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Regulation of life-long neurogenesis in the decapod crustacean brain

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Abstract

This article provides an overview of our understanding of life-long neurogenesis in the decapod crustacean brain, where the proliferation of sensory and interneurons is controlled by many of the same factors as is neurogenesis in the mammalian brain. The relative simplicity, spatial organization and accessibility of the crustacean brain provide opportunities to examine specific neuronal pathways that regulate neurogenesis and the sequence of gene expression that leads to neuronal differentiation.

Keywords: Cell proliferation; Circadian rhythms; Serotonin; Neuron precursors; NOS; Olfactory pathway

1. The context: continuous growth

1.1. Overview

The life-long addition of new neurons has been documented in many regions of the vertebrate and invertebrate brain, including the hippocampus of mammals (Altman and Das, 1965; Eriksson et al., 1998), song control nuclei of birds (Alvarez-Buylla et al., 1990), and olfactory pathway of rodents (Lois and Alvarez-Buylla, 1994), insects (Cayre et al., 1994) and crustaceans (Harzsch and Dawirs, 1996; Sandeman et al., 1998; Harzsch et al., 1999; Schmidt, 1997; Schmidt, 2001). One of the primary goals of current research in these systems is to understand the regulatory mechanisms that control the timing and rate of neurogenesis. Hormonal cycles (Rasika et al., 1994; Harrison et al., 2001b), serotonin (Gould, 1999; Brezun and Daszuta, 2000; Benton and Beltz, 2001a; Beltz et al., 2001), physical activity (Van Praag et al., 1999), living conditions (Kempermann and Gage, 1999; Sandeman and Sandeman, 2000; Scotto-Lomassese et al., 2000), seasonality (Barnea and Nottebohm, 1994; Huang et al., 1998; Ramirez et al., 1997; Dawson et al., 2001) and the day-night cycle (Chiu et al., 1995; Goergen et al., 2002; Jacobs, 2002) all influence the rate and timing of neuronal proliferation and survival in a wide variety of organisms, suggesting that mechanisms controlling life-long neurogenesis are conserved across a range of vertebrate and invertebrate species.

Unlike insects that progress through larval and nymph or pupal stages before becoming adults that no longer grow, most of the larger species of crustaceans attain their adult form early in their development when they are still very small and then grow indeterminately, increasing in weight by as much as 10,000 times from juvenile through adult stages (Wolff, 1978; Beltz et al., 2003). Small juveniles are sexually immature but their basic anatomy, musculature and central and peripheral nervous systems are essentially miniaturized versions of the adult form (Herrick, 1895; Sandeman and Sandeman, 1991). Their increase in size over many years is accomplished by periodic molting. New receptor neurons are added to cope with the increased surface area of the body, length of the appendages, or size of the compound eyes; these send new afferent axons into the central nervous system that must be received by an established inventory of interneurons, many of which are known to be unique in their individual architecture, function and connections (Letourneau, 1976; Sandeman, 1982; Laverack, 1988). To some extent, such identified interneurons accommodate the new incoming afferents by an increase in the size of the input arborization of the cell. This in turn leads to an increase in the overall size of the brain.

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Measurements from lobsters and crayfish show that the volume of specific neuropils of the brain increase linearly with the length of the animals' carapace (Helluy et al., 1995; Sandeman et al., 1998). However, brain growth in the crustaceans also occurs by the continuous addition of new neurons to certain areas. Cell clusters 9 and 10 on each side of the brain contain local and projection interneurons, respectively, that are associated with the olfactory (OL) and accessory (AL) lobes (Fig. 1) (Sandeman et al., 1992). These neurons are characterized by being small (about 10 μm in diameter) and in having the same size and packing density in small and large individual animals. The volumes of the cell clusters increase linearly with the size of the animal's body leading to the inescapable conclusion that new cells are added to these cell clusters throughout the lives of the animals. Bromodeoxyuridine (BrdU) studies confirm that these clusters are indeed the only sites of cell proliferation that persist in the midbrain of adults (Fig. 2) (Schmidt, 1997; Sandeman et al., 1998; Harzsch et al., 1999; Schmidt and Harzsch, 1999). Thus, the assumption that the numbers of neurons in the central nervous systems of decapod crustacea are fixed throughout adult life (Purves, 1988) can be laid to rest.

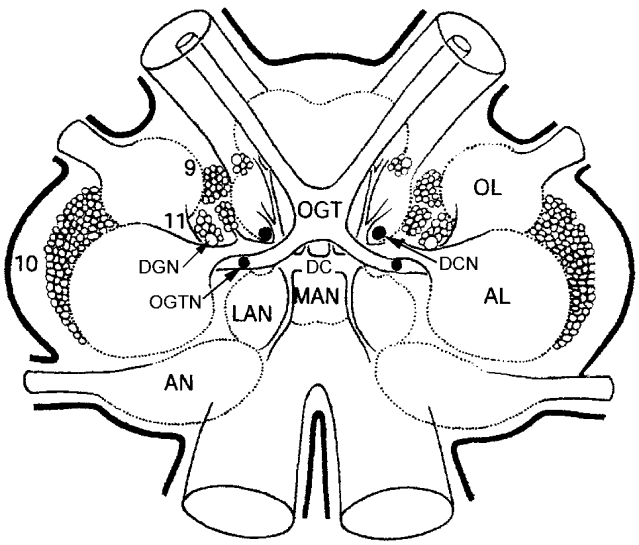

Fig. 1. Diagram of the brain of the crayfish *Cherax destructor* showing the locations of the cell body clusters and neuropils. The lateral protocerebrum and the optic neuropils are contained in the eyestalks (not shown). The paired olfactory lobes (OL) receive, probably exclusively, the entire afferent input from the olfactory receptor neurons (ORNs) associated with the aesthetasc sensilla on the lateral antennular flagellum. The larger accessory lobes (AL) lie caudal to the OLs and receive their main input from high order neurons, the DC neurons located in cluster 11. Cell bodies of local interneurons in cluster 9 (9) lie on the ventral surface of each side of the brain, medial to the OL. The projection neuron cell bodies of cluster 10 (10) lie lateral and ventral to the ALs and the OLs. Cluster 11 (11) lies dorsal and medial to the OLs. A group of local interneurons with their cell bodies in cluster 11 carry information from centers in the protocerebrum, deutocerebrum and tritocerebrum and end exclusively in the glomeruli in the ALs. The dorsal giant neuron (DGN) also has its cell body in cluster 11. AN, antenna II neuropil; DC, deutocerebral commissure; DCN, deutocerebral commissure neuropil; LAN, MAN, lateral and median antennular neuropils; OGT, olfactory globular tract; OGTN, olfactory globular tract neuropil. (Modified from Sandeman and Sandeman, 2003).

1.2. The crustacean deutocerebrum

A common ground plan of the deutocerebrum or midbrain can be recognized in all decapod crustaceans (Sandeman and Scholtz, 1995). The deutocerebrum in the nervous systems of adult crayfish and lobsters contains two areas where persistent neurogenesis has been found. Anatomical details of the midbrain are provided in Fig. 1 for reference. The deutocerebrum receives the afferent inputs from the antennules (1st antennae). The axons of the olfactory receptor neurons (ORNs) arrayed in the olfactory organs on the outer flagella of the antennules project to the OL, penetrate the lobe from its periphery and terminate in olfactory glomeruli. Axons from the mechanoreceptors and chemoreceptors on both flagella and basal segments of the antennules project to the lateral antennular neuropils (Schmidt and Ache, 1996; Cate and Derby, 2001), which, with the median antennular neuropils also house the motor neurons that control the movements of the antennules. The deutocerebrum contains the ALs which, in lobsters and crayfish, are large spherical neuropils that lie caudal to the OLs. The ALs do not receive the terminals of primary afferent axons, nor do they contain motor neurons. Two smaller neuropils, the olfactory globular tract neuropil (OGTN) and the deutocerebral commissure neuropil (DCN), complete the known complement of the deutocerebrum (Sandeman and Mellon, 2001).

Three bilateral clusters of interneuronal cell bodies, labeled clusters 9, 10 and 11 (Sandeman et al., 1992), are associated with the OLs and ALs. The neurons in cluster 9 are local interneurons that lie medial to the OL and project ipsilaterally to the ALs and OLs. Neurons in cluster 10 (the projection neurons) lie lateral to the OLs and ALs. They branch extensively in the OLs and ALs and their axons leave the OL/AL complex in the olfactory globular tract (OGT) that projects bilaterally to neuropils in the lateral protocerebrum in the eyestalks (Sullivan and Beltz, 2001a,b). Persistent neurogenesis in the deutocerebrum has been found only within clusters 9 and 10. Cluster 11 contains interneurons that have their axons in the deutocerebral commissure (DC) and that terminate in the ALs. Cluster 11 also houses the cell body of the dorsal giant neuron (DGN), which innervates the ipsilateral OL and AL.

2. Developmental neurogenesis

Knowledge of the mechanism of developmental neurogenesis among arthropod species has relied heavily on the study of insect systems, where the sequence of events from the first interactions of the proneural genes, to the delamination of the typical asymmetrically dividing stem cells (neuroblasts), to the production of ganglion mother

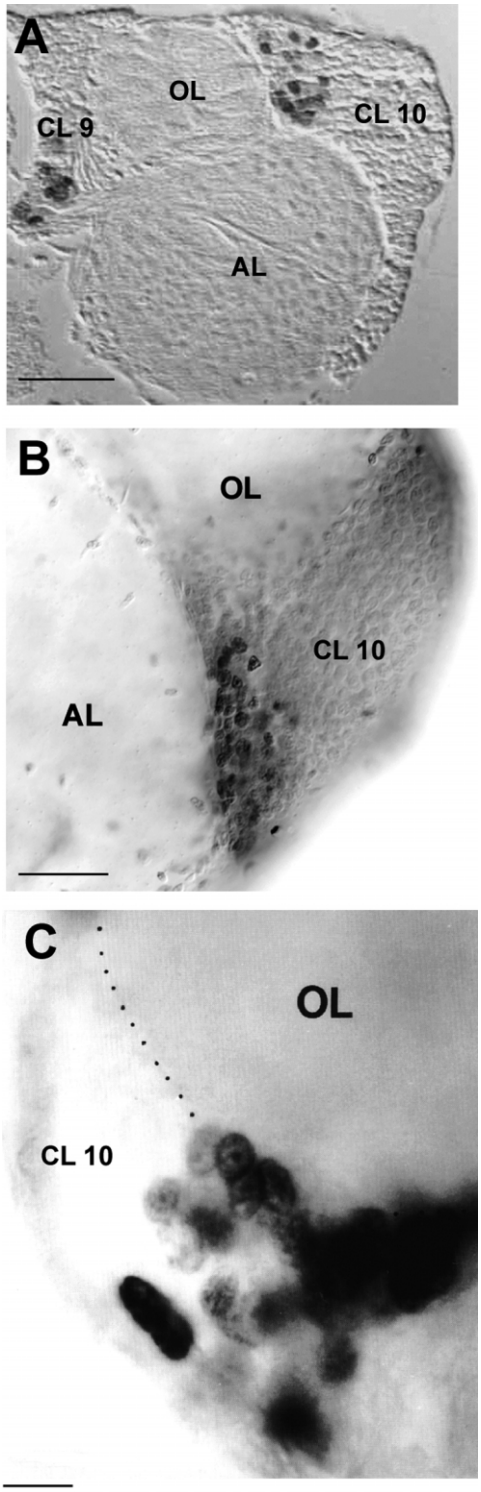

Fig. 2. Proliferation zones in the brains of *Cherax destructor* (A), *Homarus americanus* (B) and *Hyas araneus* (C). Cells that went through the S phase of the cell cycle during the period of BrdU incubation are darkly labeled against unstained tissues. (A) Section through the proliferation zones of clusters 9 (Cl 9) and 10 (Cl 10) in the brain of a juvenile *C. destructor*. (B) The cluster 10 proliferation zone of *H. americanus*, shown in a whole mount of a juvenile lobster brain. (C) BrdU-labeled cells in cluster 10, adjacent to the olfactory lobe (OL), are seen in a megalopa (day 17) of *H. araneus*. AL, accessory lobe; OL, olfactory lobe. Scale bars (A) 50 μm; (B) 50 μm; (C) 10 μm. (A modified from Sandeman and Sandeman (2000); B modified from Goergen et al., 2002; C modified from Harzsch and Dawirs, 1996).

