Patterns of Neurogenesis in the Midbrain of Embryonic Lobsters Differ from Proliferation in the Insect and the Crustacean Ventral Nerve Cord

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ABSTRACT: **Neurogenesis persists throughout life in the olfactory pathway of many decapod crustaceans. However, the relationships between precursor cells and the temporal characteristics of mitotic events in these midbrain regions have not been examined. We have conducted studies aimed at characterizing the sequence of proliferative events that leads to the production of new deutocerebral projection neurons in embryos of the American lobster, *Homarus americanus*. *In vivo* bromodeoxyuridine (BrdU) labeling patterns show that three distinct cell types are involved in neurogenesis in this region. Quantitative and temporal analyses suggest that the clearing time for BrdU is 2–3 days in lobster embryos, and that the sequence of proliferative events in the midbrain is significantly different from the stereotypical pattern for the generation of neurons in the ventral nerve cord ganglia of insects and crustaceans. The unusual pattern of proliferation in the crustacean midbrain may be related to the persistence of neurogenesis throughout life in these regions.** © 2002 Wiley Periodicals, Inc. J Neurobiol 53: 57–67, 2002

Keywords: **neurogenesis; arthropod; crustacean; clearing time; bromodeoxyuridine (BrdU)**

INTRODUCTION

Life-long neurogenesis has been documented among sensory and interneuronal populations in the olfactory pathway of a variety of decapod crustacean species (Harzsch and Dawirs, 1996; Sandeman and Sandeman, 1996; Schmidt, 1997, 2001; Sandeman et al., 1998; Harzsch et al., 1999; Schmidt and Harzsch, 1999; Steullet et al., 2000). Persistent neurogenesis of functionally analogous groups of neurons has also been demonstrated in the olfactory system of adult vertebrates (Graziadei and Monti Graziadei, 1986; Lois and Alvarez-Buylla, 1994). While the crustacean olfactory pathway appears to have a functional anatomy that is parallel to the vertebrates (Strausfeld and Hildebrand, 1999), its organization provides experimental advantages because neuronal somata are arranged in anatomically distinct clusters according to their function. Thus, the somata of local interneurons are found in clusters medial to the primary olfactory processing area, the olfactory lobe (OL) (clusters 9 and 11; Sandeman et al., 1992), while projection neurons are located lateral to the OLs in a distinct cluster [cluster 10; Fig. 1(A)]. This anatomical organization has been exploited in recent studies that have begun to define the regulatory mechanisms that control neurogenesis. Hormonal influences during the molt cycle (Harrison et al., 2001), serotonin levels (Benton et al., 1997; Benton and Beltz, 2001; Beltz et al., 2001), the day-night cycle (Bagay et al., 2001; Goergen et al., 2002), and environmental living conditions (Sandeman and Sandeman, 2000) all have been shown to affect the timing and rate of neurogenesis in the crustacean olfactory pathway.

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In spite of this relatively rapid progress in defining some of the factors that influence neurogenesis in the crustacean brain, relatively little is understood about the sequence or timing of proliferative events that lead to the production of new neurons in these areas (Harzsch and Dawirs, 1996; Schmidt, 2001). In stud-

