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Neurogenesis in the insect brain: cellular identification and molecular characterization of brain neuroblasts in the grasshopper embryo

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SUMMARY

Brain neuroblasts in the embryonic grasshopper were studied by toluidine blue staining, BrdU incorporation, and immunocytochemistry in whole-mounts as well as by reconstruction of stained serial sections. Large dividing neuroblasts are observed by the 25% stage. During early neurogenesis these neuroblasts generate their progeny through mechanisms similar to those that occur in the segmental ganglia; each neuroblast divides asymmetrically to produce a chain of ganglion mother cells, and each ganglion mother cell divides symmetrically to produce a pair of neurons. Approximately 130 mitotically active, large neuroblasts are found in each brain hemisphere at the 30-45% stages. Through morphogenetic movements that occur between the 30-35% stages these neuroblasts become located in positions which are predictive of the major brain regions that they give rise to. Many of the brain neuroblasts can be identified as individuals based on their stereotyped position in the neurogenic array. Immunocytochemical experiments with antibodies against, engrailed, fasciclin I and TERM-1 show that brain neuroblasts can also be characterized by their expression of cell-specific molecular labels. These studies indicate that many features of the complex mature insect brain derive from a surprisingly simple and stereotyped set of neuronal precursor cells. Thus, many of the concepts and methods that have been used to study neurogenesis in the simpler segmental ganglia may also be applicable to the insect brain.

Key words: neurogenesis, brain neuroblasts, central nervous system, *engrailed*, fasciclin I, TERM-1, insect, embryo, *Schistocerca*

INTRODUCTION

The embryonic central nervous system of the grasshopper has been an useful model system for studying neuronal development in insects. The cell biological mechanisms of neurogenesis, axonal pathfinding and synapse formation have been analyzed in detail (for reviews see Goodman and Bate, 1981; Goodman et al., 1984; Bastiani et al., 1985; Doe et al., 1985). Insight into the molecular signals that control these developmental processes has also been gained, especially through the use of hybridoma technology (Harrelson and Goodman, 1988; Snow et al., 1988). Much of this progress has been possible because the neurons and neuronal precursors in the relatively large grasshopper embryo are individually identifiable. Furthermore, since the homologs of many of these identified neurons in the grasshopper embryo can also be found in *Drosophila*, a combined cell biological and molecular genetic analysis of neuronal development in insects has become possible (Thomas et al., 1984; Grenningloh et al., 1990; Goodman and Doe, 1993).

Most of the investigations of neurogenesis in the grasshopper central nervous system have been carried out on the accessible and easily identifiable cells in the segmental ganglia. These neurons are generated by a set of highly stereotyped and segmentally repeated neuroblasts and midline precursor cells (Bate, 1976; Goodman and Spitzer, 1979; Bate and Grunewald, 1981; Taghert et al., 1984; Doe and Goodman, 1985a). Each neuroblast divides repeatedly in asymmetrical fashion to produce ganglion mother cells. These, in turn, divide one more time to produce a pair of sibling neurons. In this manner as many as 100 neurons can be generated by each neuroblast. The identity of each neuron is largely determined by the rank of the parent ganglion mother cell in the lineage of the founding neuroblast as well as by an interaction between sibling neurons (Doe and Goodman, 1985a,b; Doe et al., 1985; Taghert and Goodman, 1984; Kuwada and Goodman, 1985).

In contrast to the wealth of knowledge about the development of the segmental ganglia, very little is known about the embryonic development of the grasshopper brain. Gross anatomical observations suggest that the neurons of the three major parts of the embryonic brain might be generated by neuroblasts in a manner comparable to that observed in the segmental ganglia (Roonwal, 1937). However, neither the number of brain neuroblasts, nor the arrangement of these neuroblasts in the three major parts of the embryonic brain, nor the cell biological mechanisms by which these neuroblasts generate their progeny are known. This lack of basic

knowledge about neurogenesis in the grasshopper brain has become a major impediment for understanding the development of this most complex part of the insect's nervous system. In this report, we characterize a set of neuroblasts that give rise to the grasshopper brain. We describe 130 large neuroblasts that are arranged in a stereotyped pattern in the protocerebrum (excluding optic lobes), deutocerebrum and tritocerebrum of each brain hemisphere in the early embryonic brain. Moreover, we provide evidence that these neuroblasts can be identified as individuals on the basis of position in the neurogenic array and expression of specific molecular labels. These studies indicate that much of the complex neuronal assembly of the mature grasshopper brain derives from a remarkably simple and stereotyped set of neuronal precursor cells.

MATERIALS AND METHODS

Animals

Schistocerca gregaria (Acrididae, Cyrtacanthacridinae) eggs were collected from a crowded laboratory culture and kept in moist aerated containers in an incubator at 30°C. Staging of the embryos of *Schistocerca* was performed according to the criteria established by Bentley et al. (1979).

Toluidine blue staining

In order to visualize neuroblasts in the living nervous system, a toluidine blue stain was used. Embryos were opened dorsally in phosphate-buffered saline (PBS) and the yolk removed. Most embryos were processed as whole mounts. Staining procedures were according to Altman and Bell (1973). After staining, the tissue was dehydrated in an increasing ethanol series (15 minutes at each concentration), cleared in xylene (1 hour) and mounted on slides in a solution of 90% glycerol and 10% PBS.

BrdU labelling

DNA synthesis in neuroblasts and their progeny was monitored by the incorporation of 5-bromodeoxyuridine (BrdU) and the subsequent immunocytochemical visualization of incorporated BrdU with a monoclonal antibody (Gratzner, 1982; Shepherd and Bate, 1990). The BrdU incubation was performed in an embryo culture system. Eggs were first cleared and sterilized in a sodium hypochlorite solution for 30-60 seconds. After washing, the embryos were dissected out of the egg and embryonic membranes into a solution of filter-sterilized Mitsuhashi and Maramorosch insect medium supplemented with NGS (normal goat serum; 10%), bovine insulin (7.8 mg/ml), Penicillin-Streptomycin (5 μl/ml) and 20-hydroxyecdysone (150 μg/l) to which BrdU had been added in quantities necessary for a final BrdU concentration of 10^{-2} M. All components of this incubation medium were from Sigma. After dissection, the embryos were staged carefully and transferred to a single drop of incubation medium in a covered plastic dish. The plastic dish was placed on an orbital shaker and incubated at 25-30°C for various periods of time ranging from 6-24 hours. After incubation, the embryos were again staged to assess growth, then fixed in Pipes-FA (100 mM Pipes, 2.0 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde; pH 6.8) for 1 hour. Following fixation, embryos were washed thoroughly in PBS for at least 1 hour with a minimum of 6 wash changes. Then, excess tissue, such as yolk and stomodeal tissue was removed in order to increase the accessibility of the nervous system to further solutions. Thereafter, the embryos were incubated in a solution of 2 N HCl in PBS for 20-30 minutes at 25-30°C and then washed thoroughly in PBS as above.

Immunocytochemistry

For BrdU immunocytochemistry, embryos were placed in a preincubation solution of 0.4% PBT (PBS plus 0.4% Triton X-100), 5% NGS and 0.2% BSA (bovine serum albumin) for 45 minutes at 25-30°C. A mouse anti-BrdU antiserum (Becton Dickinson) was added at a dilution of 1:200 and the embryos were then incubated for 24 hours (embryos younger than 35%) or 48 hours (embryos 35% and older) at 4°C with gentle agitation. Following incubation in the primary antibody, the embryos were washed thoroughly in 0.025% PBT (PBS plus 0.025% Triton X-100). The embryos were then incubated for 24 hours or 48 hours with a peroxidase-coupled goat anti-mouse secondary antibody (Jackson Immunoresearch Labs) diluted 1:250 in a solution of 0.025 % PBT, 2% NGS. Thereafter, the embryos were washed for 1 hour in PBS with several changes and stained with DAB using standard protocols (Bourne, 1983). The embryos were then cleared and mounted in a solution of 90% glycerol and 10% PBS. In some cases, the above immunocytochemical procedures were also carried out on 20 μm paraffin sections and 20 μm cryotome sections.

For immunocytochemistry with other antibodies, embryos were dissected out of the egg and embryonic membranes, carefully staged, fixed in a 3.7% Pipes-FA solution for 30 minutes, washed in PBS several times for 30 minutes and then preincubated in a solution of 0.4 % PBT, 5% NGS for 30-60 minutes. The primary antibody (cell culture supernatant) was then diluted with preincubation solution as follows: anti-engrailed (mAb 4D9) 1:2; anti-fasciclin I (mAb 3B11) 1:2; anti-TERM-1 (mAb 4G5) 1:20. Embryos were incubated in primary antibody solution for 6 hours at 25-30°C or overnight at 4°C. Following incubation in the primary antibody, the embryos were washed in PBS and then incubated with peroxidase-coupled secondary antibody as described above. They were then washed and stained with DAB using standard protocols (Bourne, 1983), with the addition of 0.02% $NiCl_2$ for peroxidase signal intensification. Immunostained preparations were cleared and mounted in a solution of 90% glycerol and 10% PBS.

Histological sectioning and reconstruction

For precise cell counts and for an accurate spatial representation of the neuroblast pattern in the developing brain, serial sections of osmium-ethyl gallate-stained embryonic brains were carried out and anatomical reconstructions performed according to Williams (1972, 1975). For this, embryos were staged, dissected and fixed for 3-12 hours in Karnovsky's fixative and then washed thoroughly in H_2O for 1 h. Subsequently, embryos were placed in a 2 % OsO_4 solution in H_2O for 24 hours and then washed again thoroughly in H_2O for 1 hour. They were then incubated for 12 hours in a saturated solution of ethyl gallate, with several solution changes, washed thoroughly in H_2O and dehydrated through an ascending ethanol series (30 minutes at each concentration). Dehydrated preparations were cleared and stored in methyl benzoate for a minimum of 12 hours. The embryos were then embedded in Epon and cut into 16 μm sections. Serial reconstructions of the sectioned material in different orientations were performed according to Pusey (1939). In order to facilitate the identification of each neuroblast, a three dimensional spatial coordinate system was used to characterize each neuroblast. The three coordinates employed were distance from the midline, anterior (rostral)-posterior (caudal) position, and depth (ventral or dorsal) relative to the neuraxis.

