Development 121, 3989-3996 (1995)
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DEV5015



# Evolution of neuroblast identity: *seven-up* and *prospero* expression reveal homologous and divergent neuroblast fates in *Drosophila* and *Schistocerca*

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## SUMMARY

**In the *Drosophila* CNS, early neuroblast formation and fate are controlled by the pair-rule class of segmentation genes. The distantly related *Schistocerca* (grasshopper) embryo has a similar arrangement of neuroblasts, despite lack of known pair-rule gene function. Does divergent pair-rule gene function lead to different neuroblast identities, or can different patterning mechanisms produce homologous neuroblasts? We use four molecular markers to compare *Drosophila* and *Schistocerca* neuroblast identity: seven-up, prospero, engrailed, and fushi-tarazu/Dax. In both insects some early-forming neuroblasts share key features of neuroblast identity (position, time of formation, and temporally accurate gene expression); thus, different patterning mechanisms can generate similar neuroblast fates. In contrast, several later-forming neuroblasts show species-specific differences in position and/or gene expression; these neuroblast identities seem to have diverged, suggesting that evolution of the insect central nervous system can occur through changes in embryonic neuroblast identity.**

Key words: *Schistocerca*, *Drosophila*, *seven-up*, *prospero*, neuroblast, phylotypic stage, CNS

## INTRODUCTION

A central goal in developmental biology is to understand how development has evolved to generate diversity among organisms. We are addressing this question by comparing early neurogenesis in *Drosophila* and *Schistocerca* (grasshopper) embryos. These evolutionarily distant insects are most similar at completion of a segmented germband during embryogenesis, the phylotypic stage for these organisms, while both earlier and later events are different (Sander, 1976; Patel et al., 1989, 1992; Dawes et al., 1994). In *Drosophila*, the formation and fate of early CNS precursors (neuroblasts; NBs) are controlled by the pair-rule class of segmentation genes, including *fushi tarazu* (*ftz*) and *even-skipped* (*eve*) (Skeath et al., 1992). In *Schistocerca*, homologues of *eve* and *ftz* have been cloned, and neither is expressed in pair-rule stripes (Patel et al., 1992; Dawes et al., 1994). The differences in pair-rule gene function between *Drosophila* and *Schistocerca* raise an interesting question in CNS evolution: do differences in pair-rule gene function lead to different cell fates among early NBs, or can different patterning mechanisms produce homologous NBs?

In both *Drosophila* and *Schistocerca*, a similar geometrical array of approximately 60 NBs forms in each segment (Bate, 1976; Doe and Goodman, 1985; Doe, 1992). Although position is one aspect of a NB's identity, equivalent cell fate or cell lineage cannot be inferred based on positional correspondence alone. Here, we present a molecular comparison of the NB pattern in *Drosophila* and *Schistocerca* using position, time of formation and expression of four molecular markers to assay NB identities. In *Drosophila*, patterns of gene expression in subsets of NBs have provided the basis for unambiguous identification of individual NBs (Doe, 1992). Expression of homologous genes in *Schistocerca* NBs offers a more accurate method for comparing NB identity. Known gene expression patterns can be used as molecular markers for NBs in both insects. The genes involved include *engrailed* (*en*; Condron et al., 1994) and a *ftz*-related gene, *Dax* (Dawes et al., 1994). To increase our number of NB markers, we have cloned the *Schistocerca seven-up* (*svp*) gene, which is dynamically expressed in NBs in both insects (Doe, 1992; Broadus et al., 1995). In addition, we use an antiserum to the prospero (pros) protein (Matsuzaki et al., 1992), which labels the MP2, MP1 and median NB in *Drosophila* (Doe, 1992; Broadus et al., 1995); this antiserum cross-reacts with the *Schistocerca* pros protein and labels the same CNS precursors.

*svp*, named for its role in *Drosophila* eye development, encodes a predicted steroid receptor transcription factor that shows high homology to the human COUP-TF (Mlodzik et al., 1990). Homologues to *svp* have been identified in several other organisms including grasshopper (this study), zebrafish (Fjose et al., 1993), sea urchin (Chan et al., 1992), chick (Lutz et al., 1994) and mouse (Jonk et al., 1994). A common feature in all organisms examined is *svp* expression in the developing nervous system. While *svp* is expressed in all *Drosophila* and *Schistocerca* NBs, it is especially useful as a marker for identifying individual NBs because the onset of *svp* expression is precisely regulated in each NB; some NBs are *svp*-positive at formation, whereas other NBs show *svp* expression at a specific time after formation. In contrast, the *pros*, *en*, and

*ftz/Dax* genes show early and persistent expression in identified subsets of NBs.

Using these four molecular markers (seven-up, pros, en, and ftz/Dax), we find that some early NBs are homologous between insects: they have similar position, time of formation, and time of gene expression. These data show that different embryonic patterning mechanisms (with or without the pair-rule functions of *ftz* and *eve*) can generate similar NB fates. In contrast, several later-forming NBs show differences in position and/or gene expression, suggesting that their identity has diverged. Therefore, evolution of the insect CNS has occurred in part through altering NB pattern and fate.

## MATERIALS AND METHODS

### Cloning and sequence of *Schistocerca seven-up*

The *Schistocerca* cDNA library (provided by Michael Bastiani, University of Utah) was constructed from embryos at 40% of development using the λZAP vector (Stratagene). A 2 kb DNA fragment, which included the entire *Drosophila svp* coding region and short flanking 3′ and 5′ untranslated regions, was radioactively labeled and used as probe. 600,000 plaques were screened at low-stringency conditions (McGinnis et al., 1984). Following secondary screening, selected positives were excised from the λZAP vector as PBS-KS clones according to the Stratagene protocol. Excised clones were sequenced on both strands using automated fluorescent cycle sequencing with Taq DNA polymerase (University of Illinois DNA Sequencing Center). GenBank accession number: BankIt 16159 U36622.

### RNA in situ hybridization to *Schistocerca* embryos

*Schistocerca* embryos were provided by Michael Bastiani (University of Utah) and Melody Seigler (Emory University). Embryos were staged according to Bentley et al. (1979). Embryos at 20-30% development were removed from egg chambers into 1× PBS, and the amnion surrounding the embryo was dissected away. Embryos were fixed 50 minutes in 1× PBS, 50 mM EGTA, 9.25% formaldehyde. Embryos not intended for immediate use were dehydrated through methanol series, and stored at –20°C in ethanol. For use, embryos were rehydrated in a 1:1 mixture of methanol and 5% formaldehyde in PTw (1× PBS, 0.1% Tween-20), refixed for 25 minutes in 5% formaldehyde in PTw, and then washed in PTw 5× 5 minutes. Embryos were incubated 10 minutes in a 1:1 mixture of PTw and prehybridization solution (50% formamide, 4× SSC, 250 μg/ml tRNA, 0.1% Tween-20, 1× Denhardt's solution, 5% dextran sulfate) followed by 10 minutes in prehybridization solution. Freshly denatured herring sperm DNA was added at a concentration of 500 μg/ml, and prehybridzation was allowed to proceed for >1 hour at 55°C.