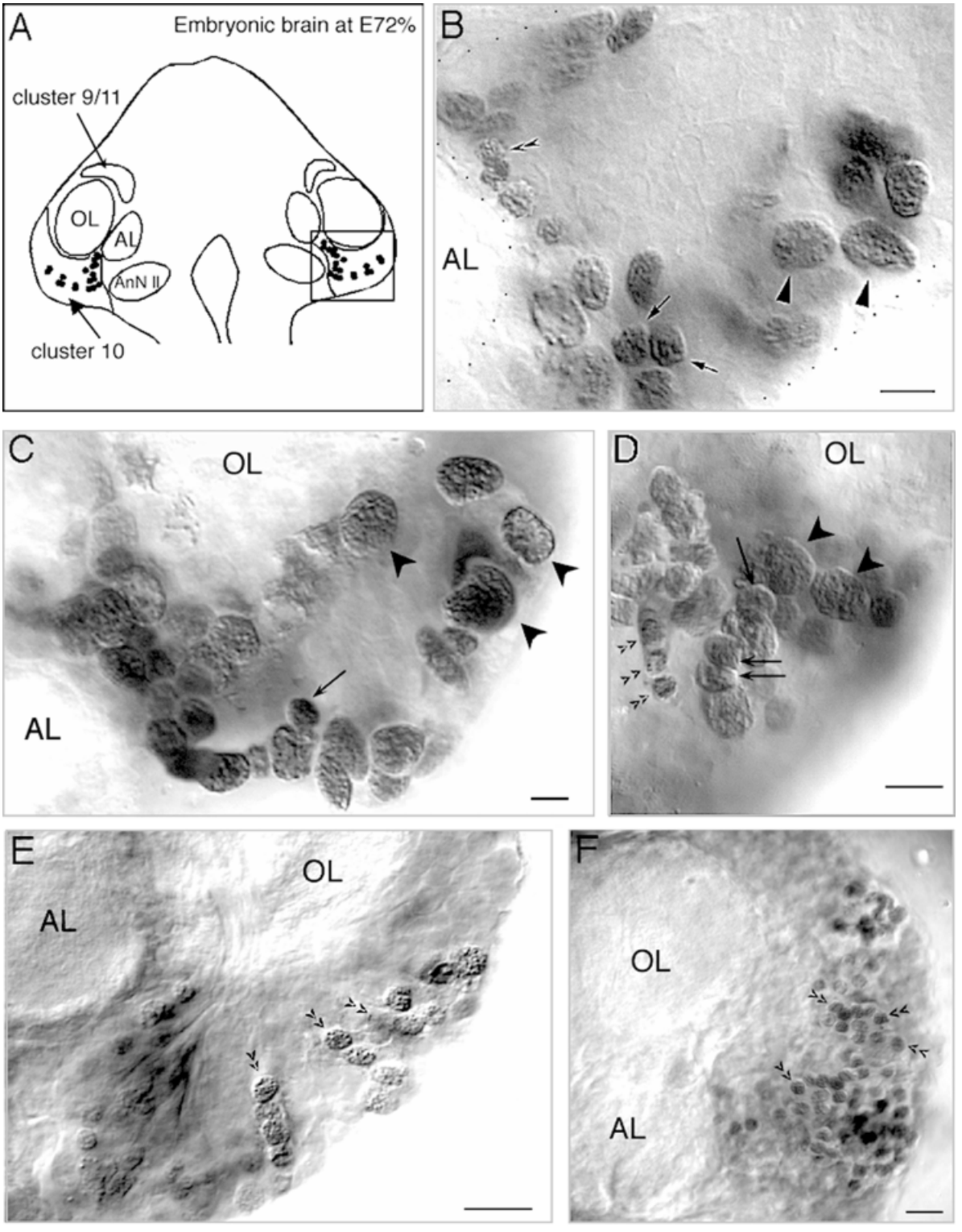

Figure 1

ies with the proliferation marker bromodeoxyuridine (BrdU), the number of labeled profiles (BrdU-positive nuclei) generally depends on the total number of proliferating cells, on the duration of the cell cycle, and on the availability of the labeling reagent. The design and interpretation of future experiments depend upon an understanding of these relationships. Therefore, we have conducted quantitative studies in embryonic lobsters in order to characterize the mitotic activity of progenitor cells from which deutocerebral projection neurons emerge. The characteristics of BrdU staining described in the present report suggest both a time frame and sequence for the generation of projection neurons from progenitor cells, aspects of which are significantly different from what is known about the generation of central neurons in insects (Doe and Skeath, 1996; Reichert and Boyan, 1997; Matsuzaki, 2000).

Our understanding of neurogenesis among arthropod species has relied heavily on the study of insect model systems where the sequence of events, from the first interactions of the proneural genes, to the delamination of the typical asymmetrically dividing stem cells (the neuroblasts), to the production of ganglion mother cells and neurons, has been carefully described (Doe et al., 1991, 1998; Campos-Ortega, 1995; Doe and Skeath, 1996; Reichert and Boyan, 1997; Matsuzaki, 2000). Studies suggest that the numbers and arrangement of neuroblasts, as well as the single division of ganglion mother cells to form a pair of neurons, are features that are highly conserved among insect species (Doe and Skeath, 1996; Campos-Ortega and Hartenstein, 1997; Truman and Ball, 1998; Matsuzaki, 2000). It has been questioned whether this conservation in structure and mechanism also extends to other arthropods such as crustaceans. In studies where divisions of neuroblasts and ganglion mother cells have been observed in the ventral nerve cord of crustacea, the pattern of neurogenesis typical of insect species has been proposed (Scholtz, 1990, 1992; Harzsch and Dawirs, 1994, 1996; Whitington, 1996; Dohle and Scholtz, 1997; Harzsch et al., 1998; Gerberding and Scholtz, 1999, 2001; Harzsch, 2001a). Large neuroblasts delaminate from the neuroectoderm and undergo unequal divisions repeatedly to produce rows of ganglion mother cells (Scholtz, 1992; Harzsch and Dawirs, 1994; Harzsch, 2001a). The single division of ganglion mother cells to produce a pair of neurons, as observed in insects, has not been challenged, although early studies in crayfish expressed some doubt about the sequence of divisions at this level (Scholtz, 1992).

The data presented in the current report suggest that, at least during embryonic development of midbrain neuronal clusters in lobsters, there are three distinct classes of cell types involved in neurogenesis, as in the insects. However, the intermediate precursors of the brain neurons, unlike the model of ganglion mother cells of insects, undergo multiple divisions to generate neurons. These data suggest that the stereotyped pattern of divisions characteristic of the insects is not typical of all regions in the crustacean nervous system.

Figure 1 (A) Schematic diagram adapted from a *camera lucida* drawing illustrating the arrangement of neuropils adjacent to the projection neuron cell cluster (cluster 10 of Sandeman et al., 1992) in embryonic lobsters at 72% development (E72%). Sites of proliferation in this cell cluster are illustrated as black dots. The area in the rectangle on the right represents the proliferation zone in cluster 10 shown in (B) and (C). Arrows point to cell clusters 9 and 11 (these clusters are indistinguishable in embryos) and cluster 10. AL, accessory lobe; OL, olfactory lobe; AnN II, antenna II neuropil. (B–F) Photographs of cluster 10 in whole mount embryonic brains showing labeled profiles after post-BrdU incubation survival times of 1 (B), 2 (C), 3 (D), 6 (E), and 21 (F) days. Labeled cells along the lateral margin of cluster 10 are more dorsal than those labeled profiles occurring as a tightly packed group located in the ventral part of the cluster directly adjacent to the AL (B and C). The photograph in (C) was produced using a double exposure technique that allows visualization of the dorsal and ventral planes in a single image. Three distinct sizes of BrdU-labeled profiles are observed within 24 h of the BrdU incubation [(B): day 1]: large precursor cells (9.5–11 μm; large arrowheads); intermediate precursor cells (6–8 μm; arrows); and small neuronal cell types (3–5 μm; small double arrowheads). Dense BrdU labeling is found in the ventral region of cluster 10 in a position between the OL and AL, along the margin of the AL [(D), day 3; large precursor cells, large arrowheads; intermediate precursor cells, arrows; and presumptive neurons, double arrowheads]. The newly born presumptive neurons are arranged in a linear pattern [(E): seen by day 6], organized into rows that radiate from the lateral margin, projecting inward to the OLs and ALs (E). Presumptive neurons are the only cell type labeled on day 21 (F). In (A–F) anterior is up. Scale bars: (B–F) = 10 μm.

MATERIALS AND METHODS

Animals

Lobster embryos (*Homarus americanus*, Decapoda, Homarida, Nephropidae) from the same clutch were obtained from the Lobster Rearing and Research Facility at the New England Aquarium, Boston, Massachusetts. At Wellesley College, eggs were kept in recirculating artificial seawater at 14°C, under a light/dark cycle of 12:12. Embryos were staged according to the method of Helluy and Beltz (1991) on the basis of the length and width of the pigmented zone in the eye.