Visualization and documentation

Immunostained material was viewed in a Zeiss UEM compound microscope or a Zeiss Axioskop compound microscope, both equipped for phase contrast and differential interference contrast. For documentation, preparations were either photographed using differential interference contrast optics or drawn with a drawing

tube fitted to either a Wild M20 microscope (bright field) or a Zeiss UEM (differential interference contrast).

RESULTS

At early stages of neurogenesis in the grasshopper brain (25%-45%) individual neuroblasts in the developing protocerebrum, deutocerebrum and tritocerebrum can be characterized by using histological staining methods such as toluidine blue or antibody-based visualization of incorporated BrdU. During later stages of embryonic development, the growth of the brain and the large number of differentiating neurons make it more difficult to resolve and identify individual neuroblasts without the help of neuroblast-specific molecular labels. We therefore restrict our description of brain neuroblasts to the early stages of neurogenesis. Furthermore, we limit our investigation to the large asymmetrically dividing brain neuroblasts and do not report here on the plate-like aggregate of smaller symmetrically dividing neuroblasts which also contribute to the development of the grasshopper brain.

For developmental and comparative reasons all of the three-dimensional descriptions of the developing brain presented in this paper are related to the neuraxis, not to the body axis. As the adult grasshopper is hypognathal, maintaining the body axes as reference points would mean that the axes used to describe the organization of the brain would differ from those used to describe the ventral nerve cord. Moreover, since the entire central nervous system of the early embryonic grasshopper is flat, the brain axes would have to shift constantly at each subsequent developmental stage as the brain changes its attitude. We have, thus, decided to maintain the neuraxes such that the same reference points apply to the brain and the ventral nerve cord throughout development. According to this scheme, the front of the (mature) brain is neurally ventral, the top of the brain is neurally anterior, the back of the brain is neurally dorsal and the base of the brain is neurally posterior (for details see Boyan et al., 1993).

Toluidine blue staining reveals an array of neuroblasts in the early embryonic brain

Toluidine blue staining is relatively selective for neuroblasts and their progeny in the developing segmental ganglia of the grasshopper (Altman and Bell, 1973; Shepherd and Bate, 1990). In the early embryonic brain (25%-35% stages), toluidine blue staining reveals a set of conspicuous large cells with large nuclei and darkly staining cytoplasm. Fig. 1A shows such cells in the anterior part of the developing brain. Each of these large cells is closely associated with a group of smaller cells, which are arranged in a column-like fashion and which are also labelled by toluidine blue. We shall show below, that the large cells with prominent nuclei and darkly staining cytoplasm are asymmetrically dividing brain neuroblasts, and that the smaller associated cells are their progeny.

At the 30% stage an ordered array of these toluidine blue-stained neuroblasts is seen throughout the developing brain. Approximately 130 large cells with darkly stained cytoplasm are observed in the anlage of each brain hemisphere at this stage. All are located near the ventral surface of the embryonic brain where they form a bilaterally symmetric, multi-lobed aggregate. Through morphogenetic

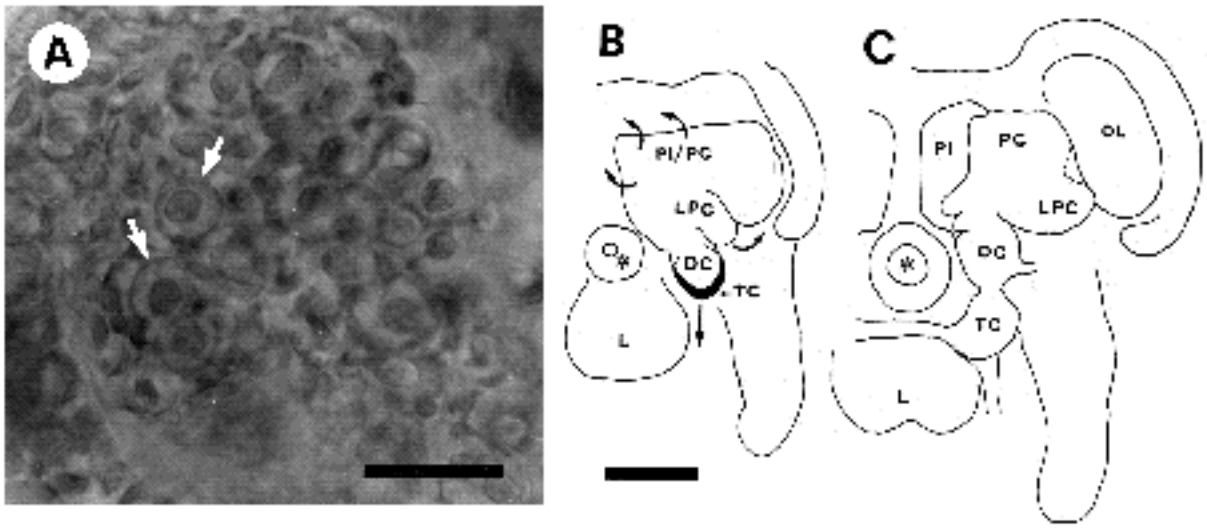

Fig. 1. (A) Embryonic brain stained with toluidine blue to visualize neuroblasts. Whole-mount preparations. At the beginning of the 25% stage of embryogenesis, large toluidine blue-positive neuroblasts with conspicuous, darkly staining nuclei are visible. Several of these (white arrows) can be seen in this single focal plane of the anterior part of the developing brain. (B,C) Morphogenetic movements of the neuroblast array in the early embryonic brain. Drawings from 30% (A) and 35% (B) stages of embryogenesis show the spatial arrangement of the principle subdivisions of the developing brain as seen from a ventral aspect. Only the right half of the brain is shown. Arrows indicate the directions of major morphogenetic movements of groups of neuroblasts that occur between the 30% and 35% stage. At the 30% stage, the neuroblasts of the pars intercerebralis and the main protocerebrum are still grouped together. Asterisk indicates the developing gut. Scale bars: A, 80 μm; B,C, 200 μm. In this and all subsequent figures the following abbreviations are used: PI, pars intercerebralis; PC, main protocerebrum, LPC, lateral protocerebrum; DC, deutocerebrum; TC, tritocerebrum; OL, optic lobe; L, labrum.

movements that occur between the 30% and the 35% stages, these neuroblasts become located in positions that are predictive of the major brain regions that they subsequently give rise to. This is shown schematically in Fig. 1B,C. In this process, the neuroblasts that will give rise to the pars intercerebralis and the protocerebrum proper move in an anterior-medial direction. As a consequence of this, the neuroblasts that were at the anterior-medial edge of the multilobed aggregate are displaced dorsally and fold over the main mass of ventrally located cells. In this way the group of cells that will generate the pars intercerebralis comes to lie near the dorsal surface of the anterior-medial part of the developing brain anlage, while the neuroblasts that contribute to the protocerebrum proper remain ventrally. The group of neuroblasts that generates the lateral protocerebrum moves in a lateral and slightly posterior direction. As a result, this cell group makes contact with the proximal part of the developing optic lobes. Medial and posterior to this cell group is the neuroblast group that gives rise to the deutocerebrum. The neuroblasts that give rise to the tritocerebrum begin to move ventrally and posteriorly along the neural axis. They come to lie posterior to the deutocerebral cells in a position appropriate for the mature tritocerebrum.

These morphogenetic movements of the neuroblasts in the brain anlage may be due in part to the on-going enlargement and medial curvature of the developing optic lobes. However, it is certainly also due to enlargement and differentiation of the columns of neuronal progeny as well as the various neuropil areas that begin to form in the embryonic brain. Moreover, the rapidly increasing size of the foregut at this stage may be partially responsible for displacing the tritocerebral cells. Morphogenetic movements of cells in the brain anlage continue until at least the 70% stage, when the first signs of neuroblast degeneration appear. However, by the 35% stage the protocerebral, deutocerebral and tritocerebral neuroblasts are already arranged along the neuraxis, the neuroblast group of the lateral protocerebrum has extended laterally and the pars intercerebralis neuroblasts have become located dorsomedially in the developing brain.

Analysis of mitotic activity in brain neuroblasts by BrdU incorporation

In order to investigate the mitotic activity of the large brain neuroblasts, we monitored DNA synthesis using the incorporation of the substituted nucleotide BrdU. For this, the embryos at stages 30%-55% were incubated for various time periods in a culture medium containing BrdU, then fixed, staged and processed for immunocytochemistry to reveal the cells that incorporated BrdU. In preparations that incorporated BrdU for 24 hours, most of the nuclei of the large neuroblasts are labelled (Fig. 2A,B). In addition to the neuroblasts, column-like clusters of smaller cells associated with each neuroblast are also labelled (Fig. 2C). Indeed, labelled neuroblasts that are not associated with a cluster of smaller labelled cells were never observed. A close inspection of the size, number and arrangement of the labelled cells suggests that the cluster of smaller cells which are associated with each neuroblast represent the progeny of that neuroblast. By shortening the duration of BrdU incorporation, and thus labelling only a subset of the neuroblasts and their progeny, it was possible to study the mechanisms by which the labelled neuroblasts produce their progeny.