Digoxigenin-labeled RNA probes were prepared according to Boehringer Mannheim's Genius System protocol. Alkaline hydrolysis of the probe in 0.1 M carbonate buffer (pH 10) for 40 minutes at 60°C was used to reduce probe size. Embryos were hybridized with probes for >24 hours at 55°C. Posthybridization washes were done in 50% formamide, 4× SSC, 0.1% Tween-20 over 12-16 hours at 55°C. Hybridization and posthybridization washes were performed without rocking. Embryos were transferred to PBT (1× PBS, 0.2% bovine serum albumin, 0.1% Triton-X) and allowed to equilibrate over 30 minutes. All subsequent steps were performed at room temperature with rocking. Alkaline phosphatase-conjugated anti-digoxigenin (Boehringer Mannheim) was diluted 1:2000 in PBT, and the embryos were incubated in the diluted antibody solution for 1-2 hours. Unbound antibody was removed by washing embryos 6× 10 minutes in PBT. Embryos were transferred to AP buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris, pH 9.5, 0.1% Tween-20) and allowed to equilibrate over 5 minutes. The alkaline phosphatase conjugate was detected by addition of 4.5 μl of 75 mg/ml NBT and 3.5 μl of 50 mg/ml X-phosphate per ml AP buffer. Development of the AP reaction product was stopped by several rinses in PBT. Embryos were cleared in glycerol and flattened under a coverslip to bring most NBs into one focal plane prior to photography.

### RNA in situ hybridization to *Drosophila* embryos

Embryos were dechorionated in 50% Clorox for 2 minutes, rinsed with water, and then fixed for 30 minutes in a 1:1 mixture of heptane and 1× PBS, 50 mM EGTA, 9.25% formaldehyde. Embryos were devitellinized by cracking in methanol. Embryos not intended for immediate use were transferred to ethanol prior to storage at –20°C. In situ hybridization of RNA probes was identical for *Schistocerca* and *Drosophila* embryos. Following staining, embryos were cleared in glycerol and flat-mounted.

The *svp* transcript can be detected earlier than the *lacZ* gene product (β-galactosidae) expressed from a *svplacZ* enhancer trap, and therefore this report documents *svp* expression in some NBs one stage prior to that described for *svplacZ* (Doe, 1992; Broadus et al., 1995).

### Immunocytochemistry

*Schistocerca* embryos were dissected in 1X PBS and then fixed for 50 minutes in PEM-FA (3.7% formaldehyde, 0.1 M Pipes (pH 6.9), 1 mM $MgSO_4$, 2 mM EGTA). Embryos not intended for immediate use were dehydrated in methanol and stored at –20°C. Immediately prior to use, embryos were rehydrated in PBT (1× PBS, 1% BSA, 0.1% Triton-X). Embryos were blocked for 1 hour in PBT + 5% normal goat serum (NGS). pros protein was detected using a 1:100 dilution of rabbit polyclonal serum generated against the 14 amino acids at the carboxyl terminus of the *Drosophila* pros protein (Matsuzaki et al., 1992). Prior to use, primary antibody was preadsorbed as a diluted solution by incubation with an excess of embryos for 1 hour at room temperature. Preadsorbed primary antibody was incubated with embryos for staining either overnight at 4°C or for 2 hours at room temperature. Embryos were then washed in PBT 6 × 10 minutes at room temperature. Biotinylated secondary antibody was diluted 1:200 in PBT + 5% NGS and incubated with embryos for 1 hour at room temperature. For histochemical detection of the antibody, we used the Vectastain ABC kit and SG chromagen (Vector Laboratories, Inc.) according to the vendor's protocols. Following staining, embryos were cleared by transfer through 50%, 70%, 90% glycerol in 50 mM Tris (pH 8). Embryos were viewed on a Zeiss Axioplan compound microscope.

### Naming of *Drosophila* neuroblasts

This paper uses a revised nomenclature for *Drosophila* NBs (Broadus et al., 1995), which is modified from Doe (1992). The changes include new names for: NB 1-1, to reflect the fact that this NB makes the aCC/pCC lineage; NB 1-2, to optimize alignment of gene expression between *Schistocerca* and *Drosophila*; and NBs 2-2 and 2-3.

## RESULTS

### Cloning and characterization of the *Schistocerca seven-up* gene

A single *Schistocerca svp* clone was isolated in a screen of 600,000 plaques (see Materials and Methods). This 3 kb clone contained 363 nucleotides of open reading frame (Fig. 1A) and about 2.6 kb of 3′ untranslated sequence. The open reading frame aligned with *Drosophila svp* in the ligand-binding domain, which encodes the C terminus of the protein. Conceptual translation of the *Schistocerca* DNA sequence shows 95% amino acid identity compared to *Drosophila* svp, and 91% amino acid identity compared to human COUP-TF (Fig. 1B). The high amino acid identity between the *Schistocerca* predicted protein, *Drosophila* svp, and human COUP-TF strongly suggests that this clone corresponds to the *Schistocerca svp* cDNA.

We used this clone to prepare RNA probes with which to examine *svp* expression in the *Schistocerca* embryo. In both

*Drosophila* and *Schistocerca*, *svp* is expressed in the central and peripheral nervous systems, in a patterned subset of cells behind the morphogenetic furrow of the developing eye, and in the fat body (Figs 2, 3; Mlodzik et al., 1990; Hoshizaki et al., 1994). These data provide additional evidence that our *Schistocerca* clone is a bona fide homologue of *Drosophila svp*.

### *seven-up* expression in identified neuroblasts in *Schistocerca*

To compare individual NB fates between insects, we used three criteria that contribute to a NB's unique identity: time of formation, position and gene expression. The position and timing of NB formation are stereotyped, and have been previously documented for *Schistocerca* and *Drosophila* (Bate, 1976; Doe and Goodman, 1985; Campos-Ortega and Hartenstein, 1985; Doe, 1992). In both insects, NBs form a grid-like array of rows and columns. *Schistocerca* NBs are aligned in orderly rows and columns, while *Drosophila* NBs are more compacted. NBs have been given number designations according to position within the segment. For example, NB 5-2 lies in row 5 and column 2 in the final NB pattern. Between insects, number identities imply positional similarity, but not necessarily molecular or lineage homology. *svp* is transiently expressed in NBs of both *Drosophila* and *Schistocerca*, and is a particularly useful marker for individual NBs because the onset of *svp* expression is precisely regulated during the generation of each NB lineage.

At approximately 2% of development after an individual segment is formed, *svp* expression is observed in a subset of NBs (Fig. 3A,C). The first *svp*-positive NBs (3-2, 4-1, 5-2, 7-4) delaminate from *svp*-positive ectodermal clusters (Fig. 3C inset). The later-arising NB 7-2 is also observed to delaminate from a *svp*-positive ectodermal cluster. Each of these NBs is *svp*-positive at formation and therefore prior to the first mitotic division. The amount of *svp* transcript is variable according to position, with NB 5-2 and the overlying ectodermal cluster consistently showing most intense expression (Fig. 3C).

NBs 2-5, 3-5, 4-4, 5-3, 6-2, and 7-1, which are among the first NBs to form, show delayed onset of *svp* expression (Fig. 3B,D). The NB progeny, GMCs, can be identified by cell morphology, position immediately dorsal to NBs, and nuclear expression of pros protein (Fig. 5B). These assays show that *Schistocerca* NBs initiate mitosis immediately following delamination. Therefore, we conclude that NBs which do not delaminate from *svp*-positive ectodermal clusters express *svp* only after at least one cell division is complete. Eventually, all NBs and some GMCs transiently express *svp*. Soon after the NB array is complete, *svp* expression is extinguished in most NBs (Fig. 3E,F). Approximately 5-8% of development later, many NBs reinitiate *svp* expression. This second burst of *svp* expression in NBs persists until at least 50% of development, although we have not characterized this later expression in detail.