BrdU Labeling

Proliferation of cells was monitored by *in vivo* labeling with BrdU (Dolbeare, 1996). Embryos at 72% of embryonic development (E72%) were incubated in BrdU (Cell Proliferation kit RPN 20; Amersham, Little Chalfont, Buckinghamshire, UK) diluted in seawater to a concentration of 0.2 mg/mL for 2 h at 14°C. Lobsters at about E70% were targeted for this study because this is the period of peak proliferation in the brain (Harzsch et al., 1999). In addition, early in embryonic life cluster 10 is not clearly delineated, but by E60% the margins of the cluster are defined by the rapidly developing accessory lobes (ALs) and OLs (Helluy et al., 1995). Many of our prior studies have focused on events that occur during these midembryonic stages (Benton et al., 1997; Harzsch et al., 1999; Sullivan et al., 2000; Benton and Beltz, 2001; Beltz et al., 2001), and the present studies therefore were intended to clarify mechanisms that are relevant to this period of time in the midbrain.

Following BrdU incubation, five embryos were sacrificed every day for the first week, and thereafter every 3 days for the next 2 weeks, providing 12 sampling points in 3 weeks. The brains were dissected from these embryos, and sequentially fixed and processed as whole mounts using standard immunocytochemical methods. Specimens were incubated for 2 h in a mouse anti-BrdU antibody (1:100; Cell Proliferation kit RPN 20; Amersham) followed by rinses in 0.1 *M* phosphate buffer with 0.3% Triton X-100 (PBTx). Preparations were then immersed overnight in biotinylated goat anti-mouse IgG antibody (1:200; Molecular Probes), followed by rinses in PBTx and overnight incubation in avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). The resulting signal was revealed using diaminobenzidine (DAB; 1 mg/2 mL PB; Sigma). The proliferation zone of the projection neuron cell body cluster was drawn using a *camera lucida* device mounted on a Nikon Optiphot compound microscope. BrdU-labeled profiles in cluster 10 were measured and the number of labeled cells in each size class was counted blindly. One-way analysis of variance (ANOVA) was used to look for significant differences among the total cell counts at the 12 time periods, followed by the Newman-Keuls multiple range test for post-test comparisons.

RESULTS

Within the first 24 h, three distinct size classes of cells are labeled in the projection neuron cluster (cluster 10; Sandeman et al., 1992) in the midbrain (deutocerebrum) of lobster embryos using BrdU methods [Fig. 1(B,C)]. These are referred to as large precursor cells (10.9 ± 1.0 μm nuclear diameter; large arrowheads), intermediate precursors of the olfactory interneurons (6.7 ± 0.6 μm nuclear diameter; arrows), and presumptive neurons, the cell bodies of which match the size of the olfactory projection neurons (3.5 ± 0.4 μm nuclear diameter; double arrowheads). In the midembryonic stages examined, neurons are generated in two contiguous regions: in posterior ventral areas of cluster 10 adjacent to the AL, and in more dorsal marginal regions of the cluster [Fig. 1(A)].

On the first two sampling days following BrdU incubation, labeled profiles are found along the lateral margin of cluster 10 in a dorsal plane [Fig. 1(B,C)] relative to BrdU-labeled cells densely packed along the posterior margin of cluster 10, near the AL [Fig. 1(C,D)]. By day 3 after BrdU incubation, counts of labeled presumptive neurons increase by 20% compared with days 1 and 2; these profiles [double arrowheads; Fig. 1(D)] are most frequently observed in the more ventral aspect of the proliferation domain in cluster 10. By day 8, a linear arrangement of labeled profiles typical of neurons in this cluster (Harzsch et al., 1999) is observed [Fig. 1(E)]. After day 11, nearly all of the labeled profiles are of the presumptive neuronal variety [Fig. 1(F)].

Graphing total numbers of cluster 10 labeled profiles of all three size categories for each of the 12 time periods assessed in this experiment illustrates that the numbers of labeled profiles appear to increase in three phases [Fig. 2(A)]. The mean number of labeled profiles for days 3 through 8 is 1.6 times greater than mean counts for days 1 and 2. The differences in the numbers of labeled cells at these plateaus are statistically significant ($p = <.001$; one-way ANOVA and Newman- Keuls post-test). After day 8 (days 8–21), the mean numbers of BrdU-labeled profiles again increase by a factor of 1.6, an increase (30%) over the mean number of cells labeled during the period from day 3 through 8 ($p = <.001$; one-way ANOVA and Newman-Keuls post-test). However, information about the relationships of the three cell types and their mitotic activity that results in these three statistically different plateaus [Fig. 2(A)] is obscured by the summation of all three BrdU-labeled cell types.

Therefore, in order to clarify the relationships among the BrdU-labeled cell types, counts are made