The large labelled neuroblasts produce ganglion mother cells through a series of unequal cell divisions. Fig. 3A shows one of these neuroblasts in the process of cell division. Both the neuroblast's nucleus and the nucleus of the nascent ganglion mother cell are labelled but final cell division has not yet occurred. In Fig. 3B the process of cell division has taken place, producing labelled neuroblasts with their associated ganglion mother cells. Fig. 3C and D show labelled neuroblasts that have sequentially generated

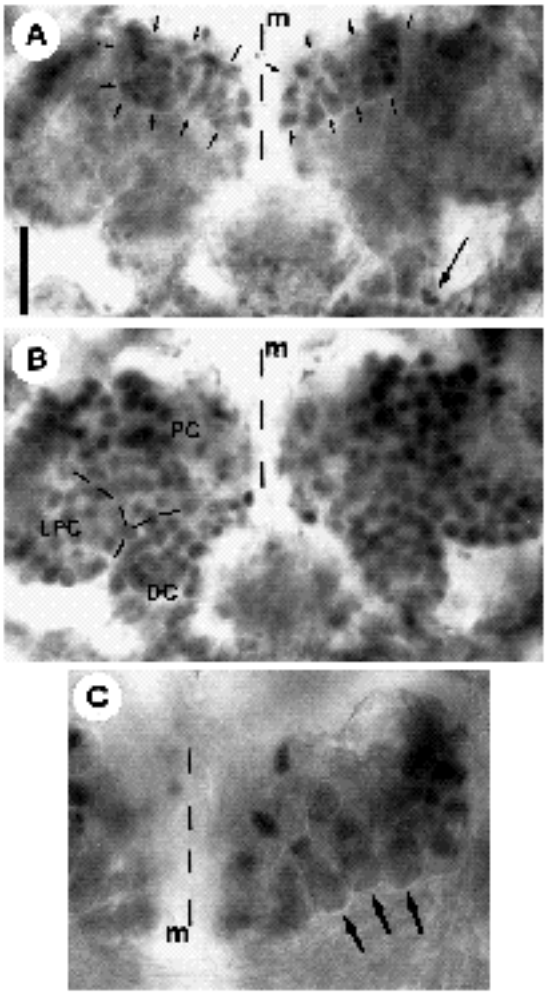

Fig. 2. Whole-mount preparations of the embryonic brain labelled with BrdU to visualize neuroblasts. (A) Total view of the neuroblasts and progeny in the pars intercerebralis. A dorsal focal plane is shown; small arrows deliminate the dorsal neurogenic region of the pars intercerebralis. Large arrow indicates dorsally located neuroblasts of the tritocerebrum. 35% stage; m: midline. (B) Same preparation as in A, with focus on the ventral neurogenic layer showing the majority of the ventral neuroblasts labelled with BrdU. Main protocerebrum, lateral protocerebrum, deutocerebrum as well as their borders are indicated. m: midline of the brain. (C) Columnar arrangement (arrows) of the progeny of neuroblasts in part of the pars intercerebralis region of the developing brain. m: midline of the brain. 35% stage. A and C are from different preparations. Scale bars: A,B, 100 μm; C, 50 μm.

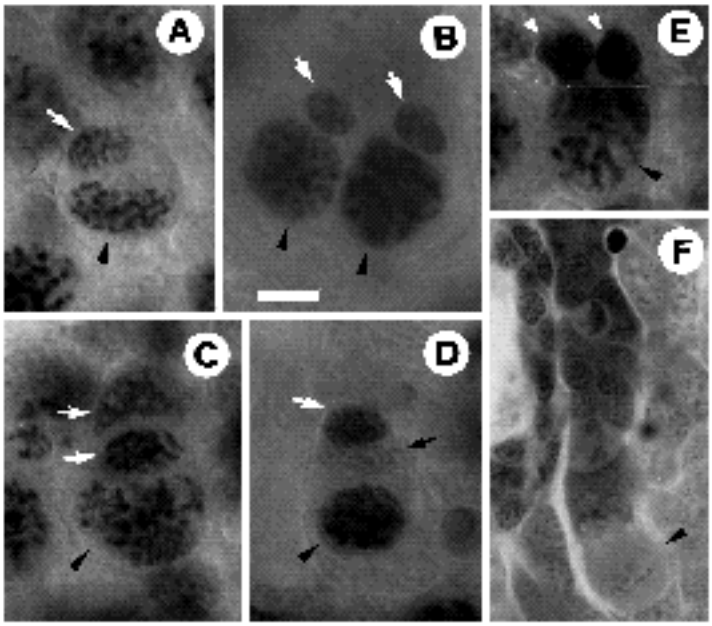

Fig. 3. Mitotic activity of neuroblasts and ganglion mother cells as seen in whole-mount preparations of 35%-40% stage embryos (A-E) and 70% stage embryos (F). (A,B) Unequal cell division of large neuroblasts gives rise to ganglion mother cells. In A, the BrdU-labelled cell nucleus in a mitotically active neuroblast has divided asymmetrically, but cell division has not yet been completed. In consequence a large nucleus (black arrowhead) and a smaller nucleus (white arrow) are still found within the same cell. In B each of two adjacent BrdU-labelled neuroblasts have divided asymmetrically and given rise to a ganglion mother cell through unequal cell division. Black arrowheads indicate the large nucleus in each of the neuroblasts and white arrows indicate the smaller cell nucleus in each of the ganglion mother cells. (C,D) Mitotically active neuroblasts generate rows of ganglion mother cells. In C the BrdU-labelled neuroblast (black arrowhead) has divided asymmetrically two times and produced a group of two ganglion mother cells (white arrows) both of which are also BrdU labelled. In D the neuroblast (black arrowhead) has also produced a group of two ganglion mother cells. Note that the first-born of the two ganglion mother cells (white arrow) has incorporated considerably more BrdU than the second-born gangion mother cell (black arrow). This indicates that the first-born ganglion mother cell has carried out significant DNA synthesis in preparation for future mitotic activity. (E) A pair of neurons are produced by symmetrical cell division of a ganglion mother cell. The BrdU-labelled neuroblast (black arrowhead) has given rise to a ganglion mother cell, which in turn has carried out DNA synthesis and then divided once to produce two BrdU-labelled neurons (white arrowheads). (F) Columnar arrangement of neuroblast progeny. Sectioned, osmium-ethyl gallate-stained material. A neuroblast (arrowhead) has produced a columnar aggregate of ganglion mother cells, most of which have already divided symmetrically to produce pairs of associated neurons. Scale bar, 10 μm.

two ganglion mother cells, which are arranged in a columnar fashion. In Fig. 3D the neuroblast as well as the more distal of the two ganglion mother cells have carried out DNA synthesis during the period of BrdU labelling, indicating the future mitotic activity of both the neuroblast and the labelled ganglion mother cell. Each ganglion mother cell divides once to produce neuronal progeny. Fig. 3E shows the pair of labelled ganglion cells (neurons) that result from an equal division of the first ganglion mother cell of the parent neuroblast. Since each neuroblast divides repeatedly to produce numerous ganglion mother cells, which then each produce a pair of neurons, this general type of neurogenic mechanism often leads to the formation of long rows or columns of neuron pairs that are still in close association with their parent neuroblast (Fig. 3F). Most of our observations were made on numerous unidentified neuroblasts, ganglion mother cells and neurons from all major brain regions. In order to confirm these observations on identified cells, we followed the general sequence of neurogenesis events explicitly for a pair of identified brain neurons, the TERM-1-expressing neurons of the pars intercerebralis (Meier et al., 1993). Using an anti-TERM-1 monoclonal antibody as a molecular label, we found that these two interneurons are generated by equal cell division of the first ganglion mother cell that is produced through unequal cell division of pars intercerebralis neuroblast number 2 (see Fig. 8 for neuroblast identification). In summary, these results indicate that each parent neuroblast produces a variable number of ganglion mother cells through a series of asymmetrical divisions and that each ganglion mother cell divides once symmetrically to produce two sibling cells that differentiate into neurons. This is very similar to the process of neurogenesis in the segmental ganglia (Bate, 1976; Goodman and Spitzer, 1979; Doe and Goodman, 1985a).

The morphological identification of individual neuroblasts in the early embryonic brain

Although toluidine blue staining and BrdU incorporation in whole-mount reveal most of the large neuroblasts in the early embryonic brain, neither method is suitable for a precise cell count or an accurate three dimensional localization of all of the large brain neuroblasts. Toluidine blue staining in whole-mounts does not reveal all of the brain neuroblasts, particularly in later stages of development when the relative size of the neuroblasts decreases compared to their enlarging progeny. The BrdU labelling technique shows inconsistencies in whole-mount, especially in the degree of penetration of the anti-BrdU and secondary antibodies into the tissue. Thus, in order to construct an accurate map of the large neuroblasts in the early embryonic brain, we carried out and analysed serial sections of osmium-ethyl gallate-stained preparations.

Fig. 4 shows examples of such serial sections. At early stages of embryonic development (30%-45%), the neuroblasts are easily identified in these sections as the prominent cells with large polymorphic nuclei and darkly stained cytoplasms. The neuroblasts lie close to the brain surface and give rise to columnar aggregates of neuronal progeny (Fig. 4A). Often the neuroblasts and their progeny in a given part of the brain such as the deutocerebrum are clearly segre-

gated from the neuroblasts in the other main parts of the brain (Fig 4B). This is probably due to the non-neuronal support cells that delimit the developing different major regions of the brain. After identification of the neuroblasts in the serial sections, a three dimensional coordinate system was used to reconstruct carefully the overall pattern of individual brain neuroblasts in a brain hemisphere. Seven reconstructions of serial sections were made from 45% stages. For comparative reasons, one reconstruction was also made from a 30% stage and one reconstruction was made from a 70% stage.