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 arrangements 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, and the cell lineage that leads to the formation of the first neuroblasts in the ventral nerve cord has been studied in a variety of crustaceans (reviewed in Scholtz and Dohle, 1996; Dohle and Scholtz, 1997; Harzsch, 2001). In common with insects, crustacean neuroblasts undergo unequal divisions to produce ganglion mother cells which later divide again to give birth to neurons (Fig. 3), however, at least in the lobster brain, there are some interesting differences.

Our understanding of the sequence of cell divisions leading to the production of neurons in the antennule and olfactory midbrain is far less secure than in the ventral nerve cord. Studies of deutocerebral interneurons in embryos of the lobster *Homarus americanus* demonstrate that three distinct cell types (large precursors, intermediate precursors, and neurons; based upon size and shape) are involved in neurogenesis, as in the insects (Fig. 4A) (Benton and Beltz, 2002). However, the intermediate precursors of the brain neurons undergo multiple divisions to generate neurons, in contrast to the single division of the ganglion mother cells of insects and the crustacean ventral nerve cord. This conclusion is based on counts of large and intermediate precursor cells and neurons in cluster 10 of *H. americanus* embryos on sequential days after BrdU administration (Fig. 4B). 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 neurons. Although quantitative studies have not been conducted in the local interneuron cluster 9, it is clear that the same size categories of cells participate in the proliferation sequence in embryos (Benton and Beltz, 2001a). Three size categories of cells also are observed in the antennules of the lobster *Panulirus argus* (Harrison et al., 2003), but the relationships between these precursor cells have not yet been clarified.

The characteristics of BrdU labeling shown in Fig. 4B also suggest a time frame for the proliferation of olfactory projection neurons in embryonic lobsters (Benton and Beltz, 2002). Three to four days following BrdU exposure, large precursor cells are no longer labeled; this time frame indicates 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

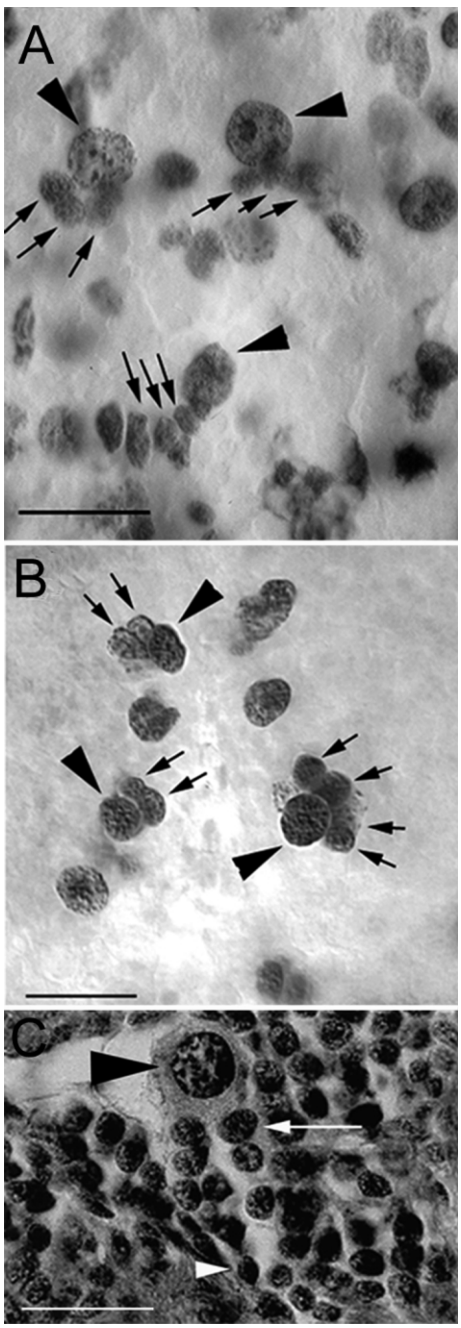

Fig. 3. BrdU labeling in the embryonic ventral nerve cord (A) and brain (B) of the lobster, *H. americanus* (whole mount preparations). (A)Third and fourth thoracic ganglia (neuromeres 6 and 7) at E20%. BrdU-labeled large precursor cells (arrowheads) with rows of progeny, the intermediate precursor cells (small arrows). (B) BrdU-labeled large precursor cells (arrowheads) and associated progeny (small arrows) in the midline of the anterior protocerebrum at E72%. (C) Ten micron sections of a silver impregnated embryonic *C. destructor* brain at E80%. The area shown is of cluster 9 cells that lie between the median protocerebrum and the olfactory lobe. A large stem cell in interphase is shown (black arrowhead) at the top of a column of progenitor cells that have budded from it. Intermediate precursor cells are noted with a white arrow, and presumed cluster 9 neurons with a white arrowhead. Scale bars: (A) 50 μm; (B) 25 μm; (C) 20 μm. (A, B modified from Benton and Beltz, 2001); C modified from Sandeman and Sandeman, 2003).

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. Studies in lobster embryos (Harzsch et al., 1999) have shown that neuronal proliferation occurs at a high rate during the developmental period examined in these experiments. Therefore, the lack of the largest labeled profiles on day 4 (Fig. 4B) is likely to indicate the time

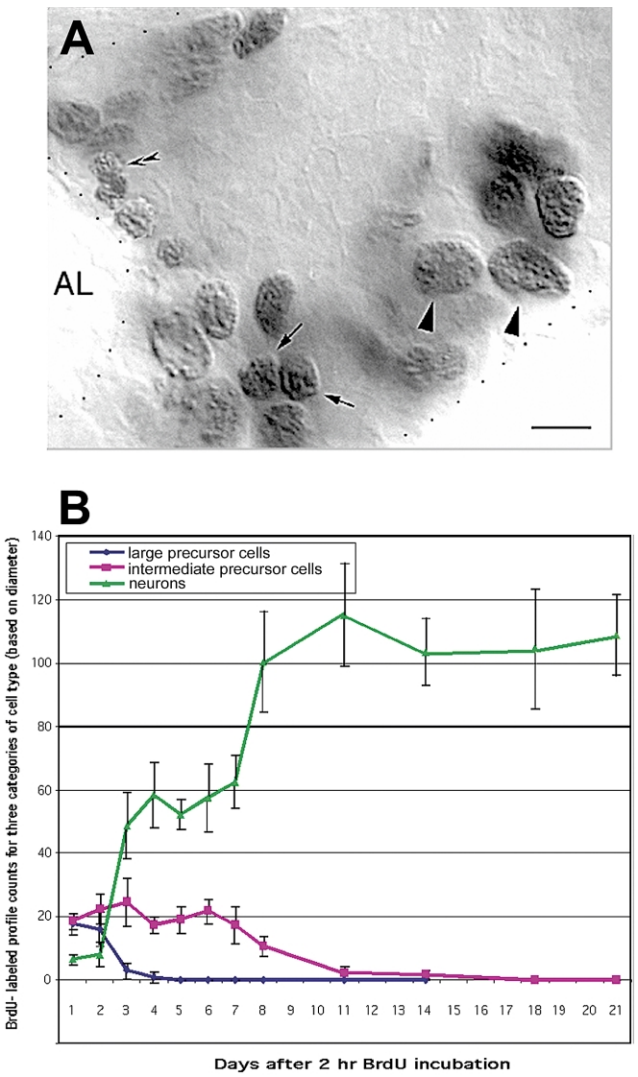

Fig. 4. (A) Photograph of cluster 10 in a whole mount embryonic lobster brain (E72%) showing labeled profiles after a 1 h survival time following a 2 h BrdU incubation. Three distinct sizes of BrdU-labeled profiles are observed: large precursor cells (9.5–11 μm; large arrowheads); intermediate precursor cells (6-8 μm; arrows); and small neuronal cell types (3–5 μm; small double arrowheads). (B) Graph of BrdU-labeled profile counts for the three cell types: large precursors, intermediate precursors and neurons. Large (blue) and intermediate (magenta) precursor cells predominate during the first 3 days after a 2 h 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. Scale bar: (A) 10 μm (modified from Benton and Beltz, 2002).

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.

It is not clear whether the divisions of the large precursor cells in cluster 10 are asymmetric, as are the vast majority for neuroblasts in both insects (Bossing et al., 1996; Campos-Ortega and Hartenstein, 1997; Doe et al., 1998; Ceron et al., 2001) and in the crustacean ventral nerve cord (Scholtz, 1992; Harzsch and Dawirs, 1994; Harzsch et al., 1998). In the brains of lobster embryos that have been exposed to BrdU, the typical image of large asymmetrically dividing precursors with associated columns or clusters of smaller cells (Fig. 3) is not seen in either the local or projection neuron clusters (Benton and Beltz, 2001a, 2002). The small differences between the counts of the BrdU-labeled large precursors and intermediate precursors in cluster 10 also suggest that the large precursors may be dividing only once, which also would indicate a symmetrical mode of division (Benton and Beltz, 2002). The pattern of cell divisions that generates neurons in the midbrain of lobster embryos is, therefore, different from the standard pattern in the crustacean ventral nerve cord and insect systems.

When the brains of juvenile and adult lobsters are examined, the large precursor cells that are typically observed in the embryonic brain are no longer seen (Benton and Beltz, 2002). This finding is in agreement with results in adult *P. argus* (Schmidt, 2001), where precursors to the projection neurons could not be distinguished on the basis of size and shape. A reduction in the numbers of large precursors also has been reported in late embryonic stages of *Cherax destructor* (Fig. 5) (Scholtz, 1992; Sandeman and Sandeman, 2003) and *Hyas 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, 2001). Alternatively, as the cell cycle slows down with periods of quiescence, the size of nuclei in the proliferative cells may decrease, reflecting the more condensed and inactive DNA. Therefore, although life-long neurogenesis has been documented in the local and projection neuron clusters of lobsters and other malacostracans, the progenitor cells that persist during the later life stages may represent a different type of proliferative cell than has been described in embryos.

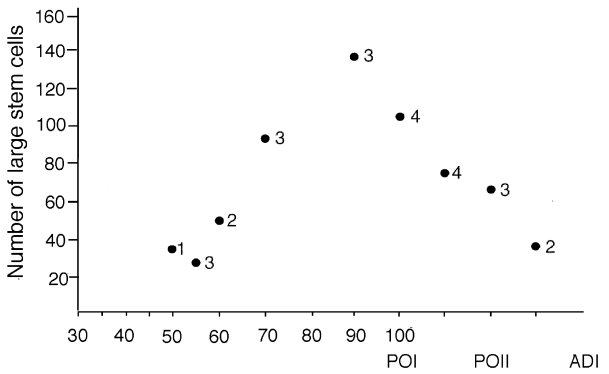

Fig. 5. Graph displaying means of large stem cell counts from toluidine blue stained brains of crayfish at different stages of development. The number of animals counted appears next to each point. The first large stem cells can be reliably identified in *C. destructor* at 50% of embryonic development and the number of stem cells then increases steadily to reach a peak at hatching (POI). From this point their numbers decline, although some can still be found in ADIV animals (not shown) (modified from Sandeman and Sandeman, 2003).