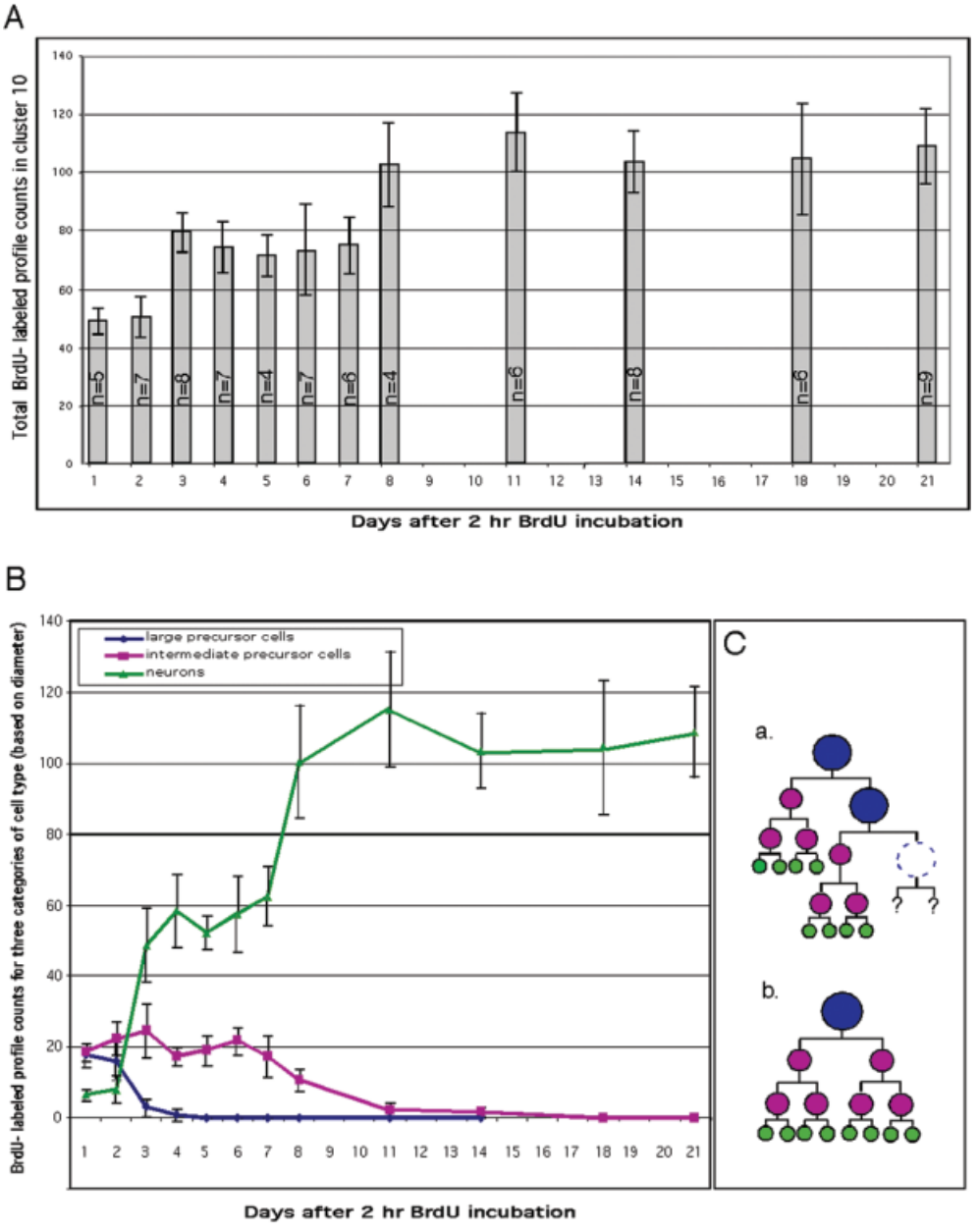

Figure 2 (A) Bar graph showing mean counts ± the standard deviation of BrdU-labeled profiles in cluster 10 following a 2 h BrdU pulse at E72%. Twelve time points were assessed during the subsequent 3 weeks: daily counts of BrdU-labeled cells for the first 8 days, and then counts every 3 days for the next 2 weeks. (B) Graph of BrdU-labeled profile counts for each of the three cell types. Large (blue) and intermediate precursor cells (magenta) predominate during the first 3 days after a 2 hr BrdU pulse. From days 4–8, intermediate (magenta) and neuronal (green) cells are observed. The BrdU "clearing time" occurs between day 2 and 3, when the numbers of large precursor cells are dramatically reduced, presumably because the BrdU labeling reagent is no longer available. (C) Two possible lineage maps are proposed, based upon the ratios of counts of the three BrdU-labeled cell types and the proliferation sequence suggested by the graph in (B). It is not clear from our studies whether the large precursor cell divisions are *asymmetric* [see (a): large precursor cell divides producing another large precursor cell (blue) and an intermediate precursor cell (magenta), which then produces at least two divisions of neuronal type cells (green)] or *symmetric* [see (b): cell division of a large precursor cell (blue) produces two intermediate precursor cells (magenta), which divide to produce presumptive neurons (green)]. Intermediate cells must undergo more than one cell division, given the number of neuronal cells (green) produced by the intermediate cells (magenta).

of labeled profiles for each size class over the 3 week period of this experiment [Fig. 2(B)]. Twenty-four hours after BrdU incubation, all three size classes of cells are labeled, although the two larger classes predominate [Fig. 1(B), day 1; Fig. 1(C), day 2]. On day 3 after BrdU exposure, the numbers of labeled large precursor cells have decreased dramatically, the numbers of intermediate precursor cells have increased slightly, and the numbers of presumptive neurons have increased sharply [Fig. 1(D), day 3; and Fig. 1(E), day 8]. By day 11 following BrdU exposure, virtually all labeled profiles are of the small presumptive neuronal variety [Fig. 1(F), day 21].

It is not clear whether the large precursor cells generating the projection neurons undergo asymmetric divisions, as do most insect and crustacean neuroblasts (large precursor cells). The largest profiles in cluster 10 have not been seen in close association with columns of intermediate precursor cells or neurons, such as are typically seen when neuroblasts divide asymmetrically to form ganglion mother cells (intermediate precursor cells). The small differences between the counts of the BrdU-labeled large precursors and intermediate precursors [Fig. 2(B)] also suggest that the large precursors may be dividing only once, which would indicate a symmetrical mode of division. Peak neurogenesis in cluster 10 is reached by E80% and decreases dramatically towards hatching; neurogenesis resumes during larval life but only in the ventral posterior region of cluster 10 (Harzsch et al., 1999). Therefore, we speculate that symmetric divisions [Fig. 2(Cb)] may occur in midembryonic life in the more dorsal precursor cells along the margin of cluster 10, resulting in a burst of neuronal differentiation and the termination of this stem cell population. This issue is still open, as suggested by the alternative lineage diagrams presented in Figure 2.

One approach to this question is to compare the pattern of proliferation in cluster 10 of the midbrain [Fig. 1(A,C)] with the pattern of neurogenesis that occurs in the ventral nerve cord of *H. americanus* [Fig. 3(A)]. BrdU labeling in the thoracic ganglia reveals large asymmetrically dividing precursor cells that regenerate themselves and bud off a smaller progeny, an intermediate precursor cell. In the thoracic and abdominal ganglia a 4 h BrdU pulse consistently revealed strings of smaller progeny trailing away from the large BrdU-labeled profiles [Fig. 3(A)], a pattern stereotypical of neurogenesis in insects. BrdU labeling in the midline in the same brain preparation described in Figures 1 and 2 also reveals the more characteristic large, unequally dividing precursors with progeny [Fig. 3(B)]. These cells display the more lightly condensed DNA typical of asymmetric division (Doe et al., 1998), in contrast to the large precursor cells along the dorsal margin of cluster 10 [Fig. 1(B,C)], where no individual lightly stained large precursors or associated intermediate precursors are observed. Proliferation of marginal precursors in cluster 10 may therefore provide an example of cells dividing once to produce two intermediate precursor cells.