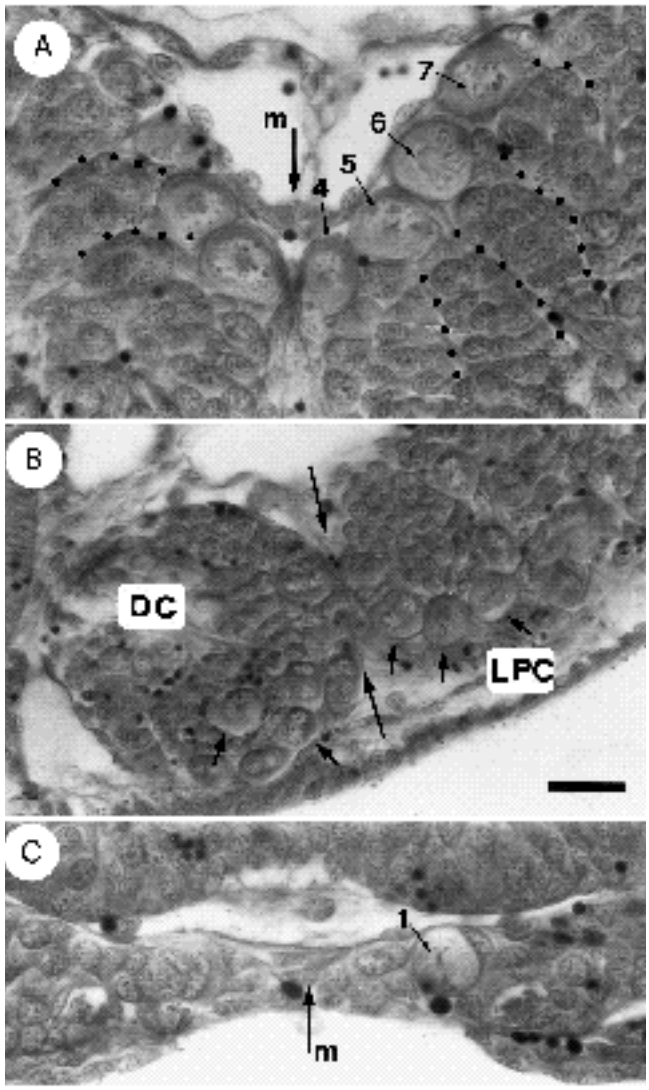

Fig. 4. Serial sections of the embryonic brain. Sections taken at three different levels in an anterior-to-posterior sequence along the neuraxis. Osmium-elthyl gallate-stained 16 μm sections of a 45% stage embryonic brain show neuroblasts as conspicious cells with large polymorphic nuclei. (A) Section through the pars intercerebralis region showing the neuroblasts 4, 5, 6, and 7 (for numbering of neuroblasts see the general mapping scheme given in Fig. 9). The brain midline (m) is indicated by a long arrow. Dotted lines delimit the columnar array of progeny produced by a given neuroblast. (B) Section showing the developing deutocerebrum and lateral protocerebrum in the right half of the brain. The transition zone between the two groups of neuroblasts is flanked by long arrows. Several ventrally located neuroblasts can be seen in this section and some are indicated by short arrows. (C) Section through the posterior part of the tritocerebrum. An identified posterior neuroblast of the tritocerebrum (1, small arrow) is located near the midline (m, long arrow). Scale bar: 25 μm.

At the 45% stage of embryonic development, the protocerebrum, deutocerebrum and tritocerebrum are already visible as discrete structures. In four of the reconstructions at this stage, a total of 130 large brain neuroblasts was found. In the other 3 reconstructions a total of 132 large brain neuroblasts was found. We do not know the reason for this discrepancy. It is unlikely to be due to counting errors since repeated reconstructions from the same preparation always yielded the same number of neuroblasts. It is possible that the difference in neuroblast number is due to sexual dimorphisms. However, we did not investigate this further since it is not possible to determine the sex of early embryonic grasshoppers. In the following we limit our description to the 130 brain neuroblasts that were found in all of the embryonic preparations.

Fig. 5A shows a schematic representation of the relative position of the 130 neuroblasts in a brain hemisphere as viewed from a ventral aspect. To reduce overlap, the pars intercerebralis neuroblasts, most of which are dorsally located, are shown separately from the anterior and ventrally located neuroblasts of the protocerebrum. At this stage, each neuroblast can be attributed to either pars intercerebralis, main protocerebrum, lateral protocerebrum, deutocerebrum or tritocerebrum. The main (medial and anterior) protocerebrum contains 41 neuroblasts. The lateral protocerebrum has 25 neuroblasts, two of which are located dorsally and the rest ventrally in the developing brain. The deutocerebrum has 32 neuroblasts. The tritocerebrum contains 12 neuroblasts, one of which is isolated from the rest and is always found at the site of exit of the tritocerebral commissure near the midline (see also Fig. 4C). The pars intercerebralis contains 20 neuroblasts; these are arranged in several groups and have a complex spatial distribution.

For a more realistic representation of the arrangement of the 130 large brain neuroblasts of the 45% stage embryo, serial sections were also reconstructed to produce the two ‘half-brain’ views shown in Fig. 5B. In this figure the embryonic brain is represented as split into an anterior and a posterior part and then viewed from a posterior aspect. Most of the brain neuroblasts are included in this representation, however, a few neuroblasts are not shown since they are occluded by other neuroblasts. In the anterior ‘half’ of the brain the pars intercerebralis neuroblasts are found in a dorsal-medial layer. The neuroblasts of the main protocerebrum are located mainly in a ventral-lateral aggregate. In the posterior ‘half’ of the brain, the majority of the neuroblasts of the lateral protocerebrum are grouped in a lateral and ventral cluster; two cell pairs are located dorsal-laterally. The deutocerebral neuroblasts are found more medially in a ventral crescent-shaped aggregate. Most of the tritocerebral neuroblasts lie in a lateral cluster, however, a single pair of tritocerebral neuroblasts is found more medially near the tritocerebral commissure.

In contrast to the 45% stage, at the 30% stage of embryonic development all of the brain neuroblasts are

still closely packed. They form a curved, sheet-like structure in which the neuroblasts of the pars intercerebralis have not yet moved to occupy their final position near the dorsal surface of the developing brain. Moreover, the neuroblasts of the deutocerebrum and the tritocerebrum have not yet moved apart along the anterior-posterior axis. Despite the more compact nature of the brain neuroblast aggregate at this stage, a reconstruction of serial sections through a brain hemisphere at the 30% stage was possible and revealed a total of 130 large brain neuroblasts.

At the 70% stage of embryonic development the complexity of the brain has increased enormously as compared to the 45% stage. The brain has enlarged greatly due to the large number of neurons that have been generated. Numerous axon fascicles and axon commissures have formed and prominent neuropil areas have developed. Accordingly it is much more difficult to carry out accurate reconstruction of all of the brain neuroblasts. Nevertheless, in the reconstruction from a 70% stage we found a total of 127 large brain neuroblasts in a brain hemisphere. Clear signs of degeneration (see Bate, 1976) were seen in two of these neuroblasts.

In serially sectioned material of early embryonic stages (30%-45%), many of the brain neuroblasts can be identified as individuals on the basis of their three dimensional spatial coordinates. However, this is not a practical identification method since it involves the serial sectioning of fixed and stained material as well as the time-consuming and neuroanatomically demanding spatial reconstruction of these sections. Fortunately, at the 45% stage a number of brain neuroblasts can be unambiguously identified in whole-mount preparations on the basis of their position (Figs 5A, 9). This is possible for most of the neuroblasts in the pars intercerebralis and the tritocerebrum and for many of the neuroblasts in the lateral protocerebrum. In the protocerebrum proper and the deutocerebrum some of the more peripherally located neuroblasts can be identified in whole-mount on the basis of their stereotyped position. However, for many of the neuroblasts in these two brain regions other