### *seven-up* expression in identified neuroblasts in *Drosophila*

*svp* expression during *Drosophila* neurogenesis has a spatial and temporal pattern similar to that observed in *Schistocerca*. Early in embryonic stage 9 (staging according to Campos-Ortega and Hartenstein, 1985) the first 10 (S1) NBs form in each hemisegment (Doe, 1992). Among this first group, NBs 3-2, 5-2, and 7-4 express *svp* at formation, each having delam-

**A**

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ATCTTCCAGGAGCAGGTCGAGAAGCTCAAGGCGCTGCATGTCGACTCCGCCGAATAC
 I  F  Q  E  Q  V  E  K  L  K  A  L  H  V  D  S  A  E  Y

TCCTGCCTCAAGGCCATCGTCCTCTTCACCACAGACGCGTGTGGCCTGTCGGACGTG
 S  C  L  K  A  I  V  L  F  T  T  D  A  C  G  L  S  D  V

GCGCACATCGAGGGACTGCAGGAGAAGTCGCAGTGCGCGCTCGAGGAGTACTGCCGC
 A  H  I  E  G  L  Q  E  K  S  Q  C  A  L  E  E  Y  C  R

ACGCAGTACCCCAACCAGCCGACGCGCTTCGGCAAGCTGCTGCTGCGCCTTCCCTCG
 T  Q  Y  P  N  Q  P  T  R  F  G  K  L  L  L  R  L  P  S

CTGCGGACGGTCAGCTCGCAGGTGATCGAGCAGCTGTTCTTCGTGCGGCTGGTGGGC
 L  R  T  V  S  S  Q  V  I  E  Q  L  F  F  V  R  L  V  G

AAGACGCCCATCGAGACGCTGATACGGGACATGCTGCTGAGCGGCAGCAGCTTCAGC
 K  T  P  I  E  T  L  I  R  D  M  L  L  S  G  S  S  F  S

TGGCCCTACATGTCCACCATGTGA
 W  P  Y  M  S  T  M  *
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**B**

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Schistocerca svp        IFQEQVEKLKALHVDSAEYSCLKAIVLFTTDACGLSDVAHI
Drosophila svp type 1   ------------------------------------T--
human COUP-TF           ----------------------------S-------A---

Schistocerca svp        EGLQEKSQCALEEYCRTQYPNQPTRFGKLLLRLPSLRTVSS
Drosophila svp type 1   -S-----------------------------------------
human COUP-TF           -S------------V-S------S-----------------

Schistocerca svp        QVIEQLFFVRLVGKTPIETLIRDMLLSGSSFSWPYMSTM*
Drosophila svp type 1   ---------------------------N------LPS-*
human COUP-TF           S----------------------------N-----IQCS*
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**Fig. 1.** *Schistocerca seven-up* is a member of the seven-up/COUP family. (A) DNA sequence and conceptual translation of a partial *Schistocerca svp* cDNA. (B) Conceptual translation of the *Schistocerca svp* DNA sequence aligns with *Drosophila* svp (95% amino acid identity) and COUP-TF (91% amino acid identity) over the C-terminal half of the ligand-binding domain.

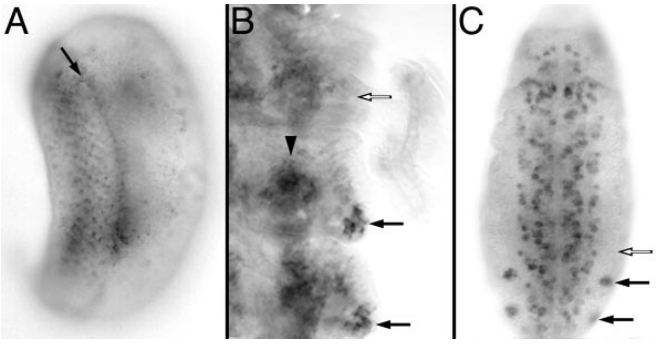


**Fig. 2.** *Schistocerca* and *Drosophila seven-up* are expressed in the eye, peripheral nervous system, and fat body. (A) A *Schistocerca* eye at 50% of development. The morphogenetic furrow (arrow) has advanced approximately one-third of the distance across the eye. *svp* is expressed in a patterned subset of cells posterior to the furrow, similar to *svp* expression in the *Drosophila* developing eye disc (Mlodzik et al., 1990). (B,C) In both *Drosophila* and *Schistocerca*, presumptive PNS structures express *svp* posterior to A1, but do not express *svp* in A1 and segments anterior to A1. (B) Ventral view of a *Schistocerca* embryo at about 42% of development. In the lateral body wall, *svp*-positive cell clusters are present in A2 and A3 (solid arrows), but are absent from A1 (open arrow) and segments anterior to A1. *svp* expression is also observed in the developing fat body (arrowhead, slight out of focus), which has been previously documented in the *Drosophila* embryo (Hoshizaki et al., 1994). (C) Ventral view of a stage 11 *Drosophila* embryo showing *svp*-positive cell clusters of the PNS in A2 and A3 (solid arrows), but absent from A1 (open arrow) and segments anterior to A1.