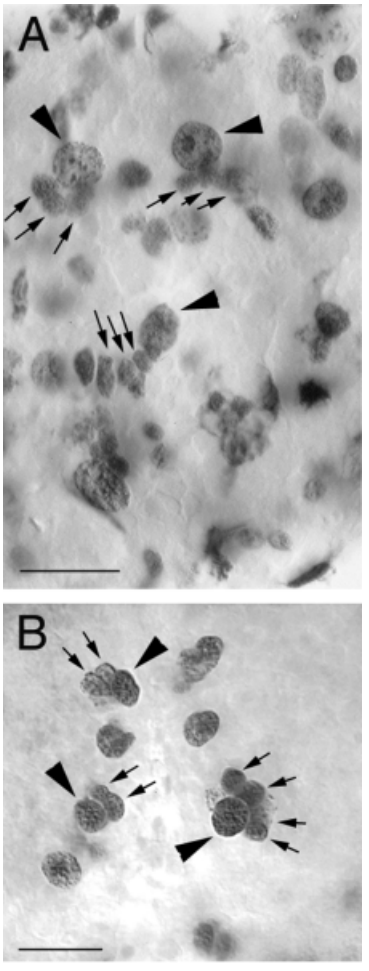

Figure 3 BrdU labeling in the embryonic (A) ventral nerve cord and (B) supraesophageal ganglion (brain) of the lobster, *Homarus americanus* (whole-mount preparations). (A) Third and fourth thoracic ganglia (neuromeres 6 and 7) at E20%. BrdU-labeled large precursor cells (arrowheads) with rows of progeny, intermediate precursor cells (small arrows). (B) BrdU-labeled large precursor cells (arrowheads) and associated progeny (small arrows) in the midline of anterior protocerebrum at E72%. Scale bars: (A) = 50 μm; (B) = 25 μm.

The primary focus of the present study is embryonic tissues, but comparisons of the spatial layout of proliferative regions have been made between these specimens and larval, juvenile, and adult lobsters (Fig. 4). Two important developmental distinctions

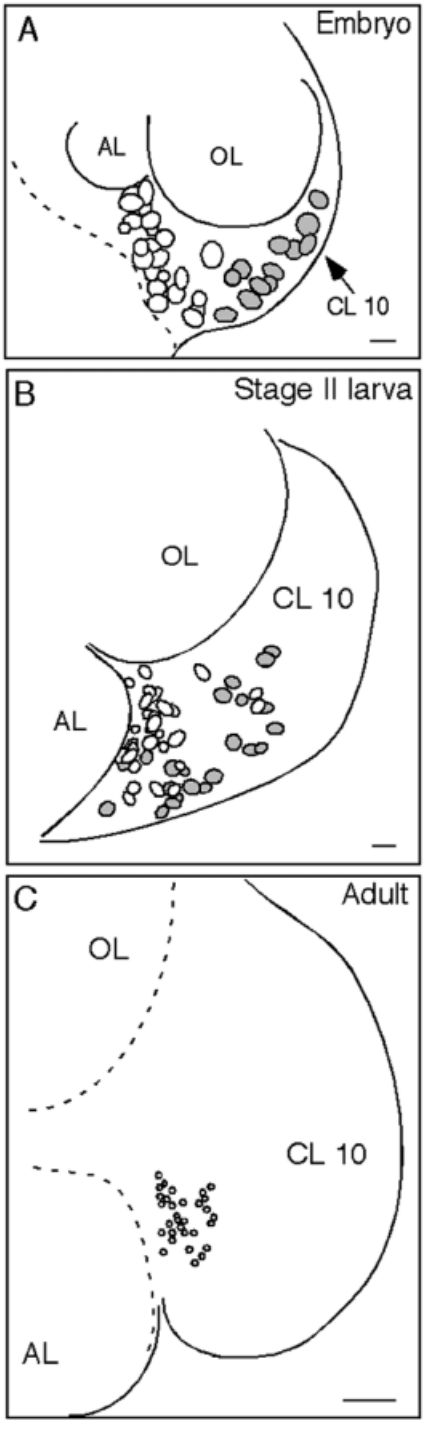

Figure 4 Spatial layout of BrdU-labeled cell profiles in cluster 10 of embryonic (A), 2nd stage larval (B), and adult (C) lobster brains. BrdU incubation times utilized for each of these stages are (A) 2 h for embryos, (B) 3 h for larvae, and (C) 4 h for adults. Two contiguous proliferation zones are observed in embryos and larvae: a dorsal and marginal region of proliferation [gray filled circles in (A) and (B)], and a more ventral region at the anterior margin of the AL [open circles in (A) and (B)]. In the adult brain (C), only the more ventral, posterior proliferation domain persists. The large precursor cells labeled in embryos are not observed after larval life. Scale bars: (A) and (B) = 10 μm; (C) = 50 μm.

emerge from these comparisons. The sequence of proliferation that we describe here, with three distinct cell types contributing to the generation of neurons, is not recognizable after larval life in lobsters [Fig. 4(C)]. In later life stages the large precursor cells have not been documented, suggesting that a different mode of cell proliferation may emerge by juvenile life. Evidence for the three sizes of cells is not found in the adult [Fig. 4(C); Harzsch et al., 1999]. It is also clear that there are two contiguous regions of neurogenesis in the projection neuron cluster of embryonic and 1st and 2nd stage larval lobsters: large precursors that are located dorsally near the lateral margin of the cell cluster, producing progeny towards the center of cluster 10 [Fig. 4(A,B); gray-filled circles] and large precursors that lie more ventrally and posteriorly, near the boundary of the AL [Fig. 4(A,B); open circles]. However, the size of the large precursor cells is greatly reduced in larvae (unpublished observations). Furthermore, the dorsal BrdU- labeled profiles are not seen after larval life, while the proliferation domain located more ventrally near the ALs remains active throughout juvenile and adult stages in lobsters.