3. Adult neurogenesis

3.1. Peripheral systems

Crustaceans add new receptor cells to their olfactory organs (and lose them) throughout their lives (Mellon and Alones, 1993; Sandeman and Sandeman, 1996; Steullet et al., 2000). Both juvenile and adult animals generate new receptors at the proximal end of the lateral flagellum. In large adults, older, distal-most segments of the antennules are shed during molting, along with the ORNs that they carry (Sandeman and Sandeman, 1996). There is nevertheless a net increase in the total number of receptors on the antennule because more neurons are added at the proximal end of the receptor array than are lost from the tip. The crustacean olfactory organ resembles a conveyer belt in which the new ORNs (about 300 ORNs are associated with a single aesthetasc sensillum) develop at the base in the 'proliferation zone' and the older ones jettisoned at the tip of the antennular flagellum, the 'senescence zone' (Fig. 6). In between the proliferation and senescence zones, ORNs mature and become odor responsive (the 'maturation zone') (Steullet et al., 2000). Thus, the crustacean olfactory organ has a proximal-distal axis of ORN birth, differentiation and death; in spiny lobsters (*P. argus*) ORNs live for ~2 years (Steullet et al., 2000). This process resembles the well-known 'turnover' phenomenon found in adult mammals in which individual ORNs die and are replaced from below with new cells (Moulton, 1974; Farbman, 1991; Brunjes, 1992). The developmental axis in the crustacean antennule provides a system unlike other prominent invertebrate models such as *Drosophila melanogaster* and *Caenorhabditis elegans* which lack post-embryonic turnover of olfactory neurons. Derby and collaborators (Harrison et al., 2001a) are taking advantage of the spatio-temporal wave of cell proliferation and differentiation in the olfactory organ of lobsters in studies aimed at identifying the genes associated with the generation and maturation of new ORNs (Stoss et al., 2001; Johns et al., 2002; Hollins et al., 2003).

3.2. Central systems

The first evidence for an extended period of neurogenesis in the crustacean central nervous system was provided by Harzsch and Dawirs (1996), who showed that neurogenesis

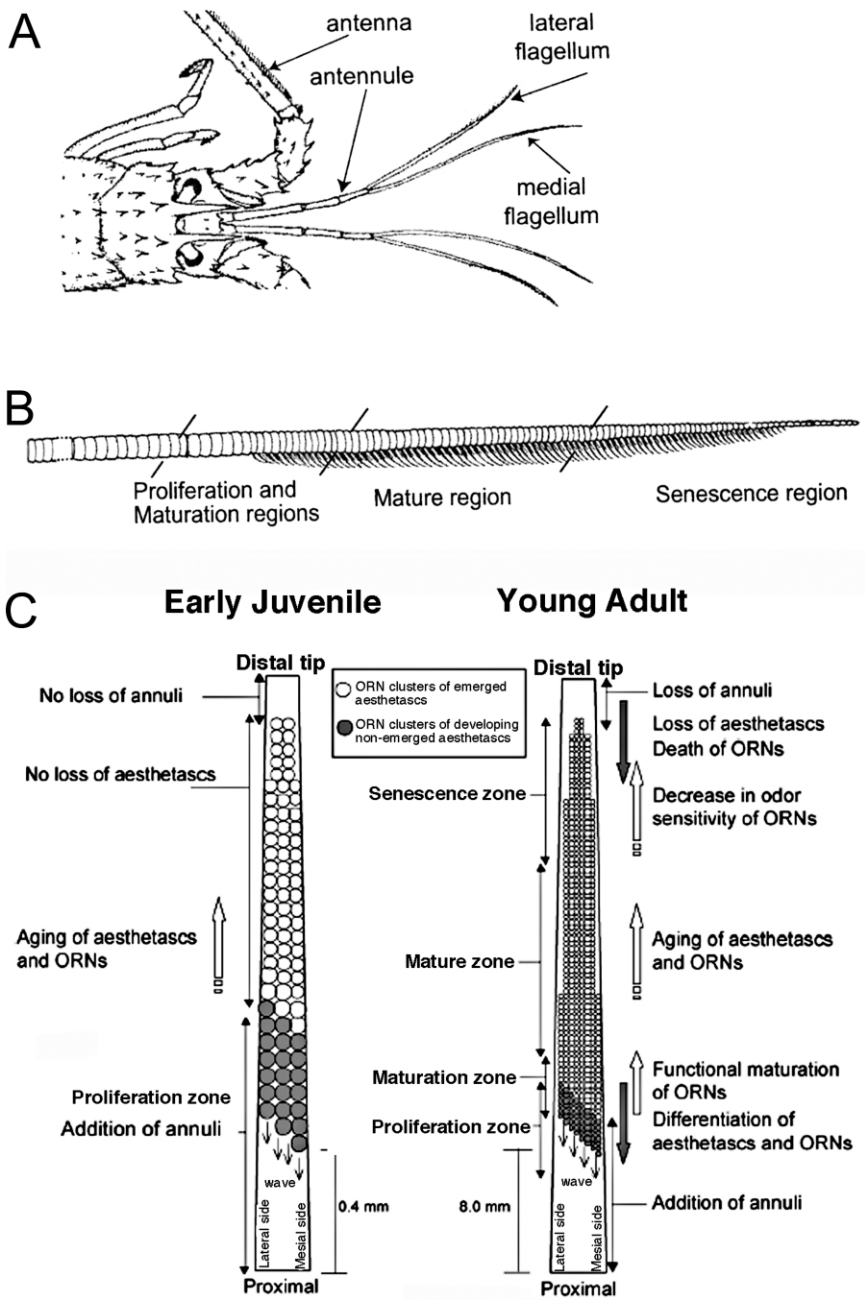

Fig. 6. The olfactory organ of *P. argus*. (A) Olfactory sensory units are located on the lateral flagellum of each antennule. (B) The lateral flagellum is composed of ring-like cuticular annuli, each housing numerous types of setae. The aesthetasc array is located on the ventral surface of the distal half of the lateral flagellum. (C) Schematic summary of differences between early juvenile and adult spiny lobsters in the turnover of aesthetascs and their olfactory receptor neurons (ORNs) in the antennular flagellum. The flagella of early juveniles and adults are drawn here on different scales since they are depicted as the same size. The olfactory organs of all the decapods have the same general functional anatomy. (A, B reprinted from Harrison et al., 2003 and C, Derby et al., 2003).

continues after the second metamorphosis in clusters of interneurons in the olfactory pathway in the brain of the spider crab *H. araneus*. In adult shore crabs, *Carcinus maenas*, Schmidt (1997) then documented neurogenesis of deutocerebral projection neurons and hemiellipsoid body local interneurons, which are the targets of the projection neurons. These initial publications were followed by a series of studies using the BrdU labeling technique in a variety of decapod species representing five infraorders and six distinct families (*C. destructor*, Sandeman et al., 1998; Schmidt and Harzsch, 1999; *H. americanus*: Harzsch et al., 1999; Schmidt and Harzsch, 1999; *Cancer pagurus*, *Pagurus bernhardus*, *P. argus*, *Sicyonia brevirostris*: Schmidt and Harzsch, 1999). These wide-ranging reports provided convincing evidence of life-long neurogenesis in the adult crustacean brain, and demonstrated that there are

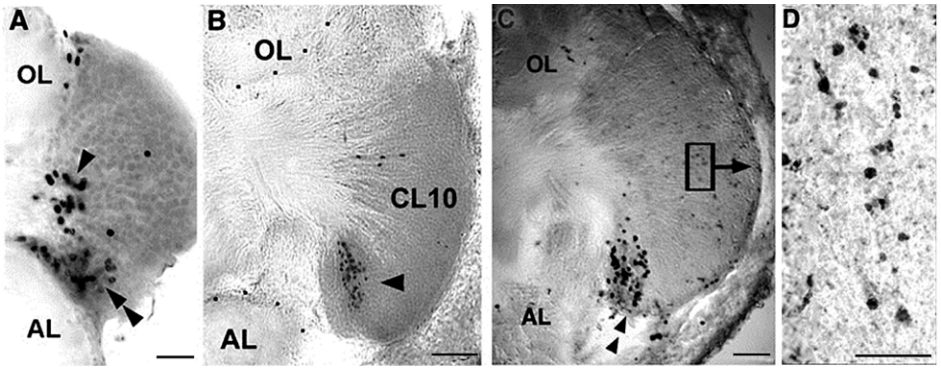

Fig. 7. Persistent cell proliferation in cluster 10, which contains the somata of the deutocerebral projection neurons, demonstrated with bromodeoxyuridine (BrdU) labeling in juvenile (A) and adult (B-D) lobster brains. Anterior is at the top of each image. (A) Newly born projection neurons are labeled in 0.5-year-old juveniles by injection of BrdU (1 mg of BrdU/30 g of body weight) 3 weeks before sacrifice and with a second pulse on the day they were killed. The proliferating cells labeled by the 24 h pulse are indicated by double arrowheads, while the cells labeled by the injection 3 weeks prior to sacrifice are indicated by a single arrowhead. As newborn cells mature, they migrate anteriorly in cluster 10. (B) BrdU injection of adult lobsters followed by sacrifice 6 weeks later demonstrates a band of labeled cells (arrowhead) that has moved anteriorly in cluster 10 from their presumed origin at the posterior margin of the cluster. (C) In an experiment where BrdU was injected into adult lobsters at 6 months and again at 24 h before sacrifice, the proliferation zone (double arrowheads) is labeled posteriorly in the cluster, while individual labeled cells have dispersed among the cluster 10 neurons. D. Higher magnification photomicrograph of labeled cells outlined in C. Scale bars: (A–C) 50 μm; (D) 20 μm (modified from Beltz et al., 2001).

features in common among these species. For example, exposure to BrdU immediately followed by sacrifice and processing, reveals the presence of proliferative zones in both clusters 9 and 10 in crayfish and lobsters (Fig. 2) (Schmidt, 1997; Sandeman et al., 1998; Harzsch et al., 1999). In contrast, neurogenesis has been demonstrated only among the projection neurons of cluster 10, not among the local interneurons of cluster 9, in shrimp and crabs (Schmidt, 1997; Schmidt and Harzsch, 1999). It may be, therefore, that continued neurogenesis in cluster 9 is associated with species with large ALs. Long survival time studies in which the exposure to BrdU is followed by a period of anything from days to months before sacrifice and processing (Harzsch et al., 1999; Schmidt, 2001), show that labeled cells are no longer confined to the proliferation zones but are dispersed throughout the cell clusters (Fig. 7) (Beltz et al., 2001). Long survival times of the labeled cells, in addition to the acquisition of differentiated properties such as transmitter accumulation (Schmidt, 2001), are taken as evidence that these cells become incorporated as functional neurons in the brain.

Studies on adult neurogenesis in the crustacean brain also report the presence of apoptosis (cell death) that occurs in parallel with proliferation among the OL and AL interneurons, suggesting turnover of these cells as a normal mechanism of structural plasticity (lobster: Harzsch et al., 1999) or in response to antennular ablation (crayfish: Sandeman et al., 1998), and potentially mirroring the turnover of peripheral ORNs. Cells in the brain that die can be detected as pycnotic profiles in toluidine blue stained sections or by treating the tissue with the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) method (Fig. 8) (Sandeman et al., 1998; Harzsch et al., 1999). A study that labeled for both cell proliferation (using the antiphospho histone H3 mitosis marker) and cell death (TUNEL method) in the lobster brain, demonstrated that many of the TUNEL-labeled profiles are localized within the proliferation zone in cluster 10 (Mahoney, 2002), suggesting that there is a culling of new neurons prior to differentiation, as in insect (Ganeshina et al., 2000) and vertebrate (Brown et al., 2001) systems. Additionally, preliminary results indicate that apoptosis, like neurogenesis (Goergen et al., 2002), may be regulated by circadian influences (Wasserman and Beltz, unpublished results). So far there is no information about whether changes in the patterns of cell death occur during the molt cycle, but there is a pronounced effect on the number of TUNEL-labeled profiles after damage, such as when an antennule has been removed from the animal (Sandeman et al., 1998).