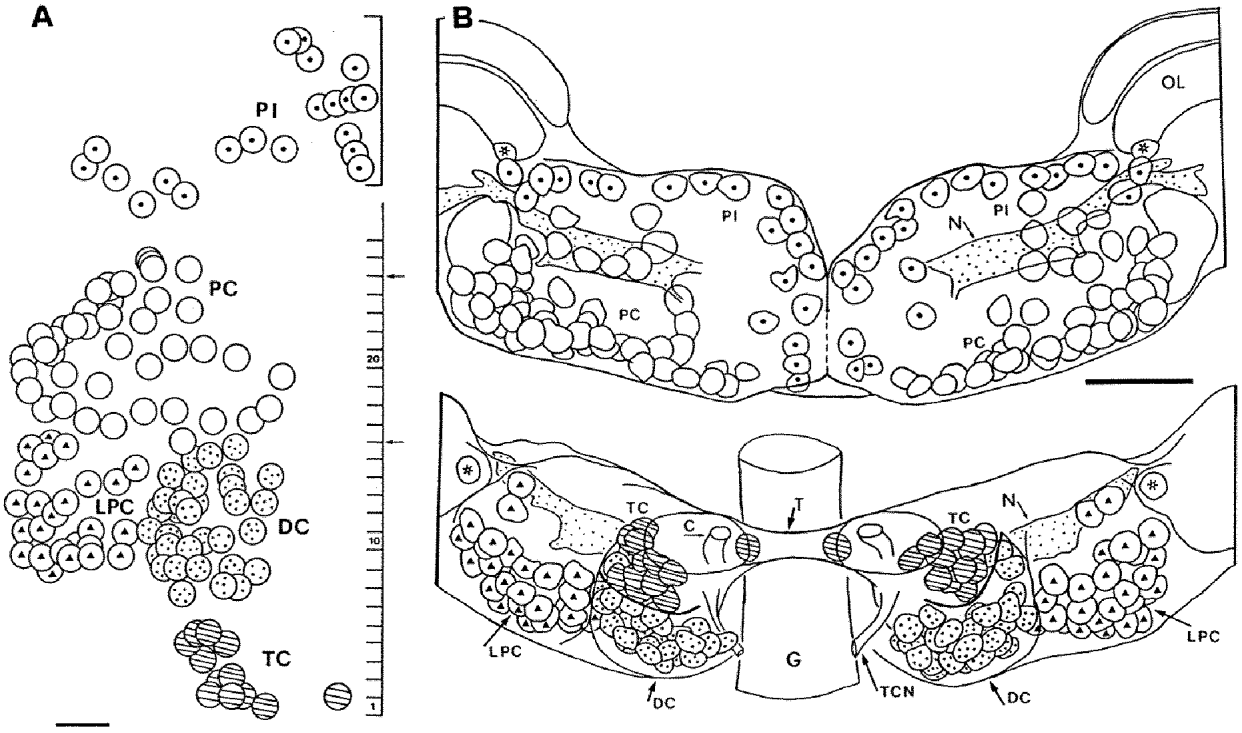

Fig. 5. Reconstructions of serial sections from a 45% stage embryonic brain. (A) The full complement of 130 neuroblasts is shown schematically from a ventral perspective. Each neuroblast is represented by a circle of standard size whose center corresponds to the actual cell center of the neuroblast in situ. The numbered scale to the right of the ventral neuroblasts indicates the planes of serial sectioning. For reasons of clarity and to reduce overlap, the pars intercerebralis neuroblasts are shown separately from the other neuroblasts. The position of the cluster of pars intercerebralis neuroblasts relative to the ventral neuroblasts can be derived by matching the bracket to the right of the pars intercerebralis neuroblasts to the sites indicated by the two arrows. Neuroblasts of the pars intercerebralis are labelled with dots, neuroblasts of the deutocerebrum are stippled, neuroblasts of the lateral protocerebrum are labelled with triangles, neuroblasts of the tritocerebrum are striped, and neuroblasts of the main protocerebrum are left blank. (B) More realistic representation of the brain neuroblasts. Reconstructed frontal brain bisection showing the brain separated into an anterior (top) and posterior (bottom) block, both viewed from a posterior aspect (e.g. as one looks anteriorly along the circumesophageal connectives). The anterior block is a reconstruction from 10 consecutive 16 μm serial sections. The posterior block is a reconstruction from 15 consecutive 16 μm serial sections. N, developing neuropile; C, circumesophageal connectives; G, gut; T, tritocerebral commissure; TCN, tritocerebral nerve; asterisks, optic lobe neuroblasts. Scale bars: A, 50 μm; B, 100 μm.

criteria for positive identification in whole-mount are needed.

Molecular characterization of neuroblasts in the early embryonic brain

Studies on neurogenesis in the segmental ganglia of insects indicate that clusters of neuroblasts and neurons can be identified in part on the basis of their patterns of gene expression. For example, a subset of neuroblasts, ganglion mother cells and neurons in each of the segmental ganglia of the grasshopper express *engrailed* protein (Patel et al., 1989a,b). Moreover, a group of neuroblasts in the segmental ganglia of *Drosophila* express fasciclin I protein (Snow et al., 1988; McAllister et al., 1992). In order to determine if the expression pattern of proteins such as these might be useful for the identification of individual brain neuroblasts, we carried out immunocytochemical studies using monoclonal antibodies against engrailed and fasciclin I as well as a monoclonal antibody against TERM-1, a brain interneuron specific molecular label (Meier et al., 1993). A total of 25 preparations were characterized with the antibody against engrailed, a total of 70 preparations were characterized with

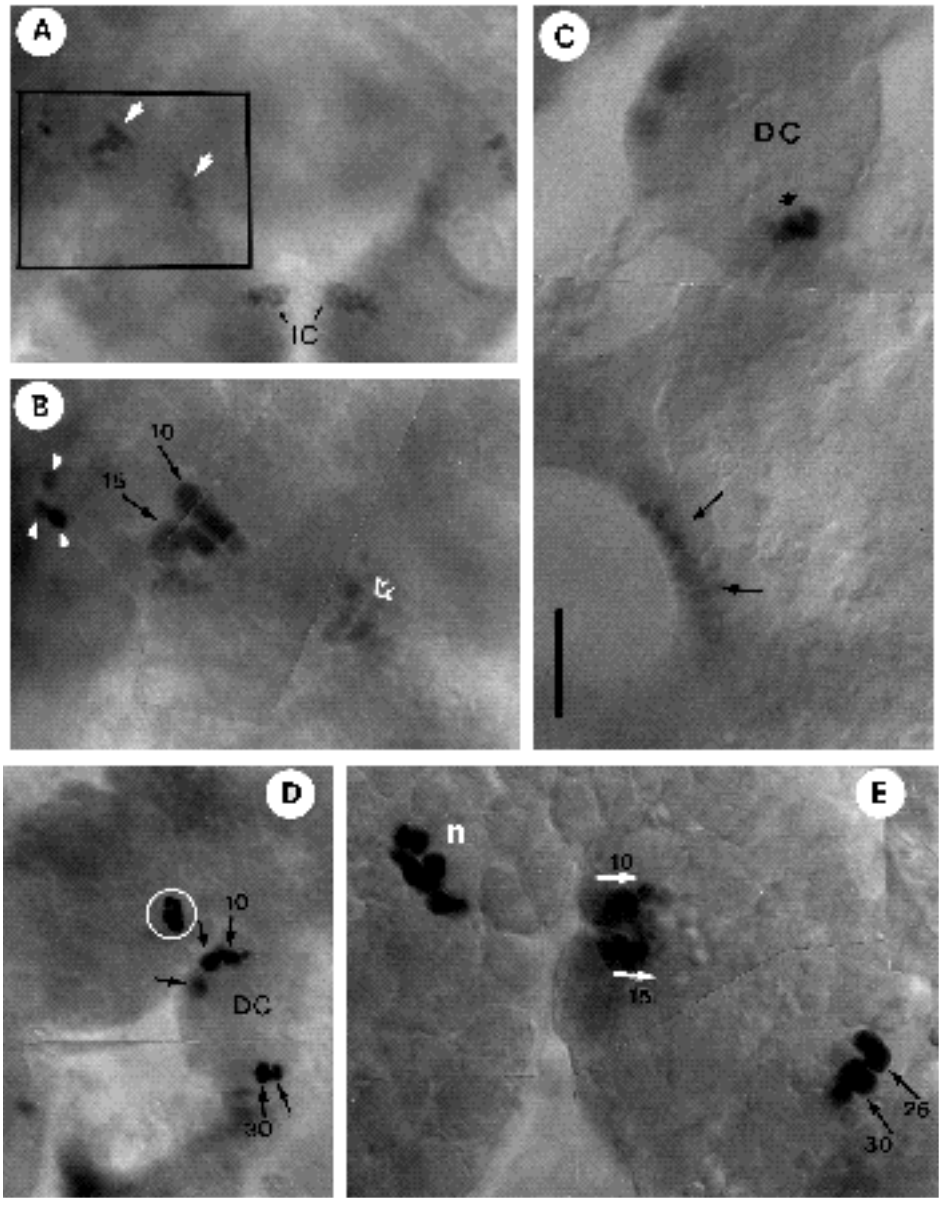

Fig. 6. Whole-mount preparations stained with anti-engrailed antibody. 30% (A,B), 33% (C), and 35% (D,E) stages. In B-E only the left half of the brain is shown. (A) Four groups of engrailed-positive cells are seen. Two of these groups are located in the deutocerebrum (arrows). A third group is located anterior and lateral to these in the lateral protocerebrum. The fourth group of cells is located at the posterior border of the intercalary segment (IC); they are non-neuronal and are not further considered here. (B) Higher magnification of the area in the rectangle in A. Arrowheads show three labelled neurons in the lateral protocerebrum. Arrows indicate two of the deutocerebral neuroblasts with their progeny. For numbering of neuroblasts see the general mapping scheme given in Fig. 9. Adjacent to neuroblast 15 and its progeny, several labelled neurons are seen, which derive from neuroblast 20. At this stage, however, neuroblast 20 is only weakly engrailed-positive. Open arrow shows the second group of weakly engrailed-positive cells in the deutocerebrum. It is not clear if these cells are related to the two engrailed-positive neuroblasts that are observed at this medial-posterior site slightly later (see C, D). (C) Thick arrow indicates a group of two neuroblasts (26, 30) and their progeny at the medial-posterior site of the deutocerebrum, that are now engrailed-positive. Thin arrows show a group of non-neuronal, transiently engrailed-positive cells at the base of the developing antenna. (D) The full complement of engrailed-positive neuroblasts and neurons in the lateral protocerebrum and deutocerebrum. In the lateral protocerebrum, the cluster of engrailed-positive neurons that are not associated with a labelled neuroblast are encircled. Arrows show the five neuroblasts with their progeny in the deutocerebrum; neuroblasts 10 and 30 are labelled as reference points. (E) Higher magnification of the same brain region. engrailed-positive neurons in the lateral protocerebrum are labelled with an n. White arrows show the direction of the columnar arrangement of engrailed-positive progeny of neuroblasts 10 and 15 in the deutocerebrum. Black arrows indicate *engrailed*-positive deutocerebral neuroblasts 26 and 30. D and E are from different preparations. Scale bar: A,D, 100 μm; B,C,E, 200 μm.

the antibody against fasciclin I, and over 100 preparations were characterized with the antibody against TERM-1.

The expression pattern of engrailed is shown in Fig. 6. The *engrailed* protein is expressed by five deutocerebral neuroblasts and their progeny in each brain hemisphere. Three of these are located anteriolaterally near the border of the protocerebrum (Fig. 6A, B, D). The two others are found posterior-medially (Fig. 6C, D, E). All five can be individually identified on the basis of their position as well as their engrailed expression. They have, in consequence, been numbered according to the general mapping scheme given in Fig. 9. The expression of engrailed in two of the neuroblasts in the anterior-lateral group precedes that in the other neuroblasts in the group, but by 35% all five neuroblasts are intensely stained by the anti-engrailed antibody. The progeny of these neuroblasts are arranged in the columnar manner, which is typical for ganglion mother cells and neurons (Fig. 6E). In each lateral protocerebrum, three engrailed-expressing neurons are observed at the 30% stage. Their neuroblast of origin does not become engrailed-positive. The number of these engrailed-expressing neurons increases further during embryonic development. (Fig. 6B, D, E). A group of non-neuronal ectodermal cells in the intercalary segment as well as a group of non-neuronal cells around the border of the developing antenna also express engrailed protein (Fig. 6A, C). These non-neuronal cells have been described (Patel et al., 1989b; Cohen and Jürgens, 1991).