DISCUSSION

The characteristics of BrdU staining suggest both a time frame and sequence for the proliferation of deutocerebral projection neurons in embryonic lobsters. Three to four days following BrdU exposure, large precursor cells are no longer labeled; we interpret this time frame to be indicative of the "clearing time" for BrdU in these embryos. This interpretation is based upon the idea that BrdU incorporated by dividing cells would be diluted with each cell division, and this dilution effect would result in a loss of labeling among the cells that are dividing most rapidly. If BrdU were still available (prior to "clearing"), then the labeling of dividing cells would be refreshed with each DNA replication; however, if BrdU levels have dropped significantly or "cleared", then no additional label is available. We know from prior studies (Harzsch et al., 1999) that neuronal proliferation is occurring at a high rate during the developmental period examined in these experiments. Therefore, the lack of the largest labeled profiles on day 4 of the

present experiment is likely to indicate the time frame when BrdU is no longer available for uptake (day 2–3), and also suggests that the largest profiles are the most rapidly dividing cells.

The loss of staining (between days 8 and 11) among the intermediate precursor cells, coincident with a steep increase in the numbers of labeled 3.5 μm presumptive neuronal cell profiles, indicates that the intermediate-sized cells are the products of the largest precursors, and also the immediate precursors of the presumptive projection neurons. The fact that only the smallest labeled profiles are still present after day 11 [Fig. 2(B)] suggests that these cells are the final products of this proliferation sequence. The numerical relationship between the intermediate precursor cells and the presumptive neurons suggests that each intermediate cell is likely to undergo two to three cell divisions to produce the final population of labeled neurons [Fig. 2(C)]. This result is obscured when the data are presented as the total number of labeled cell counts for each time period, as seen in Figure 2(A). However, in Figure 2(B), this surprising feature of multiple divisions of the intermediate precursor cells is suggested by the occurrence of a second plateau in presumptive neuron counts on day 8. The magnitude of this unexpected second plateau is likely to be determined by several concurrent processes such as differences in cell cycle times and in mode of cell division in the two proliferation domains, including a single division of the large precursors and multiple divisions of the intermediate precursors [see Fig. 2(Cb)].

The knowledge of the clearing time and patterns of neuronal proliferation in lobster embryos derived from these experiments is critical for planning future experimental protocols as well as for guiding our interpretation of data. The two to three day clearing time for BrdU suggested by these experiments is very long compared with vertebrate organisms (1 h; Böswald et al., 1990). Other parameters that influence the numbers of cells that label include the length of BrdU incubation and the postincubation survival time. In addition, any factor that changes the length of the cell cycle or regulates the numbers or activity of progenitor cells, such as temperature of the environment, hormones (Steullet et al., 2000; Benton and Beltz, 2001; Beltz et al., 2001), or living conditions (Sandeman and Sandeman, 2000) will alter the numbers of labeled cells.

The sequence of proliferative events described for the projection neurons has elements that are distinctive when compared to schemes of neurogenesis in insects and the ventral nerve cord ganglia of crustaceans. First, it is not clear that the divisions of the cluster 10 large precursor cells are asymmetric, as in the vast majority of examples in both insects (Bossing et al., 1996; Doe, 1996; Campos-Ortega and Hartenstein, 1997; Doe et al., 1998; Akiyama-Oda et al., 1999; Ceron et al., 2001) and crustaceans (Scholtz, 1992; Harzsch and Dawirs, 1994; Harzsch et al., 1998). The typical image of the large precursors with associated columns or clusters of smaller cells that is representative of these reports (also observed in the midline of the brain in these experiments) is not seen in either the local (cluster 9; Benton and Beltz, 2001) or projection neuron clusters of the embryonic lobster brain. During the development of the brain of *Schistocerca gregaria*, for example, the typical pattern of asymmetric divisions of neuroblasts to form ganglion mother cells has been reported to be the general rule (Zacharias et al., 1993). The notable exception to this pattern is found in the pars intercerebralis of the grasshopper brain, where a nest of smaller symmetrically dividing neuroblasts ultimately gives rise to the Kenyon cells of the mushroom bodies (Zacharias et al., 1993). The mushroom bodies are the one region in the insect brain where neurogenesis persists throughout the animal's life (Cayre et al., 1996, 2002).

The multiple divisions of the intermediate precursor cells also contrast with the single division reported for ganglion mother cells of the insect central nervous system (Zacharias et al., 1993; Doe, 1996; Campos-Ortega and Hartenstein, 1997; Doe et al., 1998) and in the ventral nerve cord ganglia of the crustaceans *Hyas araneus* (Harzsch and Dawirs, 1994) and *H. americanus* (Harzsch et al., 1998). However, Scholtz (1992) reported that during early neurogenesis in the thoracic and abdominal segments of the crayfish *Cherax destructor*, the number of rounds of mitoses of the ganglion mother cells was not clear. Even in the insect central nervous system, where stereotyped cell division patterns are the general rule and many lineages have been completely defined, there are exceptional cases, such as the midline precursor of the grasshopper brain that generates three daughter cells directly, not via ganglion mother cells (Ludwig et al., 1999). To what degree our findings in the brain are typical of patterns of neuronal proliferation in other crustaceans is not known; however, there are hints in the literature that multiple divisions of intermediate precursor cells in the deutocerebral cell clusters may extend across species. Schmidt (2001) reports that in the adult brain of *Panulirus argus*, there is a fourfold or more increase in the numbers of BrdU-labeled profiles, with the final doubling of labeled cells occurring between 3 and 14 months following BrdU labeling. Schmidt argues that this increase in the numbers of BrdU-positive cells cannot be due to a further round of cell

divisions, because by 3 months all BrdU-positive cells have left the proliferative zones, outside of which no mitotic nuclei were detectable. However, the numerical correspondence between our results in embryos of *H. americanus* and Schmidt's findings in adults of *P. argus* is quite striking. Although the time frame over which these divisions occurred in the adult studies was much longer than in embryos, the quantitative agreement between our data and those of Schmidt (2001) may suggest multiple divisions of intermediate precursor cells in both of these situations. Quantitative data and/or more detailed cell lineage studies will be necessary to resolve this issue.