3.3. Response to damage

Crustaceans not only grow but also have a strong regenerative capability (Skinner, 1982). There is a limit to this, however, as a lost stalked eye will not be replaced by another eye, suggesting that regeneration of the central nervous system may not be possible. Nevertheless, any appendage and its sense organs, including the antennules, can be replaced if it is damaged or lost (Fig. 9) (Laverack, 1988).

Harrison et al. (2003) have described the sequence of events that culminates in the formation of new ORN clusters in the regenerated antennule of spiny lobsters (*P. argus*). The picture they present is of a highly dynamic regulation of ORN turnover, as in vertebrates where hormones, growth factors, and CNS or ORN damage elicit a proliferative response in the stem cells generating ORNs (Brunjes, 1992; Farbman, 1992; Malun and Brunjes, 1996; Calof et al.,

1998). In the spiny lobster, following ablation of the entire antennule, including the proliferation zone, the entire organ regenerates following the formation of a blastema and the appearance of proliferating cells in patches within the antennular epithelium. If the antennule is removed distal to the proliferation zone, the rate of cell proliferation in the proliferation zone increases to compensate proportionally for the amount of tissue removed. Finally, local damage induced by shaving aesthetasc sensilla, which removes much of the ORNs dendrites, leads to the death of those ORNs but not to other cells. This ORN death induces local cell proliferation, where it normally does not occur, without affecting proliferation either in neighboring regions or in the proliferation zone. This shows that while proliferation normally occurs only in the proliferation zone, proliferation can occur anywhere along the olfactory organ, suggesting the presence of progenitor cells everywhere in the antennule, and that these progenitors are held under control unless the proper signals are present to trigger a return to the cell cycle and proliferation. From these data, Harrison et al. (2003) suggest a model for the control of stem cell proliferation that includes an inhibitory signal released by mature ORNs as well as a local positive inductive signal, an inductive signal associated with the proliferation zone, and an additional inductive signal released by the blastema that forms at the wound site following antennular damage or ablation (Harrison et al., 2001b, Harrison et al., 2003).

The consequences of antennule ablation in the central nervous system also have been explored in crayfish, lobsters and crabs (Sandeman et al., 1998; Hansen and Schmidt, 2001; Harrison et al., 2003). The unilateral nature of the projections from the antennule to the OLs enables the unoperated side to be used as a control in such studies. The expected response in the crustacean central nervous system to the removal of an antennule would be a change in the volume of the OL because of the degeneration of the central ends of the many thousands of ORN afferents that terminate in the glomeruli of the OLs. The AL does not receive any primary afferent endings and so a volume change following antennule ablation could only be due to death of the projection or local interneurons.

Neuropil volume changes following unilateral antennule ablation in adult crayfish with carapace lengths between 3.2 and 4 cm, were assessed five months after the operation and the results were in accord with the above prediction: OLs on the side of the ablation in these animals were reduced to 65 to 70% of the control volume. ALs, on the other hand, were reduced by only about 10% compared to controls (Sandeman et al., 1998). Cell proliferation in large animals that had partly reconstituted the ablated antennule was about 30% higher in clusters 9 and 10 on the operated than on the control side.

Antennular ablation in juvenile crayfish results in an increase in the number of TUNEL profiles in clusters 9 and 10 on the operated as compared to the unoperated side (Sandeman et al., 1998). Up to six times as many TUNEL profiles were found on the amputated side in comparison to the control side of the brains of the experimental animals. The implication is that damage to the afferents is transferred to second order neurons, which will also die. TUNEL profiles were found in both clusters 9 and 10 but it is not possible to determine the locations of the neurons that died as a direct result of the removal of the afferents, because of the ongoing cell death in clusters 9 and 10 in animals that have received no damage to their antennules.

The reconstitution of a lost or damaged appendage and the accompanying changes in the central nervous system occur over several molts subsequent to the injury, and in large animals this can take months due to the long intermolt period. These processes can be observed over a much shorter time span in newly hatched crayfish where the intermolt period is short. The antennules of the first postembryonic stage in crayfish were ablated and the consequences in the brain were observed by comparing the amputated with the non-amputated side. For crayfish where an antennule was ablated in the first postembryonic stage (POI), the OL volumes on the amputated side had declined to about 40% to 60% of the control side by the time they had grown to the first adult stage (ADI) and this was maintained up to the third adult stage (ADIII). By the fifth adult stage (ADV) though, the OLs were again the same size on both sides of the brain. The number of projection neurons, estimated from the measured volume of the cell cluster, dropped to between 80 and 90% of the control side and, like the OLs, did not reach the same size as the control side of the brain until ADV. The increase in the OL volume in all cases was accompanied by the reappearance of the ORNs on the antennules and one can assume that most of the increase would have been caused by the afferent axons layered around the outside of the lobe. There are no data on the rates of neurogenesis during this process but given the increase in neurogenesis that occurs with the addition of receptor neurons during normal growth, it seems likely that cell proliferation in the ADIII to ADV stages would also have been higher than normal. To restore the OL to the same size as the control side means that its growth has to be accelerated to ‘catch up’ with the non-amputated antennule. This occurs over the molts from POI to the ADV stage when symmetry is re-established (Sandeman et al., 1998).

Nitric oxide has been implicated in the initial response to antennular damage or ablation (Benton and Beltz, 2001b). In brains of lobsters and crayfish with intact antennules, various elements in the olfactory pathway stain intensely for nitric oxide synthase (NOS) using both immunocytochemical (Fig. 10) and enzyme (diaphorase) assays (not shown). Strong NOS labeling is consistently seen in the cluster 10 proliferation zone (Fig. 10A), DC and DCNs (Fig. 10C), OGTN (Fig. 10A and C) and in glomeruli in the OLs and ALs (Fig. 10C). The DGN stains dynamically for NOS (Fig. 10B), but the physiological situations that promote or inhibit NOS expression in this neuron are not understood. Because nitric oxide signaling is known in other systems to be

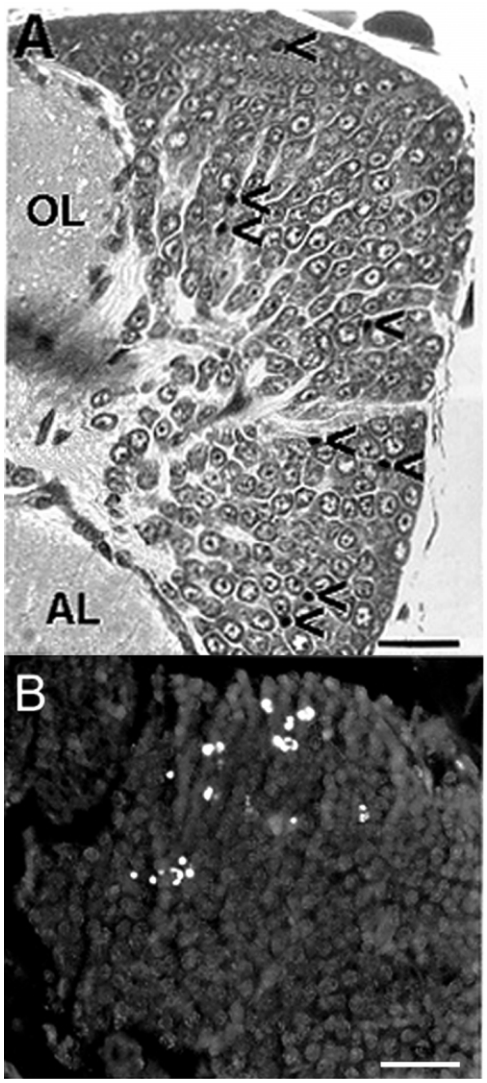

Fig. 8. Cell death in cluster 10 demonstrated in a toluidine blue-stained section (A) and by TUNEL labeling (B). (A) Cluster 10 in a brain from a postembryonic stage 4 lobster, *H. americanus*, stained with toluidine blue using standard histological methods. Darkly stained pyknotic nuclei that exhibit compaction of the nucleus and condensation of the chromatin are scattered throughout cell cluster 10 (arrowheads), often near neurons that display a regular nuclear morphology. (B) TUNEL profiles in cluster 10 of a brain from *C. destructor*. The TUNEL profiles have the typical shape and size of projection neurons residing in this cluster. Scale bars: (A) 25 μm; (B) 50 μm (A modified from Harzsch et al., 1999).

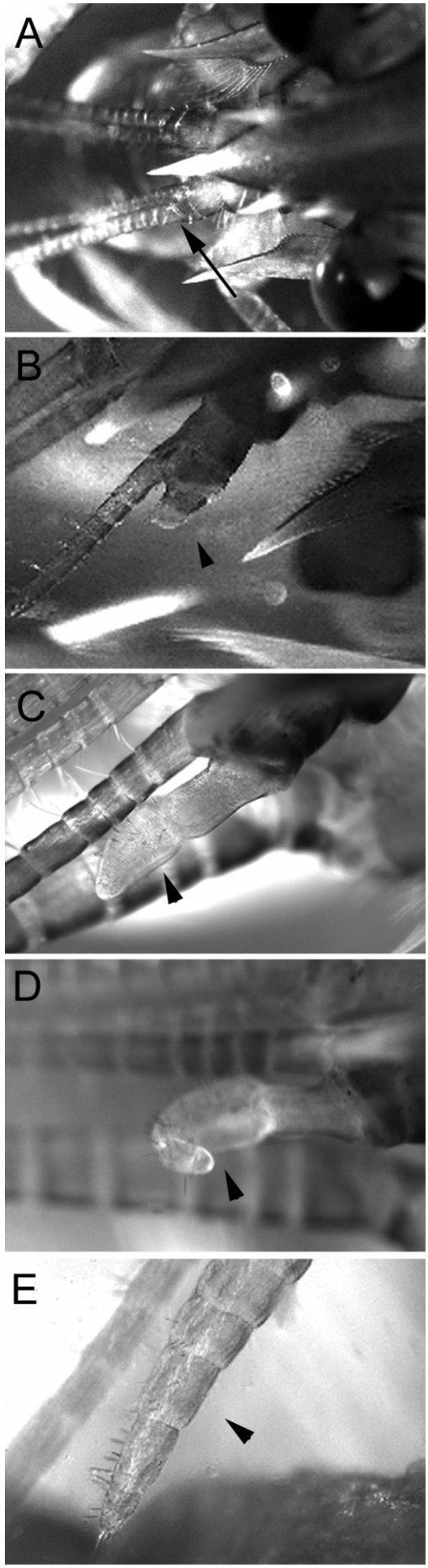

Fig. 9. Series of photos illustrating the process of antennular regrowth following ablation of the lateral flagellum in a juvenile *H. americanus*. (A) Uncut antennule, showing the position of the cut just distal to the bifurcation of the antennule. (B) After 9 days, the blastema that formed during the first few days at the site of the cut has begun to grow and a few hairs protrude from the tip. (C) By day 20 the distal end of the blastema is swollen and rounded, with the number of protruding hairs increasing. (D) By day 27 the tip of the regrowing flagellum has many dark short hairs, and the distal part of the tip moves independently, as if segmented. (E) By day 37, there are between 5 and 7 distinct segments that have regenerated at the site of the cut flagellum. Soft hairs appear at the tip.