The expression pattern of fasciclin I is shown in Fig. 7. In each pars intercerebralis, five neuroblasts express fasciclin I. Three are found along the medial edge of the pars intercerebralis (Fig. 7B, C). The two others, which express fasciclin I slightly later, are more posterior-lateral (Fig. 7D, E). Six neuroblasts in the deutocerebrum express fasciclin I (Fig. 7B,

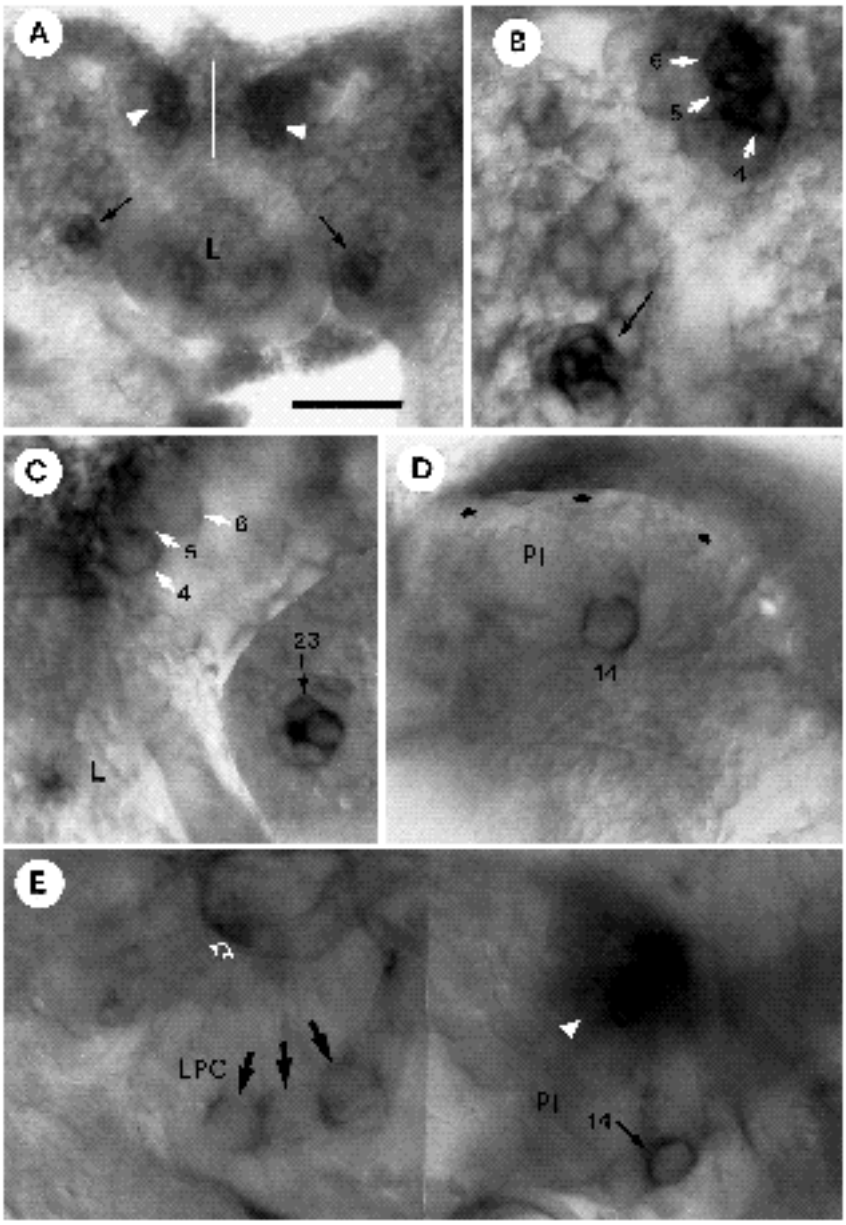

Fig. 7. Whole-mount preparations stained with anti-fasciclin I antibody. 30 % (A,B) and 35% (C-E) stages. In B,D,E the left part and in C the right part of the brain is shown. (A) Fasciclin I-positive neuroblasts and their progeny in the developing pars intercerebralis (white arrowheads) and deuterocerebrum (black arrows). White line, brain midline; L, labrum. (B) Higher magnification of the same preparation, only left hemisphere is shown. White arrows point at the fasciclin I-positive neuroblasts 4, 5 and 6 in the pars intercerebralis. For numbering of neuroblasts see the general mapping scheme given in Fig. 9. Black arrow indicates a tightly packed group of 6 neuroblasts in the deutocerebrum. (C) At the 35% stage, pars intercerebralis neuroblasts 4, 5 and 6 (white arrows) are still fasciclin I-positive. However, they do not stain as intensely as the fasciclin I-expressing neuroblasts in the deutocerebrum. The central neuroblast in this group of six cells is deutocerebral neuroblast 23. Only the right part of the brain is shown. (D) The fasciclin I-expressing neuroblast 14 of the pars intercerebralis at the 35% stage. Arrows delimit the anterior margin of the pars intercerebralis. Fasciclin I expression in the neighboring neuroblast 13 is generally much weaker than in neuroblast 14. (E) Left hemisphere of a 35 % stage embryo. In addition to neuroblast 14 (thin black arrow) of the pars intercerebralis, three neuroblasts (thick black arrows) at the posterior boundary of the lateral protocerebrum express fasciclin I. Additional fascilin I staining is due to an complex array of labelled fiber bundles (white arrow); some of these lie out of the focal plane (white arrowhead). Scale bars: A, 100 μm; B-E: 200 μm.

C). Five of these are clustered around the sixth, central neuroblast. This central neuroblast expresses fasciclin I intensely all along its cell surface; the five others have the strongest fasciclin I expression on the part of their cell surface that faces the central neuroblast. All fasciclin I-expressing neuroblasts in the pars intercerebralis and the deutocerebrum can be identified as individuals on the basis of their position as well as their specific gene expression. They have, thus, also been assigned an identifying number according to the general mapping scheme given in Fig. 9. In the lateral protocerebrum, three neuroblasts express fasciclin I (Fig. 7E), and in the main protocerebrum, four neuroblasts express fasciclin I. In both of these cases the fasciclin I-positive neuroblasts can be localized to a small set of anatomically identified neuroblasts. However, we have not been able to determine with precision which of the identified neuroblasts in these sets are the ones that express the protein. In addition to neuroblasts, a number of neurons and axon fascicles in the developing brain also express fasciclin I (Fig. 7E).

The TERM-1 glycoprotein is intensely expressed on the developing growth cones and terminal arbors of two identified brain interneurons. These interneurons both derive from the same neuroblast in the pars intercerebralis (Meier et al., 1993). Interestingly, TERM-1 is also transiently expressed by the neuroblast of origin of the TERM-1-positive interneurons (Fig. 8). This pars intercerebralis neuroblast can be identified on the basis of its position as well as TERM-1 expression. It is the only TERM-1-expressing neuroblast in the brain hemisphere.

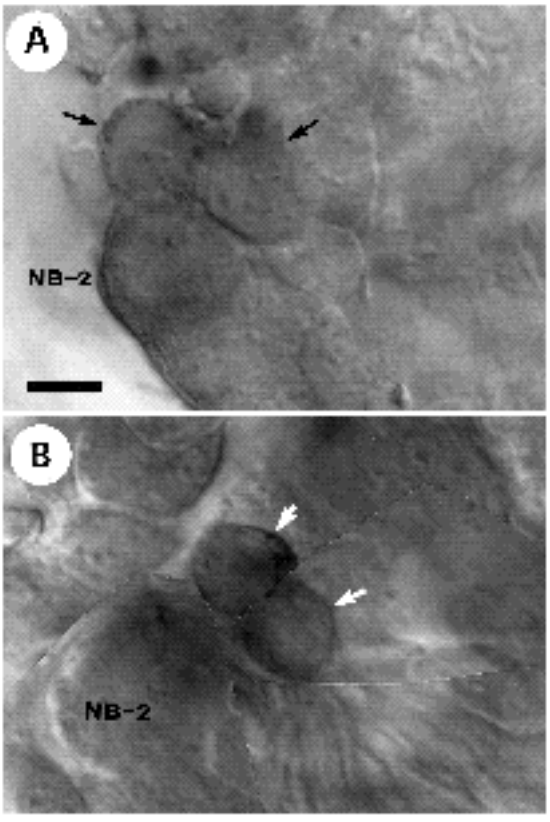

Fig. 8. Whole-mount preparations stained with anti-TERM-1. Right side of the brain. (A) One pair of interneurons (arrows) and their neuroblast of origin show TERM-1 immunoreactivity at the 35% stage. The labelled neuroblast is identified as neuroblast 2 of the pars intercerebralis according to the general mapping scheme given in Fig. 9. (B) At a slightly later stage, only the two sister-interneurons (white arrows) clearly express the TERM-1 protein. Their neuroblast of origin does not show strong immunoreactivity. Thus, TERM-1 expression in this neuroblast is transient. A and B are from different preparations. Scale bar: 10 μm.