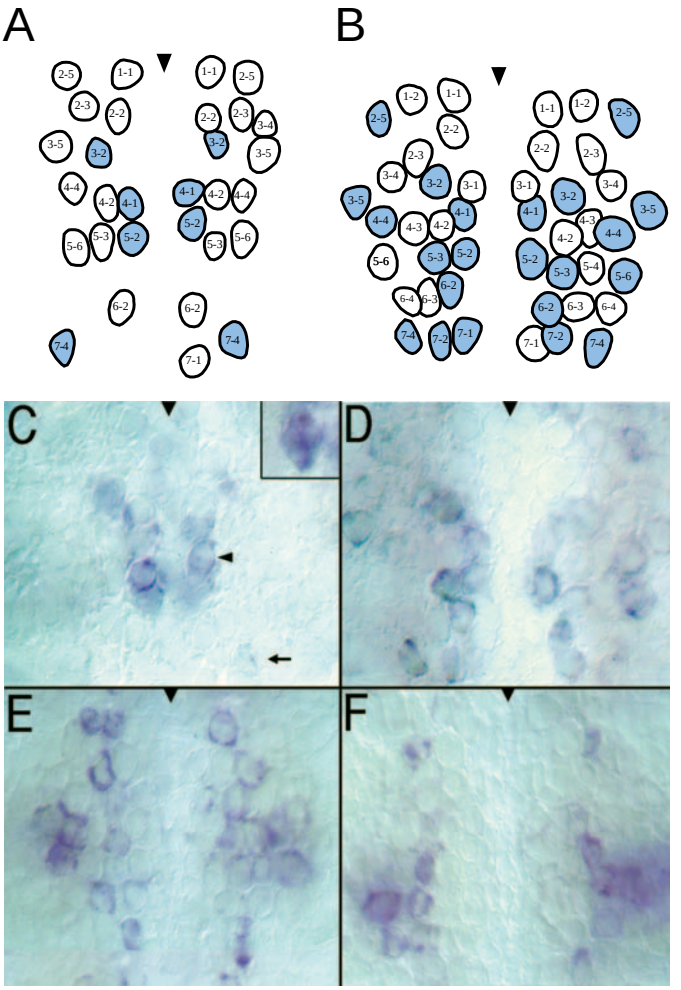


**Fig. 3.** *Schistocerca seven-up* expression in identified neuroblasts. *svp* is expressed in a dynamic sequence that includes all NBs. Some NBs are *svp*-positive at formation and therefore at the beginning of their cell lineage. Other NBs complete one or more cell divisions, then initiate *svp* expression midway through their cell lineage. (A,C) In this S1 segment, four NBs per hemisegment express *svp*: NBs 3-2, 4-1, 5-2 (arrowhead), and 7-4 (arrow). Each of these NBs delaminates from a *svp*-positive ectodermal cluster (inset) and is therefore *svp*-positive at formation and prior to the first cell division. NB 2-5, one of the first NBs to delaminate, initiates *svp* expression shortly after formation, but can be *svp*-negative during S1. (B,D) At S2, seven additional NBs per hemisegment express *svp*: NBs 2-5, 3-5, 4-4, 5-3, 6-2, 7-1, and 7-2. Among this group, only NB 7-2 is *svp*-positive at formation. The remaining NBs of this group initiate *svp* expression only after completing one or more cell divisions (see text). (E) All NBs transiently express *svp*. A majority of the NBs in this segment are *svp*-positive. (F) In a slightly older segment, most NBs in the segment no longer express *svp*. *svp* expression in NBs is extinguished shortly after the NB array is complete. Camera lucida tracings of S1 (A) and S2 (B) NB patterns (see text for description of *Schistocerca* NB stages) correspond to segments pictured in C and D respectively. Shading indicates *svp* RNA expression. Not all NBs shown in cameral lucida traces are visible in the corresponding pictures due to the thickness of the tissue. Bilateral differences in *svp* expression reflect slight variability in absolute time of NB formation and/or NB division. A single segment is shown at each stage. Anterior, up; arrowhead (top centre of each panel), ventral midline.

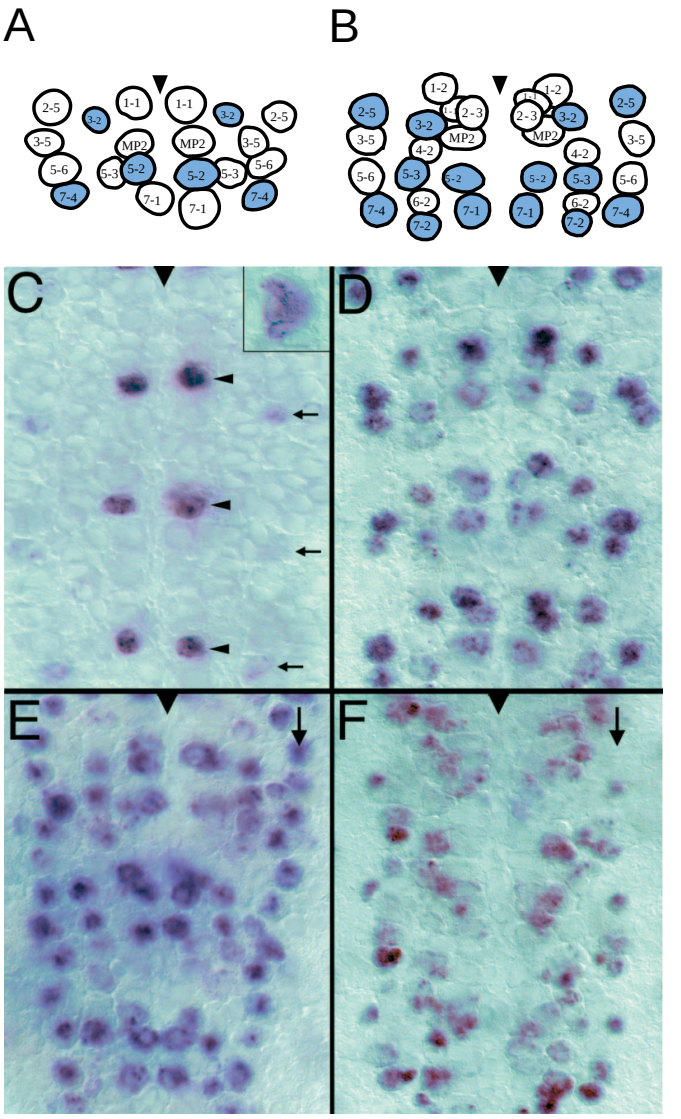


**Fig. 4.** *Drosophila seven-up* expression in identified neuroblasts. *svp* follows a dynamic sequence of expression in all NBs. Some NBs are *svp*-positive at formation and therefore at the beginning of their cell lineage. Other NBs complete one or more cell divisions, then initiate *svp* expression midway through their cell lineage. (A,C) Three S1 NBs express *svp*: NBs 3-2, 5-2 (arrowhead), and 7-4 (arrow). NB 3-2 has not delaminated in all segments. Each of these NBs delaminates from a *svp*-positive ectodermal cluster (inset) and therefore expresses *svp* at formation. (B,D) At S2, four additional NBs express *svp*: NBs 2-5, 5-3, 7-1, and 7-2. Of this group, only NB 7-2 delaminates from a *svp*-positive ectodermal cluster. The remaining NBs initiate *svp* expression after completing one or more cell divisions (see text). In some segments, a *svp*-positive cell can be seen enlarging in the ectoderm at the future position of NB 4-1. (E,F) All NBs express *svp*, but only transiently. Note that *svp*-positive NBs form a continuous lateral column at S3 (E, arrow), but that some of these NBs have terminated *svp* expression by S5 (F, arrow). Single segment camera lucida drawings of S1 (A) and S2 (B) NBs were traced from embryos pictured in C and D respectively. Shaded NBs express *svp* RNA. Several segments are shown at each stage. Anterior, up; arrowhead (top centre), ventral midline.

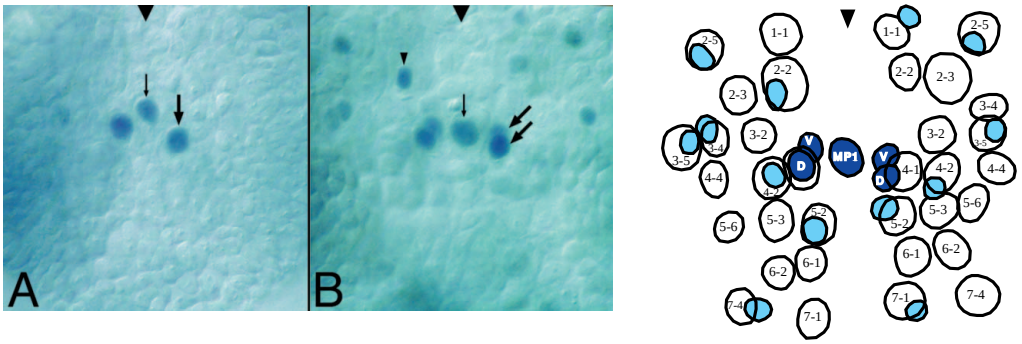


**Fig. 5.** *Schistocerca* prospero expression in MP1 and MP2. In *Schistocerca*, MP2 is identified by its characteristic position in the medial column of NBs, nuclear pros protein, and symmetrical cell division pattern. (A) Prior to the delamination of all lateral NBs, nuclear pros protein is observed in MP2 (thick arrow) and MP1 (thin arrow). (B) Shortly after its formation, MP2 divides to produce the pros-positive dMP2/vMP2 neurons (thick arrows). By this time, the earliest lateral NBs have formed and some have completed their first cell division. pros expression is observed at lower levels in newly born GMCs (e.g. arrowhead). (C) Camera lucida tracing of the segment shown in (B). Dark blue, high levels of pros protein; light blue, lower levels of pros protein. Anterior, up; arrowhead (top center), ventral midline.