A reduction in the numbers of large precursors has been reported in late embryonic stages of *C. destructor* (Scholtz, 1992), *H. americanus*, and *H. araneus* (Harzsch et al., 1998), suggesting that the numbers of mitotically active large precursor cells may vary throughout the life cycle of a particular species (Harzsch, 2001a,b). The present study in lobsters suggests not only a temporal modulation of the large precursor cell number in the brain, but also a spatial regulation of the site of neurogenesis. Thus, in embryos, neurons are generated in posterior, ventral layers as well as in more dorsal, marginal regions of the projection neuron cluster, while in juveniles and adults, neurons are born only in posterior and ventral regions of the cell cluster. Therefore, although lifelong neurogenesis has been documented in the local and projection neuron clusters of lobsters and other malacostracans (Harzsch and Dawirs, 1996; Schmidt, 1997, 2001; Sandeman et al., 1998; Harzsch et al., 1999), the stem cells that persist during the later life stages may represent a different type of proliferative cell than we have described in embryos. The large unequally dividing precursor cells have not been observed in juvenile and adult lobsters, even where a BrdU incubation and short survival time was used (Harzsch et al., 1999; Schmidt and Harzsch, 1999; Harzsch, 2001a), a protocol that always reveals large precursor cells in embryos. Large precursor cells also have not been identified in the deutocerebral clusters in adult *P. argus* (Schmidt, 2001). Alternatively, as the mitotic cycle slows down with periods of quiescence, the size of the stem cell nuclei may decrease, reflecting the more condensed and inactive DNA (Sauer, 1991; as cited by M. Jacobson, editor). Therefore, the smaller precursor cells measured in larvae may be the same large precursor cell types with greatly reduced mitotic activity. In juveniles and adults, if the stem cells and their progeny were approximately the same size as the neurons, these precursors would be difficult to identify.

The basic pattern of cell proliferation reported here for neurons in cluster 10 also appears to be typical of proliferation among the local interneurons (clusters 9 and 11) of the olfactory pathway of *H. americanus* (Benton and Beltz, 2001). Although quantitative studies such as those reported here have not been conducted in the local interneuron clusters, it is clear that the same size categories of cells participate in the proliferation sequence in embryos, and that large BrdU-labeled profiles are no longer seen after larval life in these areas.

From a developmental perspective, the most interesting aspect of these findings is that the spatial organization and type of large precursors generating projection neurons in lobsters change over the various life stages. From the evolutionary point of view, the most intriguing revelation is that the sequence of divisions observed in the deutocerebrum of embryonic lobsters is distinctive from that described in the insects. Although variations on the standard pattern of neuronal divisions in insects are seen in the generation of Kenyon cells of the mushroom bodies and daughter cells by the midline precursor in the brains of grasshoppers, in no case has multiple divisions of intermediate precursor cells been reported. It is interesting to note that the primary exceptions to the more stereotypical patterns of proliferation occur in regions where life-long neurogenesis persists.

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REFERENCES

Akiyama-Oda Y, Hosoya T, Hotta Y. 1999. Asymmetric cell division of thoracic neuroblast 6-4 to bifurcate glial and neuronal lineage in *Drosophila*. Development 126: 1967–1974.

Bagay LA, Rehm K, Benton JL, Beltz BS. 2001. Circadian control of neurogenesis in the lobster brain. Soc Neurosci Abstr 27:Program number 587.9.

Beltz BS, Benton JL, Sullivan JM. 2001. Transient uptake of serotonin by newborn olfactory projection neurons may mediate their survival. PNAS 98 22:12730–12735.

Benton J, Helluy S, Huber R, Beltz B. 1997. Serotonin depletion by 5,7-dihydroxytryptamine alters deutocerebral development in the lobster. J Neurobiol 33:357–373.

Benton JL, Beltz BS. 2001. Effects of embryonic serotonin

depletion on olfactory interneurons in lobsters. J Neurobiol 46:193–205.

Bossing T, Udolph G, Doe CQ, Technau GM. 1996. The embryonic central nervous system lineages of *Drosophila melanogaster*. Dev Biol 179:41–64.

Böswald M, Harasim S, Maurer-Schultze B. 1990. Tracer dose and availability time of thymidine and bromodeoxyuridine: application of bromodeoxyuridine in cell kinetic studies. Cell Tissue Kinet 23:169–181.

Campos-Ortega JA. 1995. Genetic mechanisms of early neurogenesis in *Drosophila melanogaster*. Mol Neurobiol 10:75–89.

Campos-Ortega JA, Hartenstein V. 1997. The embryonic development of *Drosophila melanogaster*. Berlin: Springer Verlag.

Cayre M, Malaterre J, Scotto-Lomassese S, Strambi C, Strambi A. 2002. The common properties of neurogenesis in the adult brain: from invertebrates to vertebrates. Comp Biochem Physiol B Biochem Mol Biol 132:1–15.

Cayre M, Strambi C, Charpin P, Augier R, Meyer MR, Edwards JS, Strambi A. 1996. Neurogenesis in Adult Insect Mushroom Bodies. J Comp Biol 371:300–310.

Ceron J, González C, Tejedor FJ. 2001. Patterns of cell division and expression of asymmetric cell fate determinants in postembryonic neuroblast lineages of *Drosophila*. Dev Biol 230:125–138.

Doe CQ. 1996. Asymmetric cell division and neurogenesis. Curr Opin Genet Dev 6:562–566.

Doe CQ, Chu-LaGraff Q, Wright DM, Scott MP. 1991. The *prospero* gene specifies cell fates in the *Drosophila* central nervous system. Cell 65:451–464.

Doe CQ, Fuerstenberg S, Peng C-Y. 1998. Neural stem cells: From fly to vertebrates. J Neurobiol 36:111–127.

Doe CQ, Skeath JB. 1996. Neurogenesis in the insect central nervous system. Curr Opin Neurobiol 6:18–24.

Dohle W, Scholtz G. 1997. How far does cell lineage influence cell fate specification in crustacean embryos? Sem Cell Dev Biol 8:379–390.

Dolbeare F. 1996. Bromodeoxyuridine: a diagnostic tool in biology and medicine. III. Proliferation in normal, injured and diseased tissue, growth factors, differentiation, DNA replication sites and *in situ* hybridization. Histochem J 28:531–575.

Gerberding M, Scholtz G. 1999. Cell lineage of the midline cells in the amphipod crustacean *Orchestia cavimana* (Crustacea, Malacostraca) during formation of the germ band. Dev Genes Evol 209:91–102.