Figs 9 and 10 show a summary mapping scheme that combines the data obtained from serial reconstructions and whole-mount BrdU preparations with that of the molecular characterization of brain neuroblasts. This scheme shows the relative position of the large brain neuroblasts in a 45% embryo from an idealized ventral perspective, with dorsal and ventral neuroblasts compressed into the same plane. In order to minimize overlap, some of the neuroblasts have been moved apart slightly, with care taken to preserve nearest neighbor relationships. The neuroblasts in each major part of the brain (pars intercerebralis, main protocerebrum, lateral protocerebrum, deutocerebrum, tritocerebrum) are numbered separately (Fig. 9). Whenever possible the sequence of numbering follows the row-like arrangements of neuroblasts. Members of three optic neuroblasts are shown as anatomical reference points but are not numbered. Based on this structural identification of neuroblasts, we have obtained the following molecular phenotyping of individual neuroblasts (Fig. 10). Neuroblasts 10, 15, 20, 26, 30 in the deutocerebrum express the *engrailed* protein. Neuroblasts 4, 5, 6, 13, 14 in the pars intercerebralis as well as neuroblasts 14, 17, 18, 22, 23, 24 in the deutocerebrum express fasciclin I. Fasciclin I is also expressed by four of the neuroblasts among the neuroblasts 10, 11, 16, 17, 23, 24, 25, 26 in the protocerebrum and by three of the neuroblasts among the neuroblasts 20, 21, 22, 23, 24 in the lateral protocerebrum. TERM-1 is expressed by neuroblast 2 in the pars intercerebralis.

DISCUSSION

Mechanisms of early neurogenesis in the brain and in the segmental ganglia

In recent years, investigations on insect neurogenesis have become increasingly important for analyzing the cellular and molecular mechanisms involved in neuroblast formation, neuroblast lineage, and neuronal specification (Goodman et al., 1984; Campos-Ortega and Knust, 1990; Cabrera, 1992; Goodman and Doe, 1993). Virtually all of this work has, however, been carried out on the segmental ganglia or the peripheral nervous system of insects. In consequence, much less is known about the development of the insect brain. In this study we have analyzed neurogenesis in the early embryonic brain of the grasshopper. On the basis of the results presented here, a direct comparison of some of the mechanisms of early neurogenesis in the complex brain and in the much simpler segmental ganglia is now possible.

The total number of large neuroblasts in the early embryonic brain is surprisingly small. It is not much larger than the total number of neuroblasts and midline precursors found in the three thoracic ganglia, for example (Bate, 1976;

Doe and Goodman, 1985a; Shepherd and Bate, 1990). The relative position of individual large neuroblasts in the overall neurogenic array also appears to be relatively constant in the brain from one individual to the next. In consequence, in the early embryonic brain, as in the segmental ganglia, many of the individual neuroblasts can be identified on the basis of their position (Bate, 1976; Doe and Goodman, 1985a). The cellular mechanisms by which a neuroblast gives rise to ganglion mother cells and neurons appear to be similar in the early embryonic brain and in the segmental ganglia (Goodman and Spitzer, 1979; Taghert et al., 1984; Taghert and Goodman, 1984; Raper et al., 1984). Studies on neurons in the ventral ganglia in a number of insect preparations indicate that individual neuroblasts have an invariant cell lineage at least as far as the formation of the first few ganglion mother cells and their progeny is concerned. The cell lineage of the TERM-1-expressing interneurons of the pars intercerebralis has been studied explicitly and shown also to be invariant. If this is indeed a more general phenomenon in the developing brain, then the question of how neuronal diversity in the brain is generated can be reduced, at least in part, to the question of how a given neuroblast generates its unique set of progeny.

Despite the striking similarities of some of the mechanisms of neurogenesis in the brain and in the segmental ganglia, a number of important differences are evident. Neurogenesis in the brain is much more prolonged than in the segmental ganglia. This is due to the longer life span of the brain neuroblasts. All 130 neuroblasts appear to be already present by the 30% stage. At the 70% stage of embryogenesis 127 brain neuroblasts are still visible and only 2 of these are showing signs of degeneration. In the segmental ganglia, degeneration begins much earlier and is much more widespread throughout the neuroblast population in early stages (Shepherd and Bate, 1990). For example, at the 45% stage, one half of the neuroblasts in the abdominal segments A1-A4 have degenerated. By the 70% stage only 3 of the original 29 pairs of neuroblasts are still viable in segments A1-A4

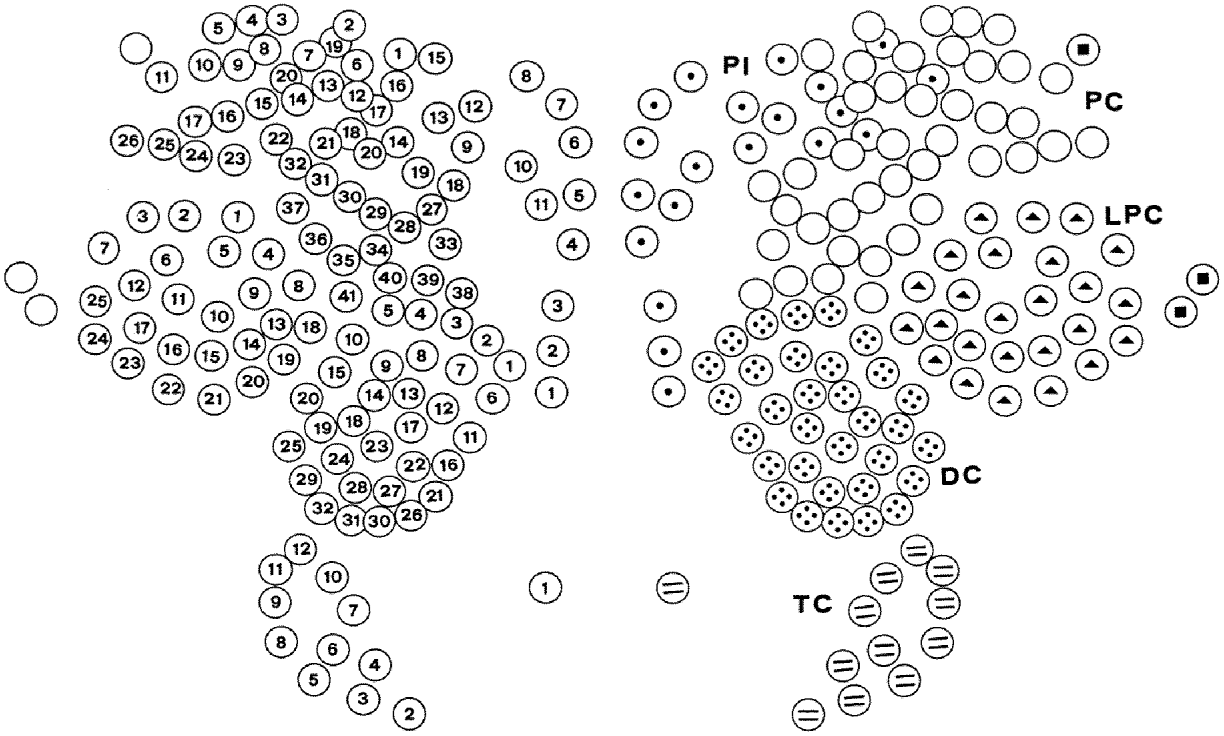

Fig. 9. Summary mapping scheme for identification of early brain neuroblasts. Anterior is at the top, both halves of the brain are shown. This scheme was derived from whole-mount BrdU preparations and confirmed by data from serial reconstructions. The relative position of all the brain neuroblasts in a 45% embryo is indicated as viewed from an idealized ventral perspective. Each neuroblast is represented by a circle of standard size which approximates to the true size of the neuroblasts. Dorsal and ventral neuroblasts are shown in the same plane. For clarity and minimization of overlap, the relative distances between neuroblasts have been increased slightly. However, nearest neighbor relationships among neuroblasts have been retained. In the right half of the Figure, the groups of neuroblasts that belong to the different parts of the developing brain are labelled by different symbols (pars intercerebralis, dots; main protocerebrum, blanks; lateral protocerebrum, triangles; deutocerebrum, stippling; tritocerebrum, stripes; optic neuroblasts, squares). In the left half of the figure, the neuroblasts in the pars intercerebralis, main protocerebrum, lateral protocerebrum, deutocerebrum, tritocerebrum are numbered. The sequences of numbers for the neuroblasts start with 1 in each of these regions and, wherever possible, follow the row or crescent-shaped arrangement of cells. Not numbered are three optic neuroblasts that are shown as anatomical reference points. Scale bar is approximately 100 μm.

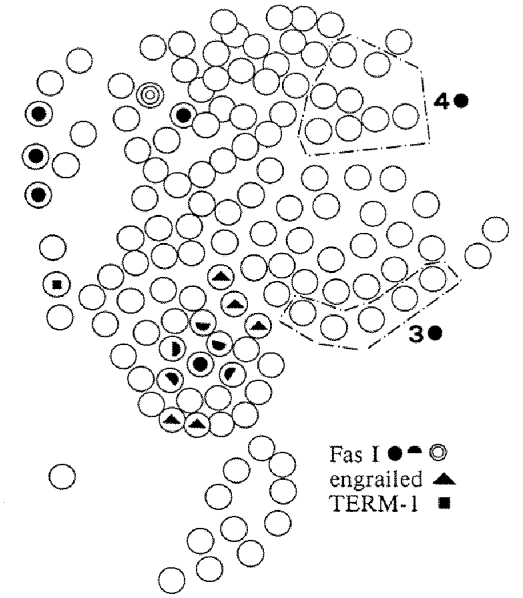

Fig. 10. Summary mapping scheme for the expression patterns of engrailed, fasciclin I and TERM-1. The schematic arrangement of neuroblasts in the right half of the brain (45% stage) is depicted as in Fig. 9. The identified TERM-1-expressing neuroblast is labelled with a square. The identified engrailed-expressing neuroblasts are labelled with triangles. The identified neuroblasts that express fasciclin I are labelled with a filled circle (intense expression on entire cell surface), half circles (expression localized) or open circles (weak expression). The two groups of neuroblasts in which some, but not all, cells express the fasciclin I label are surrounded by dotted lines and indicated by a filled circle. In the main protocerebrum 4 neuroblasts within the indicated group express fasciclin I, whereas in the lateral protocerebrum 3 neuroblasts in the indicated area are fasciclin I immunoreactive.

and by the 77% stage these 3 neuroblasts have degenerated as well. It seems likely that the markedly longer life span of brain neuroblasts will result in a pronounced increase in the average number of neurons produced per neuroblast in the brain as compared to the segmental ganglia. It also seems likely that some of the brain neuroblasts will survive embryogenesis and continue to produce neuronal progeny during larval life. This is known to occur in the developing visual system of the grasshopper (Anderson, 1978) and has been reported for holometabolous insects (e.g. Nordlander and Edwards, 1969; Ito and Hotta, 1991). A further notable characteristic of the developing brain is the extensive morphogenetic movement of the entire neuroblast array in the developing brain. These movements are so pronounced that a set of neuroblasts that was originally the anterior part of the ventral neurogenic sheet becomes displaced to the dorsal and medial surface of the brain. Due to their position after the movements have taken place, these neuroblasts of the pars intercerebralis produce a discrete group of dorsally located neurons. In the segmental ganglia, the only dorsally located neurogenic cells are the midline precursors. These cells divide only once to produce a pair of sibling neurons (Bate and Grunewald, 1981).