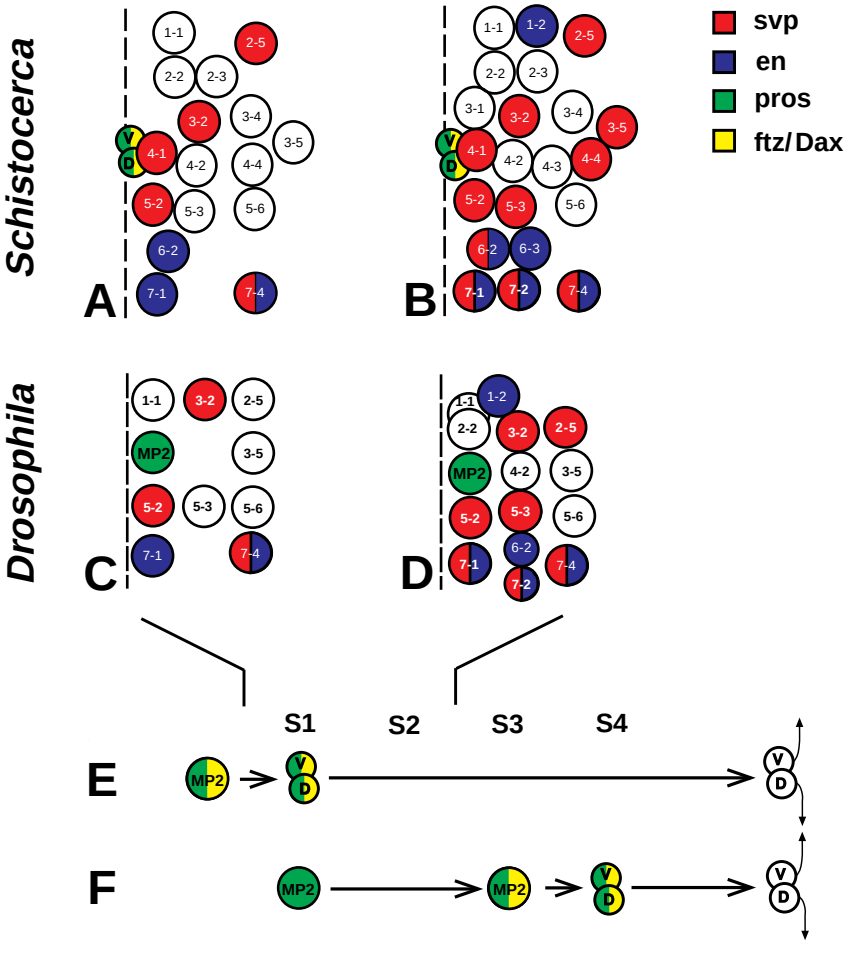


**Fig. 6.** The timing and pattern of gene expression is conserved in early-forming identified neuroblasts in *Drosophila* and *Schistocerca*. The pattern of early NBs is highly conserved between *Drosophila* and *Schistocerca*. Some differences in the position and/or timing of gene expression are also observed, especially among later-forming NBs. (A,C) In both insects, S1 NBs 5-2 and 7-4 are homologous. In both insects, NB 5-2 is *svp*-positive at formation, shows intense *svp* expression, and occupies a medial position within the hemisegment, just anterior to the *en* stripe. Similarly, in both insects NB 7-4 is *svp*-positive at formation, shows lower levels of *svp* transcript, and occupies the posterior lateral corner of the hemisegment within the *en* stripe. In contrast, NB 3-2 delaminates from a *svp*-positive ectodermal cluster in both insects, but shows different position within the hemisegment. (B,D) The *Drosophila* and *Schistocerca* S2 NBs 5-3, 7-1, and 7-2 are homologous. In both insects, NB 7-2 coexpresses *svp* and *en* at formation and is located in the posterior row of NBs. NB 5-3 in both insects delaminates at S1 just lateral to NB 5-2 and just anterior to the *en* stripe, and expresses *svp* at S2. NB 7-1 in both insects delaminates and expresses *en* during S1, is positioned at the posterior medial corner of the hemisegment, and initiates *svp* expression at S2. In contrast, *Drosophila* and *Schistocerca* NBs 3-5 are similarly positioned in the lateral NB column, but *Schistocerca* NB 3-5 is *svp*-positive at S2 while *Drosophila* NB 3-5 does not initiate *svp* expression until a later stage. A single hemisegment is shown at each stage. Dashed line, ventral midline; anterior, up. (E,F) In *Schistocerca*, MP2 forms and divides earlier than in *Drosophila*, but its position, gene expression, and cell lineage are identical in both insects. In *Schistocerca*, MP2 forms before all other NBs and almost immediately divides such that the dMP2/vMP2 progeny are present at the S1 NB stage. In *Drosophila*, MP2 delaminates at the S1 NB stage but delays cell division until the S4 NB stage. In both insects, MP2 expresses *pros* at its formation and expresses *ftz/Dax* just prior to its division. The axon projections of dMP2 and vMP2 are known to be identical in *Drosophila* and *Schistocerca*: vMP2 projects its axon anteriorly and dMP2 projects its axon posteriorly (Bate and Grunewald, 1981; Lin et al., 1994). In *Drosophila*, *pros* and *ftz* are not expressed in the mature dMP2/vMP2 (Doe et al., 1988a; Doe et al., 1991); in *Schistocerca*, *pros* and *Dax* expression in the mature dMP2/vMP2 have not been determined. Developmental time progresses from left to right.

inated from a *svp*-positive ectodermal cluster (Fig. 4A,C). Each of these NBs expresses *svp* at formation and therefore at the beginning of their cell lineage. The level of transcript in each NB and the overlying ectodermal cluster is stereotyped, with 5-2 consistently showing most intense expression (Fig. 4C).

At mid-stage 9 there are a total of 16 NBs (10 S1 NBs and 6 S2 NBs). The S2 NB 7-2 is also observed to delaminate from a *svp*-positive ectodermal cluster. In contrast, three of the S1 NBs (2-5, 5-3, and 7-1) initiate *svp* expression during the S2 stage of neurogenesis (Fig. 4B,D), at which time they have completed one or more cell divisions (Hartenstein et al., 1994). During the final three stages of NB formation (S3-S5), all NBs transiently express *svp* (Fig. 4E,F). Some NBs delaminate from a *svp*-positive ectodermal cluster, whereas other NBs express *svp* only after dividing one or more times. As *svp*-positive NBs divide, some GMCs also express *svp*. By stage 12 most CNS expression has been extinguished (data not shown). The pattern of *svp* expression in several early-forming *Drosophila* NBs is similar to that of *Schistocerca*; the comparison will be presented below, following a description of three additional NB markers: pros, en, and ftz/Dax.

### *Schistocerca* prospero shows nuclear expression in MP1, MP2, and the median neuroblast

In *Drosophila*, nuclear pros expression identifies a small subset of CNS precursors: MP1, MP2 and the median NB (Doe and Technau, 1993; Broadus et al., 1995). Here we show that an antiserum raised against the C-terminal region of *Drosophila* pros (Matsuzaki et al., 1992) cross-reacts with *Schistocerca* pros, and labels the same subset of *Schistocerca* CNS precursors. In both insects, pros is also detected in the nucleus of all newly-born GMCs (Fig. 5; Matsuzaki et al., 1992; Vaessin et al., 1991; Spana and Doe, 1995).

