig. 8E). Double labelling with antibody to Slit, which labels the midline glia (Rothberg et al., 1988), identifies the cluster of Runt-expressing cells on the midline as the midline glia (Fig. 8F).

DISCUSSION

We have shown that Runt is necessary and sufficient to induce the EL neuron fate. Only row 6 neuroblasts are competent to respond to ectopic *runt* expression by giving rise to extra EL neurons. The extra Eve-expressing cells project axons along the same route as the EL neurons. Although the structure of the protein encoded by *runt* suggests it might be secreted, Runt-GFP and anti-Runt show the protein is nuclear and that Runt acts cell-autonomously. Contrary to previous reports, Runt is expressed in neuroblast 3-3 which gives rise to the EL neurons, further supporting a cell-autonomous mode of action for Runt.

Runt is sufficient to specify the fates of neuroblasts

The loss of Runt activity during the time of neuroblast delamination leads to a loss of *eve* expression in the EL neurons (Fig. 1B,C; Duffy et al., 1991). The longitudinal fascicles appear disorganised, which could result from cell fate changes leading to altered axon projections, or pathfinding defects in certain neurons. Runt could therefore affect either the identity of neurons, or their behaviour, for example by modifying the expression of cell surface molecules involved in pathfinding. We have shown that ectopic *runt* is able to induce extra *eve* expression, but we do not know the role of Eve in these neurons. To assay another aspect of cell identity, we have labelled axonal projections with Tau-GFP. Ectopic *runt* expression not only induces ectopic Eve expression, but the Eve-expressing cells project axons in the path of the normal EL neurons. This suggests that *runt* is crucial for the identity of these neurons.

Although Runt can induce *eve* expression and induce neurons with EL neuron-like projections, this does not necessarily mean that Runt directs cell fates via *eve* as an intermediate. There could be several parallel pathways controlled by Runt, all of which are important for the production of functional EL neurons. The role of *eve* in determining cell fates has been investigated by labelling axons in *eve* mutant embryos. Temperature-shifted eve^{ts} embryos

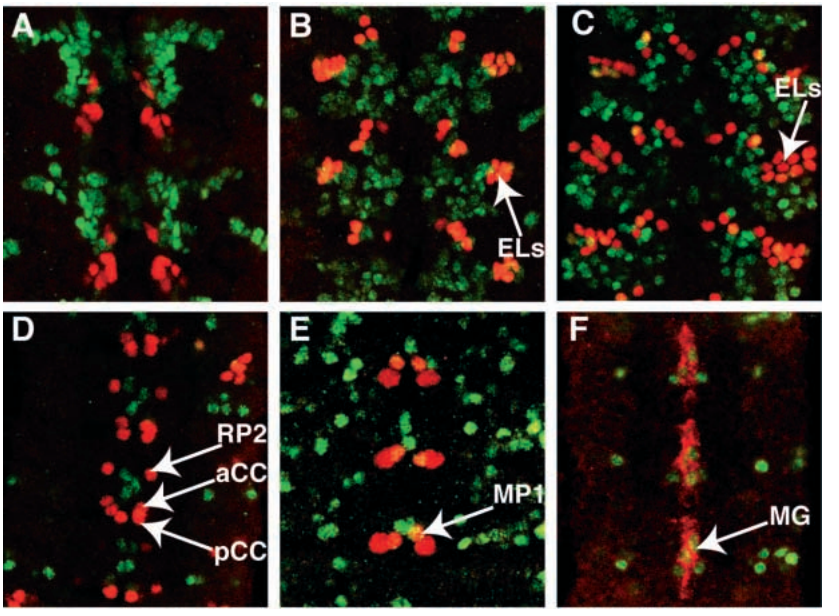

Fig. 8. Runt (green) expression in the neurons. (A-D) Eve expression in red. (A, stage 12) Runt is expressed in a subset of GMCs. (B, stage 14 and C, stage 16) There is colocalisation of Runt with Eve (yellow) in the EL neurons. (D, stage 16) Runt is not expressed by aCC, pCC or RP2. (E,F) Runt is expressed by a subset of midline cells. There is colocalisation of Runt with Odd (red) in the MP1 neurons (E, stage 16) and colocalisation of Runt with Slit (red) in the midline glia (MG) (F, stage 16).

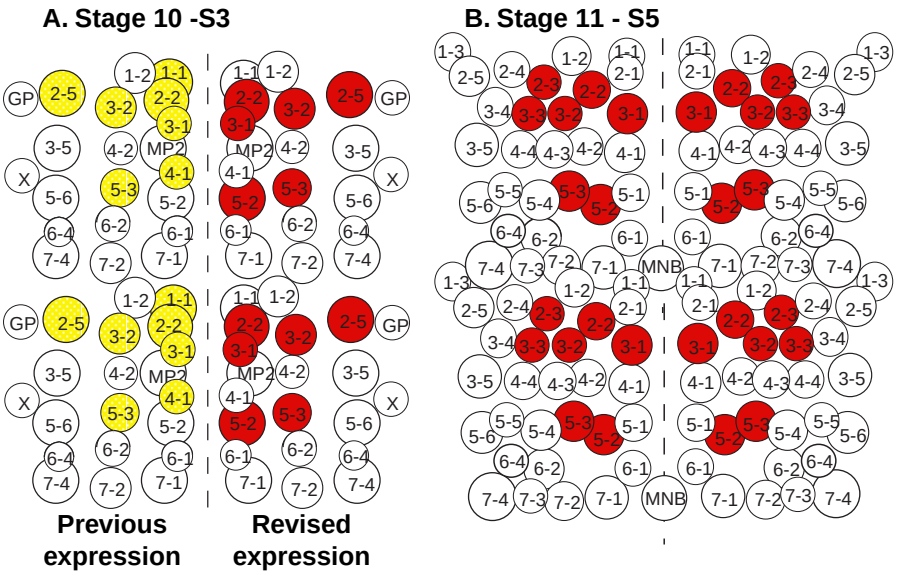

Fig. 9. The revised pattern of Runt expression in the neuroblasts. Schematic showing two segments of neuroblasts. (A) Stage 10 – S3, comparing the previously published expression pattern (yellow – left; Doe and Technau, 1993) to the neuroblasts detected by our antibody (red – right). (B) Stage 11 – S5, showing the final pattern of Runt expression in the neuroblasts as detected by our antibody.