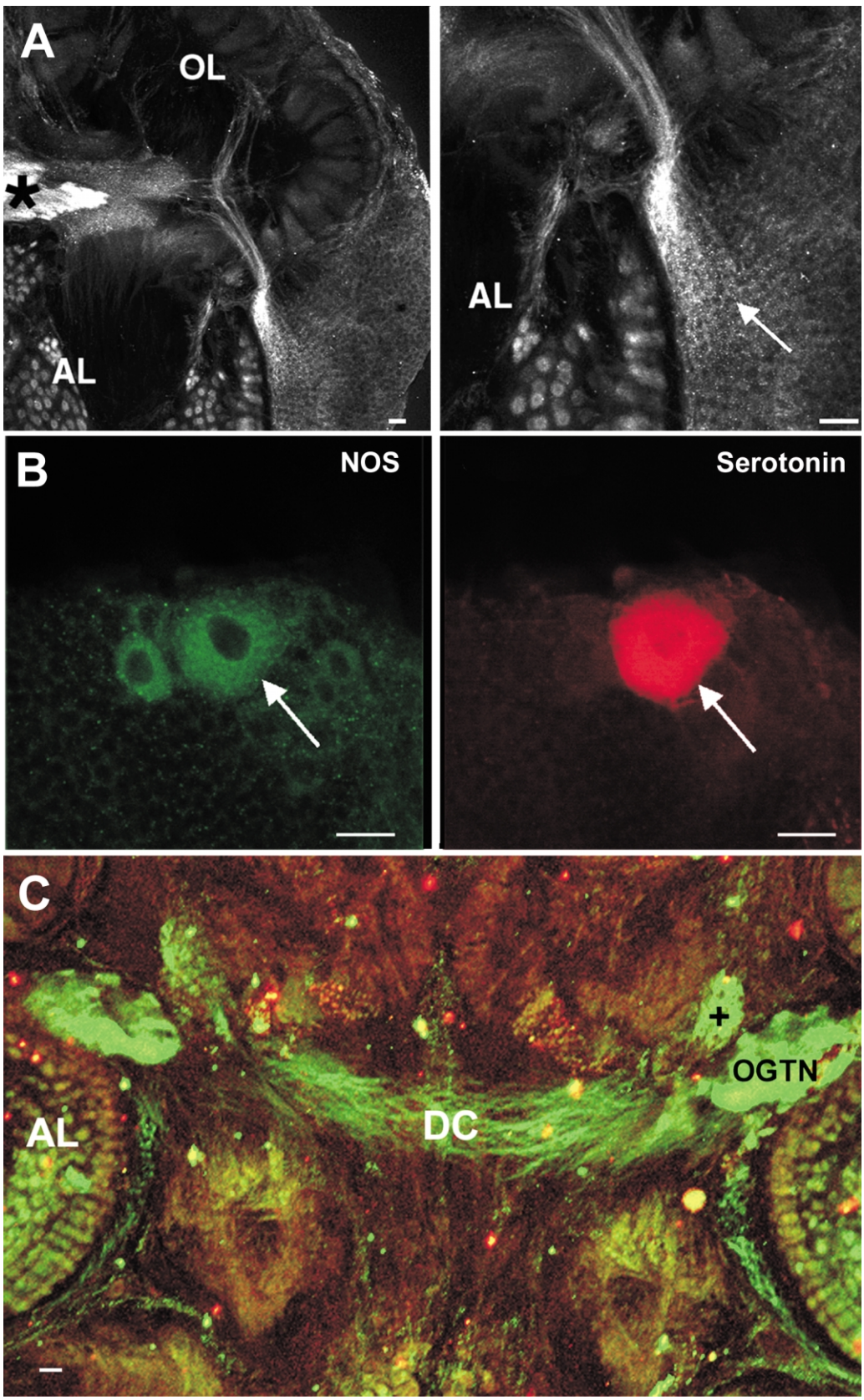

Fig. 10. Patterns of nitric oxide synthase (NOS) distribution in the deutocerebrum of the juvenile lobster, *H. americanus*, are demonstrated with immunocytochemical techniques. (A) Intense NOS labeling is consistently seen in the olfactory globular tract neuropil (asterisk, left panel) and in the cluster 10 proliferation zone (arrow, right panel). (B) The DGNs, which consistently contain serotonin immunoreactivity (right panel), also stain dynamically for nitric

involved in dynamic changes such as synaptogenesis and apoptosis (Bredt and Snyder, 1992; Roskams et al., 1994; Groc et al., 2002), staining for NOS was assessed over a month-long period following antennular ablation. Results suggest that the initial response to antennular ablation may be mediated, at least in part, by nitric oxide. This is implied by experiments in the lobster *H. americanus*, where NOS was localized in the brain at intervals following antennular ablation. During the first 24 h following removal of the lateral flagellum of the antennule, a consistent sequence of NOS labeling is observed in the olfactory pathway, culminating with intense staining in the OGT at 24 h post-ablation. Labeling on the side of the OGT ipsilateral to ablation is more intense than labeling on the non-ablated side. In contrast, the OGT in control animals that sustained no antennular damage is only slightly stained above background levels. These results suggest that NO may be part of the signaling pathway underlying the central response to antennular ablation (Benton and Beltz, 2001b).

4. Regulation of neurogenesis

One of the most exciting aspects of studying neurogenesis in crustaceans is the possibility of being able to define the control mechanisms that regulate the timing and rate of proliferative events. What factors cause a quiescent stem cell to become active, or an active stem cell to become silent? What influences the speed of the cell cycle, and the final numbers of neurons born? How can environmental circumstances or pressures impact the pathways controlling neurogenesis within an organism? Do all new neurons survive and become integrated into resident circuits? Or, as is well known in the vertebrate nervous system, are neurons overproduced and then culled by cell death? The fact of continuous growth in the crustaceans also raises several interesting questions. First, how does the hormonal cycle that underlies molting and growth influence neurogenesis? How are the loss, replacement and addition of sensory neurons in the receptor systems coordinated with the proliferation and survival of centrally located target neurons? Finally, given that the crustaceans have well-developed regenerative capabilities, what can we learn about the response of central neurons to peripheral damage and repair? How are the peripheral sensory systems reconstituted? The answers to such questions will provide insights that are of fundamental importance to our understanding of stem cells, the cell cycle, and how the controls over proliferation are altered in cancerous cells.

4.1. Rhythmic influences

Circadian regulation. In many of the studies using BrdU techniques to label mitotically active cells in crustacean systems, the BrdU incubation was conducted over a 24 h period in order to eliminate possible diurnal fluctuations and provide a measure of the daily rate of cell proliferation. The clearing time in these systems is likely to be 2–3 days in embryos (Benton and Beltz, 2002) and perhaps longer in tissues from juvenile lobsters and crayfish, ensuring that BrdU is available for uptake in dividing cells over the full 24 h before they were killed and labeled cells counted. However, when juvenile lobsters that have been maintained on a 12:12 light/dark cycle receive short (3 h) treatments with BrdU and are then sacrificed immediately at intervals throughout a 24-h period, counts of the labeled cells show a clear diurnal rhythm of neurogenesis with a peak at dusk (Fig. 11A) (Goergen et al., 2002). Similar results have been obtained with crayfish (Gorissen, 2002) and may be correlated with the habits of lobsters and crayfish, both of which are most active around dusk. In lobsters, the rhythms are maintained in constant darkness (Table 1), and when animals are exposed to a reversed light cycle for 2 weeks the peak of neurogenesis is shifted so that the largest number of BrdU profiles is generated during subjective dusk (Fig. 11B). The reversed pattern is also maintained in constant darkness for at least 3 days, suggesting that neurogenesis is regulated by an endogenous circadian oscillator that is entrained by the light/dark cycle (Table 1) (Goergen et al., 2002). Circadian changes in the rate of cell proliferation in the brain and antennules of spiny lobsters also have been documented (Horner et al., 2003). These findings are of interest because they link light-controlled rhythms and the regulation of neurogenesis. The fact that neuronal proliferation is occurring unevenly throughout the 24-h day confirms the need for 24-h exposures to BrdU in order to smooth out the diurnal variation in proliferation. Strict control over the time of BrdU administration and sacrifice is, therefore, important given that neurogenesis may be more sensitive to regulation during particular periods during a 24-h light/dark cycle.

Nothing is known to date regarding possible seasonal influences over neurogenesis in crustaceans, although a tendency towards lower numbers of labeled cells in the winter months compared with summer months has been observed in the crab *C. maenas* (Hansen and Schmidt, 2001). There is precedence for the importance of such effects in vertebrates, where effects of photoperiod and temperature have been documented (Ramirez et al., 1997; Huang et al., 1998; Dawson et al., 2001).

Molt cycle. The proliferation sequence in the antennule

oxide synthase (left panel). (C) In the deutocerebrum, the deutocerebral commissure (DC), deutocerebral commissure neuropil (+), olfactory globular tract neuropil (OGTN), and glomeruli in the OLs and ALs are consistently and strongly immunoreactive for NOS (green). Synapsin immunoreactivity is indicated in red. Scale bars: (A–C), 50 μm.

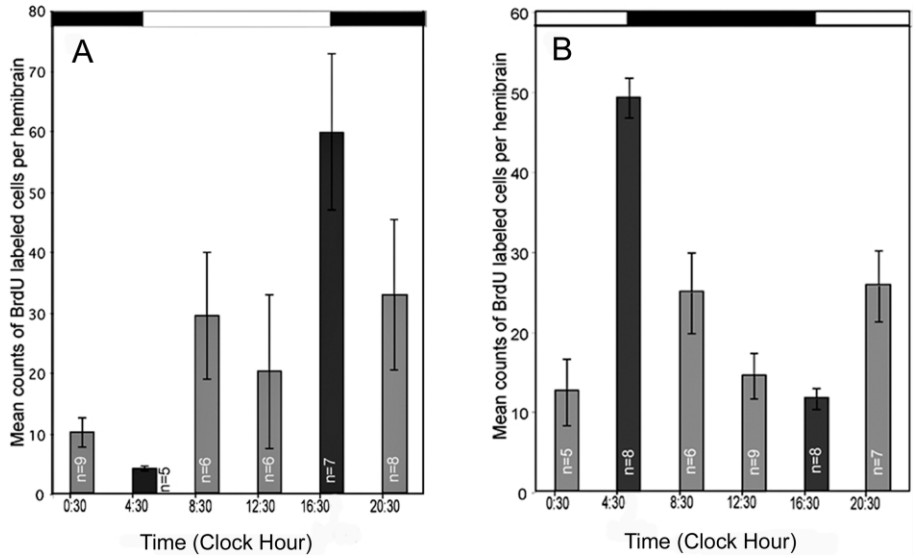

Fig. 11. The means of counts, plotted as histograms (± SEM), of BrdU-labeled neurons in the projection neuron cluster relative to time of day (clock hour) for lobsters maintained on the normal LD light/dark cycle (A), and on the reversed LD light/dark cycle (B). The darker histograms indicate the 3-h sampling periods at dawn and dusk and show that in both the normal and reversed cycle animals, the rate of neurogenesis is much higher at dusk. The numbers of samples assessed for each time point are indicated within the respective histograms. The horizontal band on top of this graph indicates illumination conditions (white, light; black, dark) (reprinted from Goergen et al., 2002).

and brain also is dependent on molt stage. The rate of neurogenesis of ORNs is greatest in premolt, lowest in intermolt, and intermediate in post-molt (Harrison et al., 2001b). Counting the numbers of BrdU-labeled cells in brain clusters 9 and 10 of crayfish over several molting stages (ADI, ADII, and ADIII) reveals that here too the rate of cell proliferation changes in relation to the stage in the molt cycle. A consistent feature is a sudden decrease in the rate of proliferation shortly before the molt, followed by a recovery in the early postmolt (Gorissen, 2002), a pattern of proliferation that contrasts with the genesis of ORNs. How the production of central interneurons and receptor cells is coordinated during the molt cycle is not known. However, these findings suggest the involvement of hormones, specifically steroids, in the regulation of proliferation of both sensory and interneuronal populations; molting is regulated by the steroid 2-hydroxyecdysone, which also is known to influence the proliferation of epidermal cells in crustaceans (Durica et al., 1999). The fact that steroids have dramatic effects on neuronal anatomy and differentiation in both vertebrates (Arnold and Gorski, 1984) and invertebrates (Beltz, 1988), makes the crustacean brain an even more compelling model system for examining regulatory mechanisms controlling neurogenesis.