In *Schistocerca*, the earliest pros expression in the CNS is in the MP2 precursor (Fig. 5A). We identify the pros-positive precursor as MP2 based on its characteristic position and symmetrical cell division. MP2 is positioned in what will be the medial NB column; it forms before any NBs have delaminated, just as ectodermal segmentation becomes visible in that segment. At approximately 2% of development following its formation, MP2 divides symmetrically to produce a pair of neurons that also express pros: dMP2 and vMP2 (Fig. 5B,C; Bate and Grunewald, 1981). The dMP2/vMP2 neurons lie immediately dorsal to the newly formed NB 4-1; this is exactly the position of these neurons in *Drosophila* (Broadus et al., 1995). pros also shows nuclear expression in the unpaired MP1 precursor and the median NB. The early-forming MP1 precursor is identified by its position precisely between the pros-positive MP2 precursors and MP2 progeny (Fig. 5). The later-forming median NB is identified as the unpaired midline NB in row 7 (data not shown).

### engrailed and fushi-tarazu/Dax label similar NBs in *Schistocerca* and *Drosophila*

The en protein is known to be expressed in a similar pattern of NBs in *Schistocerca* and *Drosophila* (Condron et al., 1994; Broadus et al., 1995). In both insects, *en* is expressed in all NBs in rows 6 and 7 and in a single NB in the first row of the next posterior segment. *en* is also expressed in GMCs and neurons lying just dorsal to the *en*-positive NBs.

The ftz/Dax proteins are also known to be expressed in a similar pattern of CNS precursors in *Schistocerca* and *Drosophila* (Dawes et al., 1994; Broadus et al., 1995). In both insects, *ftz/Dax* is expressed in MP1 and its progeny, MP2 and its dMP2/vMP2 progeny, and a stereotyped subset of GMCs and neurons. In the MP2 lineage, *ftz/Dax* is expressed just prior to division of MP2 (Fig. 6E; Dawes et al., 1994); in *Drosophila*, division of MP2 is delayed for several hours following its formation, and expression of *ftz/Dax* is similarly delayed (Fig. 6F; Doe, 1992; Spana et al., 1995).

### Timing and pattern of gene expression in identified neuroblasts is conserved between *Schistocerca* and *Drosophila*

Using molecular markers for identified NBs in both *Drosophila* and *Schistocerca*, we find that the early pattern of NBs is highly conserved. In contrast, several later-forming NBs show differences in position or timing of gene expression. In *Drosophila*, NBs segregate from the ectoderm in 5 groups or waves (Doe, 1992). In contrast, *Schistocerca* NBs delaminate in a continuous sequence (Doe and Goodman, 1985). The two *Schistocerca* NB arrays shown in Fig. 6A,B were selected as the closest comparative stages to *Drosophila* S1 and S2 based on the number of NBs and patterns of gene expression.

We find that the pattern of *svp* RNA is highly conserved in NBs of *Drosophila* and *Schistocerca*; in addition, the precise timing of *svp* expression within the cell lineage of identified NBs is often conserved between the two insects. For example, NBs 5-2 and 7-4 express *svp* at formation and therefore at the onset of their cell lineage. In both insects, NBs 5-2 and 7-4 have the same relative position (5-2 is medial and just anterior to the *en* stripe; 7-4 is lateral and within the *en* stripe), delaminate from a similar *svp*-positive cluster (5-2 delaminates from the ectodermal cluster with the highest level of *svp* expression, whereas 7-4 delaminates

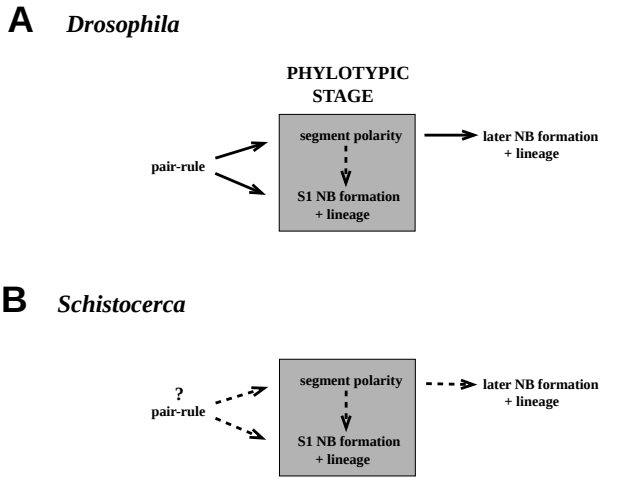


**Fig. 7.** The phylotypic stage is characterized by completion of segmentation and a partially conserved pattern of early neuroblasts. Completion of segmentation and the formation of early NBs are temporally linked events and appear to be a constrained developmental point (phylotypic stage; boxed) that is conserved in diverse species. (A) In *Drosophila*, the pair-rule genes control the formation of S1 NBs (Skeath et al., 1992). Segment polarity genes contribute to the identity of S1 NBs and regulate the formation of later NBs. (B) In *Schistocerca*, the genes controlling NB formation and specification are completely unknown. Pair-rule functions have not been identified; although *ftz* and *eve* genes have been cloned in *Schistocerca*, they are not expressed in pair-rule stripes (Patel et al., 1992; Dawes et al., 1994). Despite this difference, the earliest NBs are highly conserved between *Drosophila* and *Schistocerca*, as indicated in the boxed phylotypic stage. In contrast, the identities of several later-forming NBs appear variable. Solid arrows, direct regulatory events; dashed arrows, predicted relationships.

from an ectodermal cluster with a low level of *svp* expression), and have similar *en* expression profiles (5-2 is *en*-negative, 7-4 is *en*-positive). In contrast, NBs 5-3 and 7-1 complete at least one cell division prior to initiating *svp* expression. In both *Drosophila* and *Schisotcerca*, NBs 5-3 and 7-1 delaminate as S1 NBs, are similarly positioned (5-3 forms just lateral to NB 5-2; 7-1 forms at the posterior medial corner of the hemisegment), show similar *en* expression (5-3 is *en*-negative; 7-1 is *en*-positive), and delay *svp* expression until the S2 NB stage.

In addition to *svp*, the *pros*, *en*, and *ftz*/*Dax* genes show virtually identical expression between insects (see previous sections, Fig. 6). For example, in the *Drosophila* CNS, nuclear pros protein is observed in MP2, the unpaired MP1, the unpaired median NB, and all newly-born GMCs (Spana and Doe, 1995; Broadus et al., 1995). MP2 delaminates with the earliest forming NBs (at late embryonic stage 8); and expresses *ftz* just before it divides nearly symmetrically at stage 11 to produce two pros-positive neurons: dMP2 and vMP2 (Spana and Doe, 1995). In *Schistocerca*, MP2 forms and divides earlier than in *Drosophila*, but similarly expresses *pros* at formation and *Dax* just before dividing to produce the dMP2/vMP2 neurons (Figs 5, 6).

Using several criteria to assay NB fate (position, time of formation and temporally accurate gene expression), Drosophila and Schistocerca NBs 1-2, 5-2, 5-3, 7-1, 7-2, 7-4, and MP2 appear to be genuine homologues. The fates of other NBs have seemingly diverged or could not be assayed with the molecular markers used.

Although there are clear similarities between gene expression in *Drosophila* and *Schistocerca* NBs, there are some differences in timing and/or position of expression (Fig. 6). For example, NB 3-2 expresses *svp* at the time of its formation in both insects, but the NB initially delaminates at the anterior edge of the hemisegment in *Drosophila* and in the middle of the hemisegment in *Schistocerca*. In addition, pairs of adjacent NBs (6-2/7-1, 4-1/5-2) express *svp* in *Schistocerca*, whereas in *Drosophila* there is only one *svp*-positive NB at each position (NBs 7-1 and 5-2 respectively). Additional molecular markers, or ideally lineage analysis, will be necessary to determine the precise relationship between these NBs.