were injected with lucifer yellow to label the aCC, pCC and RP2 neurons (Doe et al., 1988). It was found that, despite the lack of Eve, pCC projects axons similar to those in wild-type embryos, whereas aCC and RP2 display abnormal projections. It seems that *eve* is only important for selected neuronal projections. The projections of the EL neurons were not examined. A distinction between expression of *eve* and axonal trajectory has also been revealed by studies of the *eagle* mutant. Eagle is expressed in the EL neurons. In *eagle* mutants, the EL neurons continue to express *eve*, but they project their axons incorrectly (Higashijima et al., 1996).

Runt can only induce neuroblasts in row 6 to form extra EL neurons. It seems that other cofactors are necessary in conjunction with Runt to specify the EL neuron fate. Runt has a novel DNA-binding domain, the Runt domain, which is also able to form protein-protein heterodimers (Kania et al., 1990). Several Runt domain-containing proteins have been identified in other organisms (Miyoshi et al., 1991; Kagoshima et al., 1993; Nucifora et al., 1993). Brother and Big Brother have been identified as partner proteins of the *Drosophila* Runt. (Golling et al., 1996). However, these proteins are expressed ubiquitously and so are unlikely to be the necessary cofactors for specification of EL neurons. Proteins expressed both by neuroblast 3-3 and by neuroblasts 6-1 or 6-2 are possible candidates for cofactors acting with Runt to induce EL neurons. Neuroblast 6-1 expresses the steroid receptor superfamily member Seven-up and neuroblast 6-2 expresses the zinc-finger transcription factor Ming (Castor) in common with neuroblast 3-3 (Broadus et al., 1995). Although Eve expression is not affected in *ming* mutants (Mellerick et al., 1992), it would be interesting to investigate whether either Ming or Seven-up contribute to other aspects of the EL neuron fate.

Previous studies have looked at the effect of Runt on other transcription factors and have not shown the impact of Runt expression on the physical characteristics of the neurons. Here we show that *runt* expression can affect the axon projections of neurons. However, we cannot tell whether these neurons connect to their correct targets, another important factor in the developing nervous system. The general disorder of the neuropile suggests that mis-expression of *runt* is having either primary or secondary effects on axonal pathfinding and/or fasciculation.

Runt acts cell autonomously to specify the fates of neuroblasts

Although previously reported data suggested that Runt might act non-autonomously, or even as a secreted signalling molecule, we found that Runt acts autonomously to specify fates in the nervous system. The N-terminal putative signal sequence is not necessary for Runt to function. On checking the expression of Runt in the neuroblasts, we found that Runt is expressed by neuroblast 3-3 and hence in the correct neuroblast to autonomously specify EL neurons.

Runt expression pattern

We have made the first detailed characterisation of the expression of *runt* in neuroblasts. Our antibody clearly detects Runt in a subset of neuroblasts at all stages of their development. We find that Runt is not expressed by neuroblasts 1-1 and 4-1, as previously described (Doe and Technau, 1993; neuroblast numbers according to Broadus et al., 1995). In addition to the neuroblasts previously described as expressing Runt, we find that Runt is also expressed by neuroblasts 2-3, 3-3 and 5-2 (Fig. 9). Recent work on the neuroblast lineages and markers may make it easier to identify unequivocally each neuroblast. Although it is possible that our antibody may detect an alternative isoform of Runt to the previous antibodies, there is no evidence for Runt existing in different isoforms.

Runt is expressed in a large number of GMCs and neurons. We find that Runt is expressed in the midline in the MP1 neurons and the midline glia. The MP1 neurons are thought to pioneer the middle FasII-expressing longitudinal fascicle (Bastiani et al., 1986; Lin et al., 1995; Hidalgo and Brand, 1997). When Runt is inactivated during the time of neuroblast delamination, the middle longitudinal fascicle is identifiable, despite disruption of the longitudinal tracts. The four pioneers of the longitudinal tracts are thought to act synergistically to

establish the longitudinal pathways, so even if loss of Runt results in a loss of the MP1, it seems likely that the other pioneers would still be able to establish the longitudinals (Hidalgo and Brand, 1997).

Concluding remarks

Runt expression in neuroblast 3-3 is required for the formation of the EL neurons. Since ectopic expression of Runt only induces extra EL neuron formation by row 6 neuroblasts, there must be other factors necessary to specify the EL neuron fate. Our description of the expression pattern of Runt clarifies the role of Runt in the formation of the EL neurons and provides a starting point for the analysis of the role of Runt in other neuroblasts and in a subset of midline cells. It should now be possible to identify factors acting in parallel with Runt to specify the EL neurons. Using the GAL4 system to express ectopically neuroblast-identity genes in different subsets of neuroblasts, and using Tau-GFP to follow the development of these cells, it should be possible to add Runt to the network of molecular interactions specifying neuroblast fates.

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