4.2. Environmental influences

Amputation of an antennule is one way to explore the

Table 1

Treatment	Light condition 6:00 18:00	Labeled profiles (4:30–7:30)	Labeled profiles (16:30–19:30)	*P*-value (dawn vs. dusk)
Normal LD		4.2 ± 0.4	55.9 ± 12.9	0.005
Normal DD		2.3 ± 0.9	48.5 ± 4.3	<0.0001
Reversed LD		49.4 ± 2.43	11.8 ± 1.32	<0.0001
Reversed DD		51.0 ± 2.7	12.0 ± 1.4	<0.0001

Summary of experiments testing the influence of the light/dark cycle on the level of neuronal proliferation. The light/dark regime and resulting mean number (+ SEM) of BrdU-labeled profiles during the dusk and dawn periods are shown. White bars indicate lights on and black bars indicate lights off. When animals maintained on a normal light cycle (normal LD) for 2 weeks were subsequently placed in total darkness for 3 days (normal DD), the pattern of proliferation typical of the normal LD brains, which have the major peak of neurogenesis at dusk, was maintained. Likewise, in animals maintained on a reversed light cycle (reversed LD) for 2 weeks and subsequently placed in total darkness for 3 days (reversed DD), the reversed pattern of proliferation in cluster 10 was maintained, with most neurons born at subjective dusk. These data suggest that the diurnal rhythm of neurogenesis observed is a product of an endogenous circadian oscillator that is entrained by the light/dark cycle.

response of the central nervous system to an environmental challenge but there is now a large body of evidence suggesting that structural changes in the brains of animals, including regulation of neurogenesis, are caused by far less invasive environmental conditions (Brown et al., 2001). When animals are confined to environments that are small and lacking in external stimuli (impoverished), or, alternatively, provided with stimulating surroundings and something to do (enriched), animals respond by down- or up-regulating the rate of neurogenesis (Kempermann and Gage, 1999; Scotto-Lomassese et al., 2000). Crayfish are no exception, and counts of BrdU labeled cells in clusters 9 and 10 of siblings kept in impoverished or in enriched environments show that after exposure to such conditions for 1–2 weeks, the brains of the confined animals contained fewer labeled neurons than those of the animals in large containers (Sandeman and Sandeman, 2000). Feeding, lighting, temperature and the water in which the two groups were kept were the same, leading to the conclusion that the animals are able to detect the quality of their environments; the response of the central nervous system is to adjust the rate of production of new neurons. Experiments in crayfish from different environments also defined the survival rate of the newly added neurons. In such experiments the animals were not killed and assayed immediately after exposure to BrdU, but instead were kept alive for at least 2 weeks after the 24-h exposure to BrdU, and then killed and assayed without further exposure to the BrdU. It was found that more cells were labeled in the brains of the animals that were in the enriched environments than in those that were in the impoverished conditions. Therefore, not only do the animals in the enriched environments make more neurons but they also keep more of them, and, presumably, incorporate more of them as functional units in the brain.

The pathways and signals that regulate this phenomenon are not known. One difference between the two populations was the three-dimensional space in which they could move. The diameter of the small containers was 36 mm and they were 10 mm deep. The large containers on the other hand were 250 mm wide, 500 mm long and 300 mm deep. While the small containers did not constrain the bodily movement of the animals, those in the large containers were able to walk comparatively long distances and swim up into the water column. Locomotor activity associated with some learning task is suspected to be a factor in the upregulation of neurogenesis in mammals (Van Praag et al., 1999), and may have been a feature of the conditions for crayfish in the large containers. Locomotory activity, however, is the end result of an internally set excitatory state, and it could be that this is what also sets the level of cell proliferation. Coincidence between bodily activity and upregulated cell proliferation is not, therefore, necessarily causally linked; they could simply be driven independently by the same source. A second difference between the impoverished and enriched environments was that the crayfish in the large containers were together and had the opportunity to interact with one another, whereas those in the small containers were individually isolated. In order to assess the various elements that contribute to differences in the rate of neurogenesis in crayfish held in impoverished and enriched environments, contributions from physical activity, social relationships and learning will need to be separated experimentally.

Lobsters and crayfish are known to engage in physical contests with one another and set up dominance relationships that appear to be stable, in the sense that the dominant animals remain dominant and the submissive animals avoid further contests with them (Huber and Kravitz, 1995; Yeh et al., 1996; Edwards and Kravitz, 1997; Kravitz, 2000; Beltz and Kravitz, 2002). The biogenic amines octopamine and serotonin have been implicated in this behavior following injection of each amine into lobsters and observing the resulting postural behaviors and neuronal outputs (Livingstone et al., 1980). Injected serotonin also alters the response of submissive lobsters in dominant-subordinate pairs. When submissive lobsters were injected with serotonin, these animals no longer retreated from the dominant individual as in control situations, but instead approached and continued to fight with the dominant animal (Huber et al., 1997a,b). Social experience also alters serotonergic modulation of the escape circuit of crayfish (Yeh et al., 1996). As serotonin levels have been shown to influence the rate of neurogenesis in lobsters (Benton and Beltz, 2001a; Beltz et al., 2001; Benton, Goergen and Beltz, unpublished results), the possibility that social interaction or status could have an effect on neurogenesis has been tested in crayfish and lobsters using two different experimental paradigms.

The influence of dynamic social interactions on the rate of neurogenesis was tested in juvenile crayfish *C. destructor* that were isolated from one another for 2 weeks and then housed in three different environments: Group 1 crayfish ($n = 4$) were housed alone in small containers, Group 2 crayfish ($n = 4$) were housed alone in large containers and Group 3 crayfish ($n = 9$), were housed in groups of three individuals in large containers which allowed each individual the same area of living space as the Group 2 animals. After 2 weeks in these conditions, the animals were exposed to BrdU for 24 h, killed and their brains then processed. Counts of the labeled cell profiles in clusters 9 and 10 showed that the animals that were alone in the large containers exhibited the highest rate of neurogenesis, those alone in the small containers, the lowest rate, and those that shared a large container with conspecifics, intermediate values (Pelz, 2001).

The animals that shared the large containers with their conspecifics (3 groups of 3) were observed during the experimental period and their agonistic encounters recorded and scored. Crayfish do not engage in ‘fights’ of the same intensity as lobsters. Instead the encounters occur when two animals confront one another head-on; one of the pair will retreat and this animal is regarded as having ‘lost’ the encounter. The rate of cell proliferation

in clusters 9 and 10 of the animals was then compared with the total numbers of 'losses' that were scored for each individual. A strong correlation was found between the numbers of labeled BrdU profiles in clusters 9 and 10 and the number of losses each animal sustained such that the animals with the most losses contained the lowest number of BrdU-labeled profiles in clusters 9 and 10 and those with least losses contained the highest counts of labeled cells ($F(1,8) = 32.01$; $p = 0.000$; $R^2 = 80.0\%$; $R^2_{adj} = 77.5\%$) (Pelz, 2001).

In order to test whether a stable social relationship influences the rate of neurogenesis, juvenile lobsters (*H. americanus*, carapace length ~1 cm) were paired such that one member of the pair was ~20% larger than its partner. Such a size mismatch ensures the establishment of a stable dominant-subordinate relationship, with the larger member of the pair quickly establishing its dominance over the smaller lobster (Scrivener, 1971). Each pair of was housed in a 5-gallon tank, where individual lobsters of each pair were separated by a porous, opaque divider except during the encounters, when they were allowed to interact. The divider was removed each day for 2 weeks. This repeated interaction was intended to reinforce the dominant-subordinate relationship which was generally established during the first encounter between two animals. Control lobsters were housed as the experimental animals, but the pairs were never allowed to interact. Experimental animals were injected with BrdU immediately after the final encounter, and sacrificed after 4 h. The basal level of neurogenesis in cluster 10 was indicated by the control lobsters that were also injected with BrdU after the 2-week period. No statistically significant differences were seen in the numbers of BrdU-labeled profiles in cluster 10 between the control, dominant and subordinate lobsters (Mazzarella, 2003).

These results on lobsters and crayfish seem to provide contradictory answers to the question of whether social relationships influence the rate of neurogenesis. However, the experimental approaches test two very different aspects of the hierarchical dominance phenomenon. The crayfish study used animals that were closely matched in size and that had not achieved a stable hierarchy at the time they were sacrificed. Hence, the measurements were made on animals still in the dynamic, and perhaps stressful, stage of their struggle to establish the social order. In contrast, the lobster study was designed to test the rate of neurogenesis after a stable dominance of one animal over the other had been established. The results suggest that it is the process of establishing the social relationship, rather than its maintenance, that may have an influence on the rate of neurogenesis.

4.3. Serotonin

Serotonin has been implicated as a developmentally important molecule in the nervous systems of vertebrate and invertebrate organisms (Lauder, 1991; Goldberg et al., 1991). The crustacean brain appeared to be a particularly appropriate system in which to examine this issue, because the OLs and ALs receive a massive serotonergic innervation early in embryonic development from identified neurons, the paired DGNs (see below, and Sandeman and Sandeman, 1987, 1994; Sandeman et al., 1995; Beltz, 1999; Benton and Beltz, 2001a; Beltz et al., 2001).

The first clue that serotonin played a developmental role in the crustacean brain came from immunocytochemical studies that examined the ontogenetic appearance of serotonin in *H. americanus* (Beltz et al., 1990). Serotonin's presence in the protocerebrum by 10% embryonic development (E10%) is precocious, far earlier than the mid-embryonic onset of staining for octopamine (~E43%; Schneider et al., 1996) and dopamine (~E50%; Cournil et al., 1995). The full complement of serotonergic somata in the brain and ventral nerve cord is labeled by mid-embryonic life, at a time when other amines are just beginning to appear in neurons (Beltz, 1999). Therefore, the acquisition of serotonin in neurons is early and is compressed into a relatively short developmental period (Beltz et al., 1990). The DGNs are the first neuronal somata to label for this amine, and the first staining in DGN neurites is temporally correlated with the emergence of the ALs (Beltz et al., 1992; Helluy et al., 1996). In contrast, the OL anlagen emerge much earlier and grow for some time in the apparent absence of serotonin.

The role of serotonergic input in the development of these brain regions was investigated in lobster embryos by pharmacological depletion of serotonin using chronic injections of 5,7-dihydroxytryptamine (5,7-DHT). A ~90% long-term reduction in serotonin levels was confirmed in toxin-treated embryos using high performance liquid chromatography (Benton et al., 1997). In these experiments, the three neuropils that are targets of the serotonergic DGNs (OLs, ALs and OGTNs) were significantly reduced in size after 5,7-DHT treatments, although glomeruli differentiated at the normal times (Benton et al., 1997; Benton and Beltz, 2001a). In contrast, the antenna II neuropil, an area that is not serotonin immunoreactive during embryonic life, was not altered in size by 5,7-DHT treatment. Therefore, the effects of 5,7-DHT in the deutocerebrum are selective for synaptic regions that receive intense serotonergic innervation.