## DISCUSSION

The phylotypic stage for arthropods is based on morphological comparisons of development in diverse species. Molecular analysis has supported the idea that complete segmentation of the embryonic germband is a highly conserved developmental stage: ectodermal expression of *en* foreshadows the formation of the posterior segmental boundary in all arthropods examined, while earlier gap and pair-rule divisions are not strictly conserved (Patel et al., 1989, 1994; Dawes et al., 1994). Do nonectodermal tissues also show a conserved developmental stage? This study presents a molecular comparison of early neurogenesis in *Drosophila* and *Schistocerca*, and we find that the pattern of early NBs is highly conserved. Therefore, the completion of segmentation and the formation of early NBs are not only temporally linked events, and both may be similarly constrained during evolution.

In *Drosophila*, the pair-rule genes regulate expression of the proneural genes in neuroectodermal clusters that foreshadow the sites of NB formation (Skeath et al., 1992). *Schistocerca* homologues of the *Drosophila* pair-rule genes *ftz* and *eve* have been cloned, and neither is expressed in a pair-rule pattern (Patel et al. 1992; Dawes et al., 1994). How is the stereotyped pattern of NB formation initiated in *Schistocerca*? Despite the absence of *eve* and *ftz* pair-rule function in *Schistocerca*, the position of NB delamination and early gene expression in NBs is highly conserved. In both insects, *svp* is expressed transiently at reproducible timepoints in identified NB cell lineages. Between insects, some NBs appear to be genuine homologues in that they occupy similar positions and share timed expression of several developmentally important genes: *svp, pros, en,* and *ftz/Dax*. Perhaps most impressive is the conserved dynamics of *svp* expression in individual NB cell lineages. In both insects, NBs 5-2, 7-2 and 7-4 delaminate from *svp*-positive ectodermal clusters and therefore express *svp* during the earliest part of their cell lineage. NBs 5-3 and 7-1 in both insects express *svp* only after completing one or more cell divisions or midway through their lineage. Therefore, both a conserved pattern of early NBs, as well as ectodermal expression of *en* in each segment, are characteristic of the phylotypic stage of embryogenesis (Fig. 7). The genes controlling early neurogenesis in *Schistocerca* are entirely unknown, but are clearly different than those used in *Drosophila* (Fig. 7).

How does neurogenesis unfold in different insects as they develop past the phylotypic stage? Previous studies have led to the proposal that there is a conserved plan for embryonic neurogenesis in arthropods (Thomas et al., 1984 but see Whitington et al., 1993). Thus, the origin of species-specific CNS differences among insects have been attributed to different modes of embryonic development: holometabolous insects undergo metamorphosis while hemimetabolous insects continue embryonic development as a miniature adult form (Truman et al., 1993). In the holometabolous *Drosophila*, NBs arrest mitotic cycles near the end of embryogenesis, and then reactivate to produce additional neurons during larval stages (Prokop and Technau, 1991). In contrast, the hemimetabolous *Schistocerca* embryo shows continuous development such that embryonic NBs undergo many more divisions than observed in *Drosophila* embryonic neurogenesis (Bate, 1976; Shepherd and Bate, 1990). Using molecular markers for individual NB identities, we detect differences in position and/or timing of gene expression, especially among later-forming NBs. Therefore, while the pattern of early NBs is highly conserved, several later-forming NBs appear to have divergent fates. Changes in embryonic NB pattern and fate demonstrate evolution of CNS differences prior to the dramatic changes that occur at metamorphosis.

Aspects of the mature embryonic CNS are known to be conserved between *Drosophila* and *Schistocerca*: gene expression in identified neurons (Bastiani et al. 1987; Dawes et al., 1994; Doe et al., 1988a,b; Patel et al. 1992; Zinn et al., 1988), neuronal position, and axon morphology (Thomas et al., 1984; Kuwada and Goodman, 1985). Differences are also observed. For example, within each hemisegment, *eve* is expressed in a single laterally positioned cluster of neurons (EL cluster) in *Drosophila*, and is expressed in two lateral neuronal clusters in *Schistocerca* (J. B., unpublished results). Differences in the pattern of neurons may arise due to changes in how individual neurons are specified. Alternatively, or in addition, differences in the pattern of neurons may reflect earlier alterations in NB identity. Lineage tracing studies of the median NB in *Drosophila* and *Schistocerca* demonstrate that some differences in the CNS are attributable to changes in the lineage generated by identified NBs (Bossing et al., 1994; Condron and Zinn, 1994). We have uncovered differences in gene expression between positionally homologous *Drosophila* and *Schistocerca* NBs, which may result in differences in NB lineages between insects.

We have compared early neurogenesis in *Drosophila* and *Schistocerca* using several criteria to assay CNS precursor identity: time of formation, position, and gene expression. Although the pattern of early NBs is highly conserved between insects, the pattern shows increasing dissimilarity as additional NBs delaminate. These data highlight two important differences between *Drosophila* and *Schistocerca* NBs: first, while many early NBs appear homologous between insects, the pattern of early NBs is established by different mechanisms; and second, the identities of some later-forming NBs appear to have diverged, demonstrating that evolution of the insect central nervous system can occur through changes in embryonic neuroblast identity.

We thank Michael Bastiani for *Schistocerca* embryos and the λZAP *Schistocerca* cDNA library; Yash Hiromi for the *Drosophila svp* cDNA; Fumio Matsuzki for anti-prospero; Melody Seigler and Barry Yedvobnick for the opportunity to work in their laboratories at Emory University; and Lisa Nagy, Jim Skeath and Jon Henry for comments on the manuscript. This work was supported by an NIH Systems and Integrative Biology predoctoral traineeship (J. B.) and NSF PYI and HHMI grants (C. Q. D.); C. Q. D. is an Assistant Investigator of the Howard Hughes Medical Institute.

## REFERENCES

**Bastiani, M. J., Harrelson, A. L., Snow, P. M. and Goodman, C. S.** (1987). Expression of fasciclin I and II glycoproteins on subsets of axon pathways during neuronal development in the grasshopper. *Cell* **48**, 745-755.

**Bate, C. M.** (1976). Embryogenesis of an insect nervous system I. A map of the thoracic and abdominal neuroblasts in *Locusts migratoria. J. Embryol. Exp. Morph.* **35**, 107-123.

**Bate, C. M. and Grunewald, E. B.** (1981). Embryogenesis of an insect nervous system II. A second class of neuron precursor cells and the origin of the intersegmental connectives. *J. Embryol. Exp. Morph.* **61**, 317-330.

**Bentley, D., Keshishian, H., Shankland, M. and Toroian-Raymond, A.** (1979). Quantitative staging of embryonic development of the grasshopper, *Schistocerca nitens. J. Embryol. exp. Morph.* **54**, 47-74.

**Bossing, T. and Technau, G. M.** (1994). The fate of the CNS midline progenitors in *Drosophila* as revealed by a new method for single cell labeling. *Development* **120**, 1895-1906.

**Broadus, J., Skeath, J. B., Spana, E. P., Bossing, T., Technau, G. and Doe, C. Q.** (1995). New neuroblast markers and the origin of the aCC/pCC neurons in the *Drosophila* CNS. *Mech. of Dev.*, in press.

**Campos-Ortega, J. A. and Hartenstein, V.** (1985). *The embryonic development of* Drosophila melanogaster. Berlin: Springer-Verlag.