Gerberding M, Scholtz G. 2001. Neurons and glia in the midline of the higher crustacean *Orchestia cavimana* are generated via an invariant cell lineage that comprises a median neuroblast and glial progenitors. Dev Biol 235: 397–409.

Goergen EM, Bagay LA, Rehm K, Benton JL, Beltz BS. 2002. Circadian Control of Neurogenesis. J Neurobiol, to appear.

Graziadei PP, Monti Graziadei GA. 1986. Principles of organization of the vertebrate olfactory glomerulus: an hypothesis. Neurosci 19:1025–1035.

Harrison PJ, Cate HS, Swanson ES, Derby CD. 2001. Postembryonic proliferation in the spiny lobster antennular epithelium: rate of genesis of olfactory receptor neurons is dependent on molt stage. J Neurobiol 47:51–66.

Harzsch S. 2001a. From stem cell to structure: Neurogenesis in decapod crustaceans. In: Wiese K, editor. The Crustacean Nervous System. Berlin: Springer Verlag, pp 417–432.

Harzsch S. 2001b. Neurogenesis in the crustacean ventral nerve cord: homology of neuronal stem cells in Malacostraca and Branchiopoda? Evol Devel 3:154–169.

Harzsch S, Dawirs RR. 1994. Neurogenesis in larval stages of the spider crab *Hyas araneus* L. (Decapoda, Brachura): proliferation of neuroblasts in the ventral nerve cord. Roux's Arch Dev Biol 204:92–100.

Harzsch S, Dawirs RR. 1996. Neurogenesis in the developing crab brain: postembryonic generation of neurons persists beyond metamorphosis. J Neurobiol 29:384–398.

Harzsch S, Miller J, Benton J, Beltz B. 1999. From embryo to adult: Persistent neurogenesis and apoptotic cell death shape the crustacean deutocerebrum. J Neurosci 19:3472–3485.

Harzsch S, Miller J, Benton J, Dawirs RR, Beltz B. 1998. Neurogenesis in the thoracic neuromeres of two crustaceans with different types of metamorphic development. J Exp Biol 201:2465–2479.

Helluy SM, Beltz BS. 1991. Embryonic development of the American lobster (*Homarus americanus*): Quantitative staging and characterization of an embryonic molt cycle. Biol Bull 180:355–371.

Helluy SM, Ruchhoeft M, Beltz B. 1995. Development of the olfactory and accessory lobes in the American lobster: An allometric analysis and its implications for the deutocerebral structure of decapods. J Comp Neurol 358:1–13.

Lois C, Alvarez-Buylla A. 1994. Long distance neuronal migration in the adult mammalian brain. Science 264: 1145–1148.

Ludwig P, Williams JLD, Lodde E, Reichert H, Boyan GS. 1999. Neurogenesis in the median domain of the embryonic brain of the grasshopper *Shistocerca gregaria*. J Comp Neurol 414:379–390.

Matsuzaki F. 2000. Asymmetric division of *Drosophila* neural stem cells: a basis for neural diversity. Curr Opin Neurobiol 10:38–44.

Reichert H, Boyan G. 1997. Building a brain: developmental insights in insects. Trends Neurosci 20:258–263.

Sandeman D, Sandeman R, Derby C, Schmidt M. 1992. Morphology of the brain of crayfish, crabs, and spiny lobsters: a common nomenclature for homologous structures. Biol Bull 183:304–326.

Sandeman R, Clarke D, Sandeman D, Manly M. 1998. Growth-related and antennular amputation-induced changes in the olfactory centers of crayfish brain. J Neurosci 18:6195–6206.

Sandeman R, Sandeman D. 2000. "Impoverished" and "enriched" living conditions influence the proliferation and

survival of neurons in crayfish brain. J Neurobiol 45:215–226.

Sandeman RE, Sandeman DC. 1996. Pre- and postembryonic development, growth and turnover of olfactory receptor neurones in crayfish antennules. J Exp Biol 199: 2409–2418.

Sauer FC. 1991. Developmental Neurobiology. New York: Plenum Press. p 44–45.

Schmidt M. 1997. Continuous neurogenesis in the olfactory brain of adult shore crabs, *Carcinus maenas*. Brain Res 762:131–143.

Schmidt M. 2001. Neuronal differentiation and long-term survival of newly generated cells in the olfactory midbrain of the adult spiny lobster, *Panulirus argus*. J Neurobiol 48:181–203.

Schmidt M, Harzsch S. 1999. Comparative analysis of neurogenesis in the central olfactory pathway of adult decapod crustaceans by *in vivo* BrdU-labeling. Biol Bull 196: 127–136.

Scholtz G. 1990. The formation, differentiation, and segmentation of the post-naupliar germ band of the amphipod *Gammarus pulex* L. (Crustacea, Malacostraca, Peracarida). Proc Roy Soc Lond B 239:163–211.

Scholtz G. 1992. Cell lineage studies in the crayfish *Cherax destructor* (Crustacea, Decapoda): germ band formation, segmentation, and early neurogenesis. Roux's Arch Dev Biol 202:36–48.

Steullet P, Cate HS, Derby CD. 2000. A spatiotemporal wave of turnover and functional maturation of olfactory receptor neurons in the spiny lobster *Panulirus argus*. J Neurosci 20:3282–3294.

Strausfeld NJ, Hildebrand JG. 1999. Olfactory systems: common design, uncommon origins? Curr Opin Neurobiol 9:634–639.

Sullivan JM, Benton JL, Beltz BS. 2000. Serotonin depletion *in vivo* inhibits the branching of olfactory projection neurons in the lobster deutocerebrum. J Neurosci 20: 7716–7721.

Truman JW, Ball EE. 1998. Patterns of embryonic neurogenesis in a primitive wingless insect, the silverfish, *Ctenolepisma longicaudata*: Comparison with those seen in flying insects. Dev Genes Evol 208:357–368.

Whitington PM. 1996. Evolution of neural development in arthropods. Seminars Cell Dev Biol 7:605–614.

Zacharias D, Williams JLD, Meier T, Reichert H. 1993. Neurogenesis in the insect brain: cellular identification and molecular characterization of brain neuroblasts in the grasshopper embryo. Dev 118:941–955.