Experiments have focused on defining which neurons in the deutocerebrum are altered by serotonin depletion, and therefore, responsible for this altered growth. As auto-regulation of growth is a phenomenon that has been demonstrated for serotonergic neurons in other systems (e.g. Diefenbach et al., 1995), experiments first tested whether the DGN is itself sensitive to serotonin depletion. However, morphometric analyses of individual DGNs in normal, control (sham-injected) and toxin-treated embryos show that the general morphology and size of the DGNs are

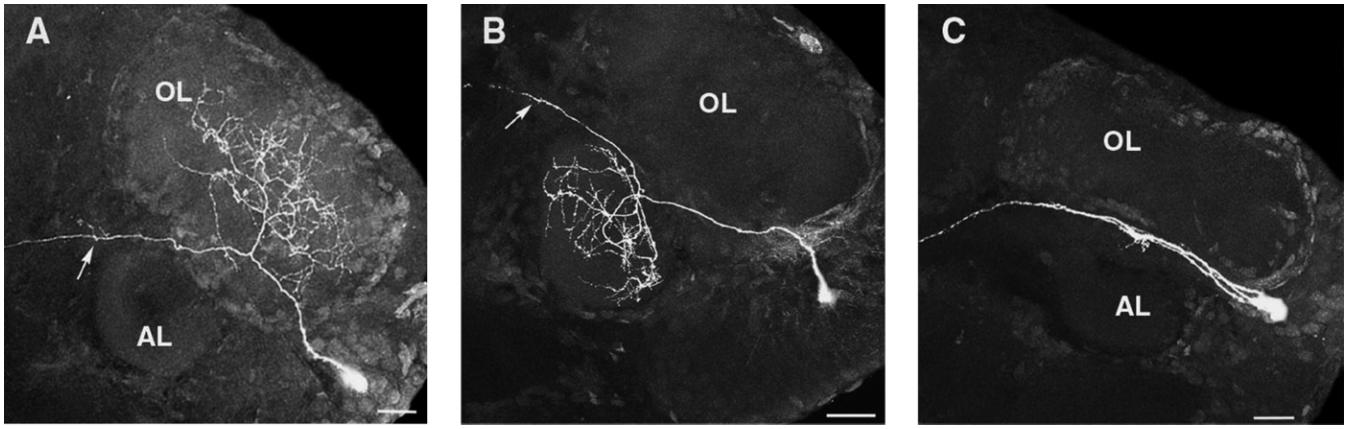

Fig. 12. Stacked confocal images illustrating the morphologies of two major types of projection neuron (A, B). Arrows indicate fine branches within the OGTN. (A) Projection neuron innervating the olfactory lobe (OL). (B) Projection neuron innervating the accessory lobe (AL). (C) Three neurons stained sequentially in a serotonin-depleted embryo. Although axons of these neurons in the olfactory globular tract (OGT) extend to the lateral protocerebrum, innervation of either the OL or AL is absent, a condition that was never observed in projection neurons of normal embryos. Scale bars: (A-C) 20 μm (reprinted from Sullivan et al., 2000).

not altered by 5,7-DHT treatment (Benton and Beltz, 2001a). Therefore, the growth reduction in the OLs, ALs, and OGTNs implicated the projection neurons as primary targets of serotonin depletion, since these interneurons innervate all three of these synaptic areas (Arbas et al., 1998; Mellon and Alones, 1994; Wachowiak and Ache, 1994; Sandeman et al., 1994; Sandeman et al., 1995). Two types of experiments then examined the effects of serotonin depletion on the deutocerebral interneurons in clusters 9 and 10: (1) neuronal proliferation and survival were assessed by immersion of serotonin-depleted and control embryos in BrdU in order to label replicating cells; (2) lucifer yellow injection of individual projection neurons also was used to examine their morphology.

The numbers of labeled nuclei in the cell clusters containing the olfactory interneurons were counted in serotonin-depleted and control embryonic brains either 24 h after BrdU immersion to determine the rate of proliferation, or 2 weeks after BrdU immersion to assess survival of newly proliferated projection neurons. The numbers of BrdU-labeled projection neurons were severely reduced in serotonin-depleted brains in both proliferation (to 65% of control numbers) and survival (to 48% of control numbers) studies (Beltz et al., 2001). Similar reductions in proliferation and survival were seen among the local interneurons (Benton and Beltz, 2001a). These effects involve regulatory mechanisms that are likely to persist throughout the animal's life, as shown by proliferation and survival assays of the olfactory interneurons in the brains of juvenile lobsters, where proliferation was dramatically reduced by serotonin depletion and where no BrdU-labeled neurons were present at the end of a 3-week assessment period (Beltz et al., 2001).

Dye fills in normal embryos showed that each projection neuron extensively innervates either the OL or AL before projecting to neuropil regions in the protocerebrum (Fig. 12A and B) (Sullivan et al., 2000). In embryos treated with 5,7-DHT, however, projection neurons were sometimes encountered (13.5%) that projected to the protocerebrum as in normal embryos, but whose branching within the lobes was either markedly reduced or absent (Fig. 12C). This condition was never observed in projection neurons of normal embryos. Olfactory projection neurons with aberrant morphologies were also encountered, though less frequently, in control embryos indicating that the sham injections can affect the development of these neurons. This observation provides insight into the nature of effects seen in control embryos in previous experiments, and may be related to a stress response in the sham-injected animals (Benton et al., 1997; Sullivan et al., 2000). These results indicate that *in vivo* serotonin depletion inhibits the branching of olfactory projection neurons and suggests, therefore, that one of the functions of serotonin during normal development is to promote the ingrowth of these neurons, either directly or indirectly, into the deutocerebral neuropils.

Therefore, in the crustacean deutocerebrum, the reduced volumes of the OLs and ALs following serotonin depletion or stress (sham-injected controls, see Benton et al., 1997; Sullivan et al., 2000) is due to a lack of dendritic arbors in these lobes as well as to decreased neurogenesis (Benton et al., 1997; Sullivan et al., 2000; Benton and Beltz, 2001a; Beltz et al., 2001). Likewise, in mammals, the reduced hippocampal volume observed in response to stress is due at least partly to dendritic retraction of the CA3 neurons, as well as to reduced cell proliferation (Magarinõs et al., 1996; Gould et al., 1997; Gould et al., 1998).

In recent studies, the effects of increased serotonin levels on the rate of neurogenesis also have been examined. Juvenile lobster brains were dissected and maintained for 3 h in culture medium iso-osmotic with lobster hemolymph, and containing BrdU with or without added serotonin

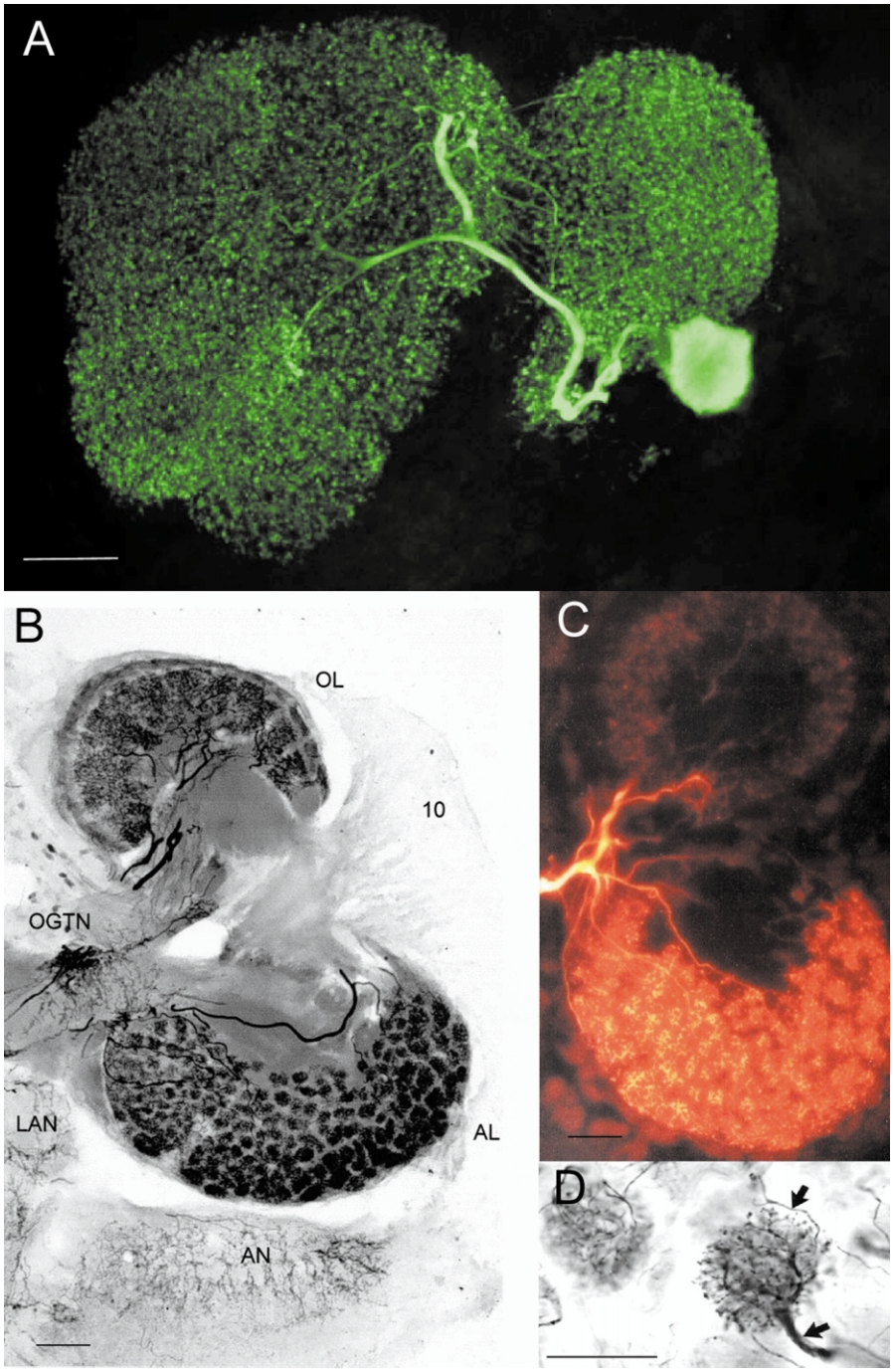

Fig. 13. Dorsal giant neurons (DGNs) in embryonic lobsters (A) and adult crayfish (B–D). The DGN cell soma is large and lies on the dorsal surface of the brain. The neurite extends from the cell body to the ipsilateral olfactory (OL) and accessory (AL) lobes, where it branches extensively into all glomeruli in each lobe (A, B, C). The main input to the AL glomeruli is through the endings of interneurons whose axons are in the deutocerebral commissure (DC, see Fig. 1). Double labeling with serotonin antibody and neurobiotin fills of the DC interneurons (D) shows that each glomerulus in the AL receives a single club-shaped ending from a DC neuron. The DGN, on the other hand, branches to all glomeruli in the AL (Sandeman et al., 1995). One arrow points to the thick fiber of the DC interneuron, while the second arrow points to the finer caliber fiber of the DGN. AN, antennal neuropil; LAN, lateral antennular neuropil; OGTN, olfactory globular tract neuropil. Scale bars: (A) 50 μm; (B, C) 100 μm; (D) 20 μm. (A modified from Benton and Beltz, 2001); B modified from Sandeman and Sandeman, 2003.)

(10^{-4} M). Brains exposed to serotonin *in vitro* showed a mean 77% increase in the numbers of BrdU-labeled neurons in cluster 10 over this 3 h period when compared with control brains that were not exposed to serotonin (two tail *t*-test; $p < 0.00001$). These experiments demonstrate that serotonin dramatically increases the rate of neurogenesis in isolated lobster brains (Benton, Goergen and Beltz, unpublished results).