**Chan, S-M., Xu, N., Niemeyer, C. C., Bone, J. R. and Flytzanis, C. N.** (1992). SpCOUP-TF: A sea urchin member of the steroid/thyroid hormone receptor family. *Proc. Natl. Acad. Sci. USA* **89**, 10568-10572.

**Condron, B. G., Patel, N. H. and Zinn, K.** (1994). engrailed controls glial/neuronal cell fate decisions at the midline of the central nervous system. *Neuron* **13**, 541-554.

**Condron, B. G. and Zinn, K.** (1994). The grasshopper median neuroblast is a multipotent progenitor cell that generates glia and neurons in distinct temporal phases. *J. Neurosci.* **14**, 5766-5777.

**Dawes, R., Dawson, I., Falciani, F., Tear, G. and Akam, M.** (1994). *Dax*, a locust Hox gene related to *fushi-tarazu* but showing no pair-rule expression. *Development* **120**, 1561-1572.

**Doe, C. Q.** (1992). Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. *Development* **116**, 855-863.

**Doe, C. Q., Chu-LaGraff, Q. Wright, D. M. and Scott, M. P.** (1991). The *prospero* gene specifices cell fates in the *Drosophila* central nervous system. *Cell* **65**, 451-464.

**Doe, C. Q. and Goodman, C. S.** (1985). Early events in insect neurogenesis I. Development and segmental differences in the pattern of neuronal precursor cells. *Dev. Biol.* **111**, 193-205.

**Doe, C. Q., Hiromi, Y., Gehring, W. J. and Goodman, C. S.** (1988a). Expression and function of the segmentation gene *fushi tarazu* during *Drosophila* neurogenesis. *Science* **239**, 170-175.

**Doe, C. Q., Smouse, D. and Goodman, C. S.** (1988b). Control of neuronal fate by the *Drosophila* segmentation gene *even-skipped*. *Nature* **333**, 376-378.

**Doe, C. Q. and Technau, G. M.** (1993). Identification and cell lineage of individual neural precursors in the *Drosophila* CNS. *Trends Neurosci.* **16**, 510-514.

**Fjose, A., Nornes, S., Weber, U. and Mlodzik, M.** (1993). Functional conservation of vertebrate *seven-up* related genes in neurogenesis and eye development. *EMBO J.* **12**, 1403-1414.

**Hartenstein, V., Younossi-Hartenstein, A. and Lekven, A.** (1994). Delamination and division in the *Drosophila* neurectoderm: spatiotemporal pattern, cytoskeletal dynamics, and common control by neurogenic and segment polarity genes. *Dev. Biol.* **165**, 480-499.

**Hoshizaki, D. K., Blackburn, T., Price, C., Ghosh, M., Miles, K., Ragucci, M. and Sweis, R.** (1994). Embryonic fat-cell lineage in *Drosophila melanogaster*. *Development* **120**, 2489-2499.

**Jonk, L. J. C., de Jonge, M. E. J., Pals, C. E. G. M., Wissink, S., Vervaart, J. M. A., Schoorlemmer, J. and Kruijer, W.** (1994). Cloning and expression during development of three murine members of the COUP family of nuclear orphan receptors. *Mech. Dev.* **47**, 81-97.

**Kuwada, J. Y. and Goodman, C. S.** (1985). Neuronal determination during embryonic development of the grasshopper nervous system. *Dev. Biol.* **110**, 114-126.

**Lin, D. M., Fetter, R. D., Kopczynski, C., Grenningloh, G. and Goodman, C. S.** (1994). Genetic analysis of fasciclin II in *Drosophila*: defasciculation, refasciculation, and altered fasciculation. *Neuron* **13**, 1055-1069.

**Lutz, B., Kuratani, S., Cooney, A. J., Wawersik, S., Tsai, S. Y., Eichele, G. and Tsai, M-J.** (1994). Developmental regulation of the orphan receptor *COUP-TF II* gene in spinal motor neurons. *Development* **120**, 25-36.

**Matsuzaki, F., Koizumi, K., Hama, C., Yoshioka, T. and Nabeshima, Y.** (1992). Cloning of the *Drosophila prospero* gene and its expression in ganglion mother cells. *Biochem. Biophys. Res. Comm.* **182**, 1326-1332.

**McGinnis, W., Garber, R. L., Wirz, J., Kuroiwa, A. and Gehring, W. J.** (1984). A homologous protein-coding sequence in *Drosophila* homeotic genes and its conservation in other metazoans. *Cell* **37**, 403-408.

**Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C. S. and Rubin, G. M.** (1990). The *Drosophila seven-up* gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell* **60**, 211-224.

**Patel, N. H., Kornberg, T. B. and Goodman, C. S.** (1989). Expression of *engrailed* during segmentation in grasshopper and crayfish. *Development* **107**, 201-212.

**Patel, N. H., Ball, E. E. and Goodman, C. S.** (1992). Changing role of *even-skipped* during the evolution of insect pattern formation. *Nature* **357**, 339-342.

**Patel, N. H., Condron, B. G. and Zinn, K.** (1994). Pair-rule expression patterns of even-skipped are found in both short- and long-germ beetles. *Nature* **367**, 339-342.

**Prokop, A. and Technau, G. M.** (1991). The origin of postembryonic neuroblasts in the ventral nerve cord of *Drosophila melanogaster*. *Development* **111**, 79-88.

**Sander, K.** (1976). Specification of the basic body pattern in insect embryogenesis. *Adv. Insect Physiol.* **12**, 125-238.

**Shepherd, D. and Bate, C. M.** (1990). Spatial and temporal patterns of neurogenesis in the embryo of the locust (*Schistocerca gregaria*). *Development* **108**, 83-96.

**Skeath, J. B., Panganiban, G., Selegue, J. and Carroll, S. B.** (1992). Gene regulation in two dimensions: the proneural *achaete* and *scute* genes are controlled by combination of axis-patterning genes through a common intergenic control region. *Genes Dev.* **6**, 2606-2619.

**Spana, E. P. and Doe, C. Q.** (1995). The prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in *Drosophila*. *Development* **121**, 3187-3195.

**Spana, E. P., Kopczynski, C., Goodman, C. S. and Doe, C. Q.** (1995). Asymmetric localization of numb autonomously determines sibling neuron identity in the Drosophila CNS. *Development* 121, 3489-3494.

**Thomas, J. B., Bastiani, M. J., Bate, M. and Goodman, C. S.** (1984). From grasshopper to *Drosophila*: a common plan for neuronal development. *Nature* **310**, 203-207.

**Truman, J. W., Taylor, B. J., and Awad, T. A.** (1993). Formation of the adult nervous system. In *The Development of* Drosophila melanogaster (eds. M. Bate and A. Martinez Arias), pp. 1245-1275. New York: Cold Spring Harbor Laboratory Press.

**Vaessin, H., Grell, E., Wolff, E., Bier, E., Jan, L. Y. and Jan, Y. N.** (1991). *prospero* is expressed in neuronal precursors and encodes a nuclear protein that is involved in the control of axonal outgrowth in *Drosophila*. *Cell* **67**, 941-953.

**Whitington, P. M., Leach, D. and Sandeman, R.** (1993). Evolutionary change in neural development within the arthropods: axonogenesis in the embryos of two crustaceans. *Development* **118**, 449-461.

**Zinn, K., McAllister, L. and Goodman, C. S.** (1988). Sequence and expression of fasciclin I in grasshopper and *Drosophila*. *Cell* **53**, 577-587.

(Accepted 18 September 1995)