In addition to the population of large, unequally dividing neuroblasts described in this paper, a nest of smaller equally dividing neuroblasts also contributes to brain neurogenesis. This group of equally dividing neuroblasts is located in the pars intercerebralis and ultimately gives rise to the intrinsic neurons (Kenyon cells) of the mushroom bodies (Boyan and Williams, unpublished data). Equally dividing neuroblasts are not found in the segmental ganglia of the grasshopper.

Cell specific gene expression in the embryonic brain

In the brain, as in the segmental ganglia, individual neuroblasts are characterized by cell-specific gene expression (Patel et al., 1989a, McAllister et al., 1992; Cabrera, 1992; Doe, 1992; Goodman and Doe, 1993). Some of these genes may have a role in the specification of neuroblast identity or neuroblast lineage. In the segmental ganglia such genes are expressed in very complex and dynamically varying patterns in groups of neuroblasts, ganglion mother cells and neurons (Doe et al., 1988 a,b). In the developing brain the expression patterns of two molecular labels, fasciclin I and engrailed, are also complex and involve clusters of neuroblasts and their progeny as well as groups of neurons without labelled precursors. Moreover, they are also likely to be dynamic. In contrast to this, the expression of TERM-1 is remarkably simple. In each brain hemisphere, it is limited to a single neuroblast and the first pair of neuronal progeny that derive from this neuroblast. The spatio-temporal expression pattern of TERM-1 in the labelled neurons suggests that the protein may play a role in cell-specific pathfinding and synapse formation (see Meier et al., 1993). The reason for its transient expression in the neuroblast remains obscure. Independent of their possible roles in neurogenesis, all of these proteins are extremely useful molecular labels for the identification of individual neuroblasts.

In the segmental ganglia of the grasshopper, the site of formation of each neuroblast in the segmental neurogenic array is reproducible. However, several neighboring cells generally have the potential to form a given neuroblast. Inhibitory cell interactions among these neighboring cells in the neurogenic regions are responsible for only one cell differentiating into a neuroblast at a given position (Doe and Goodman, 1985 a,b; Goodman et al., 1984). Studies in *Drosophila* indicate that 'proneural' genes are responsible for controlling the position at which the ectodermal cells become competent to form neuroblasts, while 'neurogenic' genes are involved in the cell interactions that prevent more than one cell from forming a neuroblast at a given site in the neurogenic region (Lehman et al., 1983; Ghysen and Dambly-Chaudiere, 1989). Given that the site of formation of specific brain neuroblasts in the neuroectoderm is also relatively stereotyped, it will now be important to determine if there are positional cues in the developing brain that confer identity on a given neuroblast and how the expression of these cues is genetically controlled.

Segmentation and neurogenesis in the insect brain

The number of segments that contribute to the head and brain of insects has long been the subject of controversy (see Sharov, 1966; Rempel, 1975; Finkelstein and Perrimon, 1991). Among investigators studying *Drosophila* embryo-

Table 1. Comparison of neuroblast numbers in the embryonic grasshopper CNS

Brain					Gnathal[1]				Thoracic[1]	Abdominal[1]		
PI	PC	LPC	DC	TC	S0	S1	S2	S3	T1-T3	A1-A7	A8	A9
20	41	25	32	**12**	**13**	24	29	29	30	29	30	29

For details see text. [1]Data from Doe and Goodman (1985a).

genesis current debate is centered on the question of whether there are 6 or 7 head segments (Cohen and Jürgens, 1991; Diederich et al., 1991; Schmidt-Ott and Technau, 1992). Although it is generally agreed that there are 3 gnathal segments each with its own neuromere, the number of pregnathal segments as well as the identity of their neuromeres are still disputed. Recently, Schmidt-Ott and Technau (1992), using molecular markers to study the number, location and orientation of brain neuromeres and their corresponding segments, postulated the existence of 4 pregnathal segments. These are (from anterior to posterior) the labral segment, the ocular segment, the antennal segment and the intercalary segment. All four of these pregnathal segments contribute cells to the neuromeres of the central nervous system, implying that in *Drosophila*, and presumably in other insects, a given head segment contributes a neuromere to the central nervous system.

In the embryonic grasshopper the number and arrangement of the large neuroblasts in the entire central nervous system is now known (Table 1). It is still unclear how the number of neuroblasts in the protocerebral structures of the pars intercerebralis (PI), protocerebrum proper (PC) and lateral protocerebrum (LPC) can be related to one or more head segments (labral, ocular). However, the 32 neuroblast pairs of the deutocerebrum are remarkably similar in number to the 29-30 neuroblast pairs found in most of the gnathal, thoracic, and abdominal segments. This supports the notion that the deutocerebrum might indeed be the neuromere of a pregnathal segment, namely the antennal segment (n.b. the insect deutocerebrum receives and processes antennal input).

The three gnathal segments S1-S3 as well as most of the posterior segments clearly have their own well defined neuromere consisting of 24-30 segmentally homologous neuroblast pairs (Doe and Goodman, 1985a). However, the identification of the neuromere of the segment that is intercalated between the antennal segment and the first gnathal segment, namely the intercalary segment, remains elusive. It is interesting that a group of 13 neuroblast pairs which do not have the typical segmental pattern are found at the anterior margin of the S1 (mandibular) neuromere (Doe and Goodman, 1985a). These are referred to as the S0 neuroblasts. It is also interesting that a further group of 12 neuroblast pairs which also do not have the typical segmental pattern are found in the developing tritocerebrum (TC). An attractive hypothesis is that the neuromere of the intercalary segment comprises 25 identified neuroblast pairs which become segregated into a tritocerebral group and an S0 group. In accordance with this notion is the observation that both the tritocerebral and the S0 neuroblasts are intimately associated with another early in embryogenesis (30% stage) and only later move apart as the gut enlarges (L. Williams, unpublished observations).

Implications for future work on neuronal development in the insect brain

The characterization of the neuroblasts presented here should contribute to a more comprehensive cellular and molecular analysis of the development of the grasshopper brain. The developmental origin of many of the groups of neurons that form the anatomical subunits of the brain can now be traced to identified neuroblasts. For example, the central complex, the neurosecretory cells and the protocerebral neurons of the PI(2) group (Williams, 1975) are probably generated by separate, discrete sets of identified pars intercerebralis neuroblasts. Similarly, based on the identification of the brain neuroblasts reported here, it should now be possible to determine the neuroblast of origin and cell lineage of all of the brain interneurons that have been identified to date (e.g. O'Shea et al., 1974; Bacon and Tyrer, 1978; Reichert et al., 1985; Hensler, 1988). This, in turn, should make it possible to determine the phenotypic characteristics that clonally related neurons in the brain have in common, much as has been done for identified neurons in the ventral nerve cord (Goodman and Spitzer, 1979; Taghert and Goodman, 1984; Raper et al., 1984). Indeed, one of the major implications of this study is that the concepts and methods that have been successfully used to analyze neurogenesis in the segmental ganglia can, in principle, be applied to study neurogenesis at the level of identified cells in the developing brain.

As more molecular markers for neuroblasts are used to characterize neuronal precursor cells in the grasshopper brain, and as further potential molecular markers of this type are discovered and become available, it may be possible to positively identify each brain neuroblast exclusively on the basis of the specific set of molecular labels that it expresses (Doe, 1992). Given the relatively small number of neuroblasts in the early embryonic brain, it is even conceivable that a neuroblast-specific molecular expression system exists in which each neuroblast is uniquely identified by a single molecular label. In either case, a more complete molecular neuroblast map for the developing brain will be a useful complement to the initial anatomical neuroblast map presented in this study.

Many features that we have described here for brain neuroblasts in the grasshopper are likely to be general in insect neurogenesis. It may even be possible to identify homologous neuroblasts in different insect groups. For example, the unique anatomical location of the neuroblast pair found near the tritocerebral commissure in the grasshopper (Figs 3C, 5B) is very similar to an equivalently positioned pair in the the stick insect *Carausius morosos* and the cockroach *Periplaneta americana* (Malzacher, 1968).

The full potential of a homology-based comparative approach to brain neurogenesis in insects can be exploited if the cellular studies in the embryonic grasshopper are

combined with molecular genetic studies in *Drosophila* (Thomas et al., 1984; Grenningloh et al., 1990; Meier et al., 1991). A great deal has already been learned about the genes involved in the formation, specification and lineage of the neuroblasts that generate the segmental ganglia in *Drosophila* (Campos-Ortega and Knust, 1990; Cabrera, 1992; Goodman and Doe, 1993). An important task will now be to extend our analysis of brain neurogenesis in the grasshopper to brain neurogenesis in *Drosophila*.

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