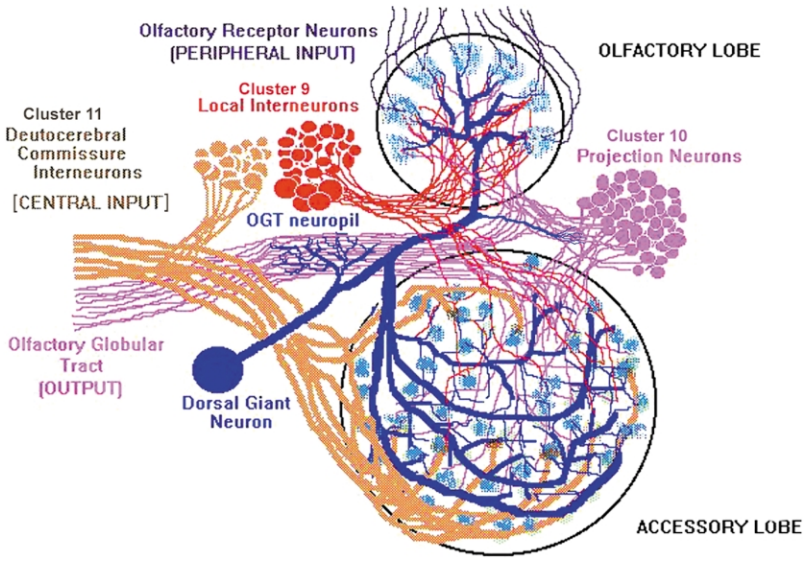

Fig. 14. Diagram of the known circuits in the OLs and ALs related to the DGN. ORNs (purple) penetrate the OL and terminate on local interneurons (clusters 9 and 11; red and brown, respectively) or projection neurons (pink) that have their cell bodies either medial (cluster 9, 11; local interneurons) or lateral (cluster 10; projection neurons) to the OLs. Each projection neuron has arborizations in either the OL or AL. Their axons leave the lobe in the OGT. Cluster 11 interneurons with axons in the deutocerebral commissure (brown) are presynaptic to neurons in the AL. The DGN has its cell body in cluster 11 on the dorsal side of the brain. Its neurite extends ventrally, giving off branches among the axons of the projection neurons in the OGT neuropil (OGTN). The DGN then branches into all glomeruli in the OLs and ALs and also in the region of the cluster 10 cell bodies.

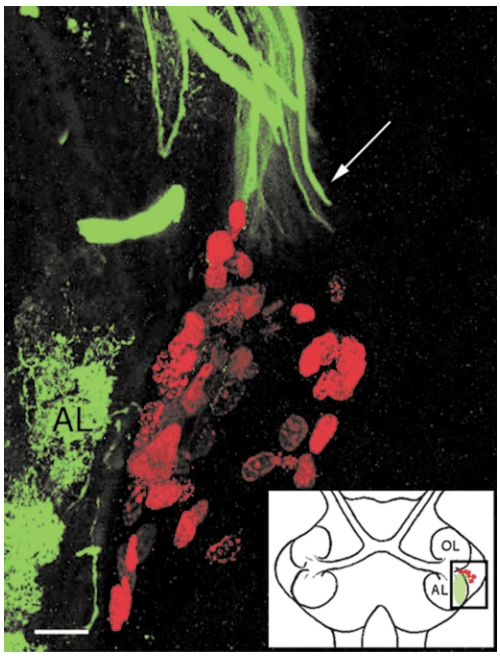

Fig. 15. Confocal image of the brain of a juvenile lobster labeled for BrdU (red) and serotonin (green) immunoreactivities. Lobsters were sacrificed 6 h after BrdU injection, and dissected brains were incubated in serotonin, rinsed, fixed and then processed immunocytochemically. BrdU-labeled nuclei are found in the cluster 10 proliferation zone immediately adjacent to the AL. Serotonin antibodies label a group of fine fibers (arrow) that terminate blindly at the proliferation zone. These fibers have been traced back to the DGN in adult, juvenile and larval brains. Inset diagram indicates the position of the image in the whole brain. Scale bar: 50 μm (modified from Beltz et al., 2001).

5. The serotonergic dorsal giant neurons

Physiological, anatomical, developmental and immunocytochemical investigations in both *C. destructor* and *H. americanus* leave no doubt that the DGNs in these two species are homologous, having the same morphology and connectivity and so, we assume, function (Fig. 13) (Helluy et al., 1993).

The DGN is of potential importance in the context of neurogenesis because (1) serotonin is known to regulate the rate of neurogenesis (Benton and Beltz, 2001; Beltz et al., 2001), (2) the DGN sends a tract of fine fibers directly to the proliferation zone of the projection neurons in cluster 10 (Figs. 14 and 15) (Beltz et al., 2001), (3) the DGN is the primary serotonergic neuron innervating the OLs and ALs, which also are innervated by the local and projection neurons that undergo life-long neurogenesis (Sandeman et al., 1995; Sandeman and Sandeman, 2000; Benton and Beltz, 2001), (4) serotonin is releasable from the terminals of the DGN in an isolated AL (Meunpol et al., 1998).

Inputs. The anatomical context of the DGN is summarized in the diagram in Fig. 14. Physiological studies in *C. destructor* have established some of the inputs to the DGN. The first of these is within the olfactory globular tract neuropil (OGTN). The OGTN is unusual in the sense that it is embedded within the through-going axons of the OGT, the only output pathway of the OLs and ALs. The OGTN, therefore, constitutes an 'en passant' region where the output of the OLs and ALs is 'tapped' by the DGN. Electrical stimulation of either the cell somata of the cluster 10 projection neurons, or their axons in the OGT, will elicit

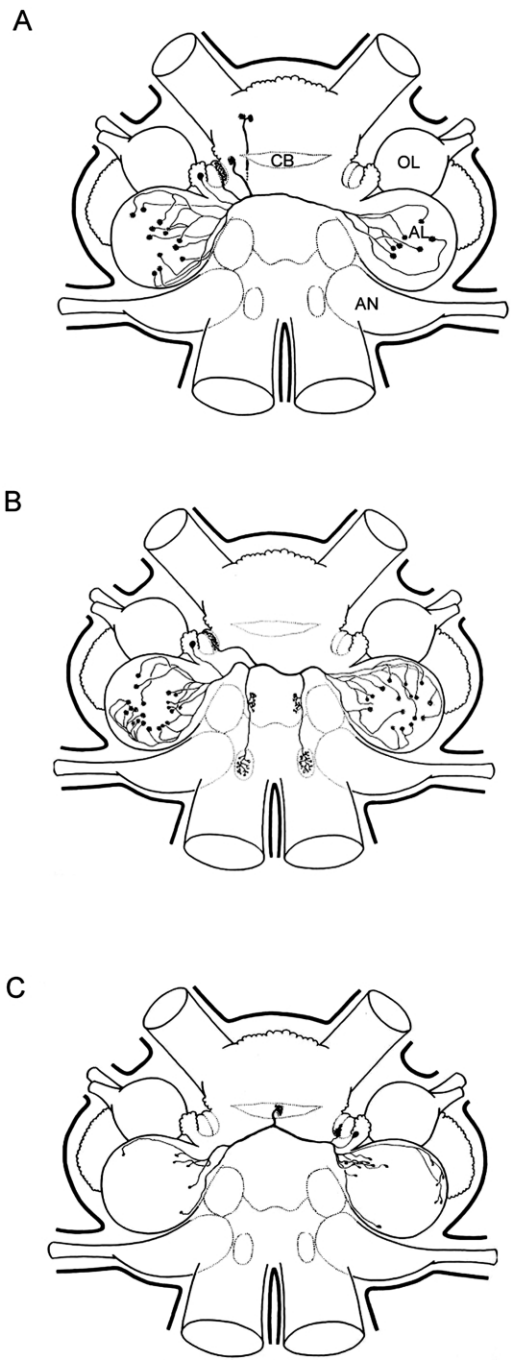

Fig. 16. Three different classes of deutocerebral commissure interneurons in which the inputs are in the anterior median protocerebrum (A), the tritocerebrum (B) and central body (C). All of these interneurons have their somata in cluster 11, and branches in a small laterally situated neuropil, the deutocerebral commissure neuropil, or DCN (see Fig. 1). Their bilateral outputs end in 6-15 glomeruli in each AL. Each glomerulus receives a single club-ending from only one interneuron and so there is no convergence between the deutocerebral commissure interneurons (modified from Sandeman et al., 1995).

an excitatory postsynaptic response in the DGN (Sandeman and Sandeman, 1994). A second input to the DGN is through the interneurons whose axons lie in the deutocerebral commissure; these DC neurons constitute the dominant input to the AL (Sandeman et al., 1993, 1995). Electrical stimulation of this pathway also produces an excitatory postsynaptic response in the DGN which differs from the projection neuron input in that the excitation is followed by a period of strong inhibition during which the DGN cannot be excited by either input. The third known input to the DGN is via the OL. Electrical stimulation of the lateral antennular flagellum excites the ORNs that terminate in the OL, and is accompanied by a inhibitory post-synaptic response in the DGN.

Outputs. The output of the DGNs is not understood because neither physiological nor ultrastructural studies have revealed clear input–output relationships of the DGN terminals in the OLs and ALs (Sandeman et al., 1994). The tract of fine DGN fibers that end within the proliferation zone in cluster 10 are the only candidates identified so far that could represent a focused output, a possibility that is supported by the immunocytochemical labelling of putative 5HT receptors in this area (Spitzer et al., 2001). An output in this region would fit well with the proposal that the DGN is a direct regulator of neurogenesis within the cluster 10 neurons (Beltz et al., 2001).

As serotonergic neurons in many species often do not have classical synapses with anatomically recognizable outputs (Descarries et al., 1975; Sandeman et al., 1994), such criteria may not be helpful in this regard; it is nevertheless assumed that the DGN will exert its influence through release of serotonin from its terminals in the OLs or ALs, or both. Given the broad anatomical arborizations of these cells in these regions, this model would imply the potential for a general and simultaneous modulation of OL and AL activity. Selectivity of the effect of the DGN could, of course, be imposed by the locality and variety of 5HT receptors. In addition, the fact that there are several anatomical and physiological classes of DC interneurons (Fig. 16) could enable a localized influence of the DGN (Sandeman et al., 1995). One class of the DC interneurons is known to carry information from the visual system, another, tactile information from the second antennae (Sandeman et al., 1995), and a third olfactory information (Sullivan and Beltz, unpublished results). The information received by the AL is, therefore, diverse, but the *projection* to the AL is highly selective: each DC interneuron terminates bilaterally in only 6 to 15 of the many thousands of glomeruli in the ALs, and no two DC interneurons have ever been seen to converge in the same glomerulus. The possibility exists, therefore, that each one of the many thousands of glomeruli is an independent integrative unit blending the input from a single DC interneuron with activity in projection and local interneurons that, with the DGN, are the other elements in each of the AL glomeruli. Hence, if one or more DC neurons is activated, this could initiate a localized (in a few glomeruli) or more general release (in many or all glomeruli) of serotonin from the DGN.

How the DC neurons regulate (or are regulated by) the release of serotonin from the DGN is the question that now needs to be addressed. By virtue of the DGN's striking

anatomical features and diverse physiological connections in the OL and AL pathways, these neurons are optimally located to receive and integrate a broad spectrum of information. We would like to know if serotonin release from these neurons is governed by the convergence of higher order interneurons carrying heterogeneous afferent signals. We also will explore how the DGNs participate in the complex chain of events that leads from the perception of the environmental state, such as living conditions and time of day, to the regulation of cell proliferation in the cluster 10 neurons. Given the accessibility of these large cells, they serve as an excellent model in which to study aspects of the serotonergic regulation of life-long neurogenesis.

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Appendix A

List of arthropod taxa discussed

Cancer pagurus (Linnaeus, 1758) (Malacostraca, Decapoda, Reptantia, Brachyura)
Carcinus maenus (Linnaeus, 1758) (Malacostraca, Decapoda, Reptantia, Brachyura)
Cherax destructor (Clark, 1936) (Malacostraca, Decapoda, Reptantia, Astacida)
Drosophila melanogaster (Meigen, 1830) (Insecta, Pterygota, Diptera)
Homarus americanus (Milne Edwards, 1837) (Malacostraca, Decapoda, Reptantia, Homarida)
Hyas araneus (Linnaeus, 1758) (Malacostraca, Decapoda, Reptantia, Brachyura)
Pagurus bernhardus (Linnaeus, 1758) (Malacostraca, Decapoda, Reptantia, Anomala)
Panulirus argus (Latraille, 1804) (Malacostraca, Decapoda, Reptantia, Palinura)
Sicyonia brevirostris (Stimpson, 1871) (Malacostraca, Decapoda, Natantia)

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