# Newborn Cells in the Adult Crayfish Brain Differentiate into Distinct Neuronal Types

**Jeremy M. Sullivan, Barbara S. Beltz**

Department of Biological Sciences, Wellesley College, Wellesley, Massachusetts 02481

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**ABSTRACT: Mitotically active regions persist in the brains of decapod crustaceans throughout their lifetimes, as they do in many vertebrates. The most well-studied of these regions in decapods occurs within a soma cluster, known as cluster 10, located in the deutocerebrum. Cluster 10 in crayfish and lobsters is composed of the somata of two anatomically and functionally distinct classes of projection neurons: olfactory lobe (OL) projection neurons and accessory lobe (AL) projection neurons. While adult-generated cells in cluster 10 survive for at least a year, their final phenotypes remain unknown. To address this question, we combined BrdU labeling of proliferating cells with specific neuronal and glial markers and tracers to examine the differentiation of newborn cells in cluster 10 of the crayfish, *Cherax destructor*. Our results show that large numbers of adult-generated cells in cluster 10 differentiate into neurons expressing the neuropeptide crustacean-SIFamide. No evidence was obtained suggesting that cells differentiate into glia. The functional phenotypes of newborn neurons in cluster 10 were examined by combining BrdU immunocytochemistry with the application of dextran dyes to different brain neuropils. These studies showed that while the majority of cells born during the early postembryonic development of *C. destructor* differentiate in AL projection neurons, neurogenesis in adult crayfish is characterized by the addition of both OL and AL projection neurons. In addition to our examination of neurogenesis in the olfactory pathway, we provide the first evidence that adult neurogenesis is also a characteristic feature of the optic neuropils of decapod crustaceans.** © 2005 Wiley Periodicals, Inc. J Neurobiol 65: 157–170, 2005

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## INTRODUCTION

Life-long neurogenesis is a characteristic feature of the brains of decapod crustaceans (see reviews in Schmidt, 2001a; Beltz and Sandeman, 2003), as it is in most mammals. While increasing evidence suggests that newborn neurons in adult mammals may be involved in learning and memory (Shors et al., 2001, 2002; Rochefort et al., 2002), the functional importance of adult neurogenesis in decapod crustaceans remains largely unknown. The first step towards understanding the significance of persistent neurogenesis in these species is to define the functional and chemical phenotype of adult-generated cells. In adult mice, for example, newborn cells in the subventricular zone have been shown to differentiate into inhibitory interneurons (granular and periglomerular cells) within the olfactory bulb (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Kornak and Rakic, 2001; Carlén et al., 2002; Petreanu and Alvarez-Buylla, 2002; Belluzzi et al., 2003), while cells born in the subgranular zone of the hippocampal dentate gyrus differentiate into granular cells, a class of excitatory interneurons (Kaplan and Bell, 1984; Markalis and Gage, 1999; van Praag et al., 2002; Jessberger and Kempermann, 2003; Kempermann et al., 2003). Comparatively little is known, however, about the functional identities of newborn neurons in the adult decapod brain.

*Correspondence to:* B. Beltz (bbeltz@wellesley.edu).

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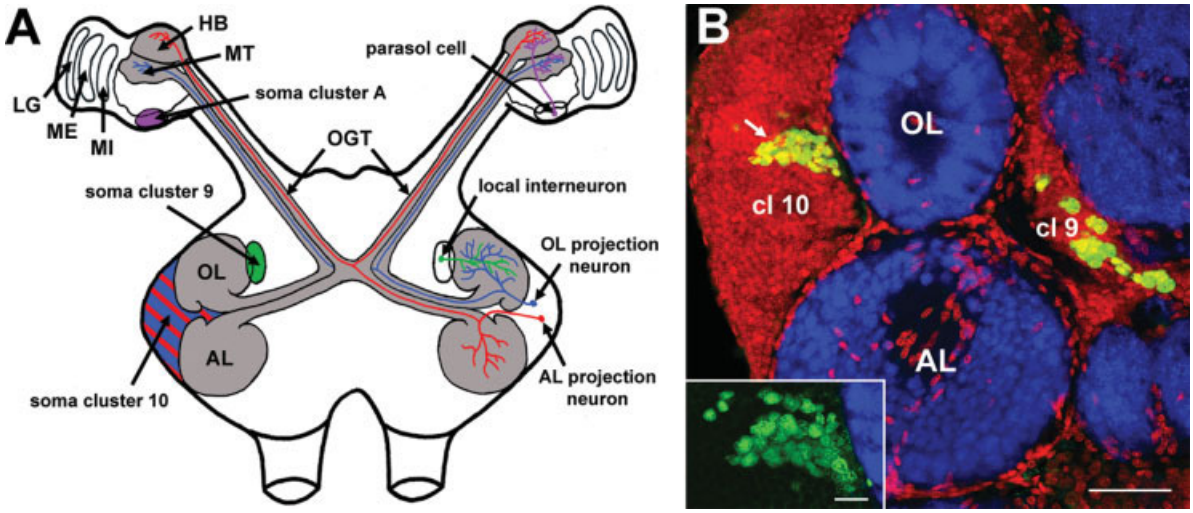


**Figure 1** Morphology of the brain of freshwater crayfish. (A) Schematic diagram outlining the olfactory pathway of the crayfish *Cherax destructor*. Olfactory receptor neurons project ipsilaterally to the brain where they terminate within the olfactory lobe. The olfactory lobe is also innervated by populations of local interneurons and projection neurons whose somata reside within soma clusters 9 and 10, respectively (terminology from Sandeman et al., 1992). Neurons with somata in these soma clusters also innervate the accessory lobe, which lies adjacent to the olfactory lobe. The main output pathway from both the olfactory and accessory lobes is provided by the projection neurons, whose axons form the olfactory globular tract. The olfactory globular tract bifurcates at the midline of the brain before projecting bilaterally to both the medulla terminalis and the hemiellipsoid body, which form the lateral protocerebrum. Dye injections into the olfactory and accessory lobes of crayfish have demonstrated that projection neurons innervating the olfactory lobe primarily target the medulla terminalis while those innervating the accessory lobe terminate within the hemiellipsoid body (Sullivan and Beltz, 2001, 2005). The hemiellipsoid body and medulla terminalis are innervated by a large population of local interneurons, known as parasol cells (Mellon, 2003), whose somata reside in a cluster, known as soma cluster A (terminology from Blaustein et al., 1988). The three optic ganglia (medulla interna, medulla externa, lamina ganglionaris) occur rostral to the medulla terminalis. (B) Horizontal section through the olfactory and accessory lobes of *C. destructor* labeled immunocytochemically for BrdU (green) and *Drosophila* synapsin (blue) and counterstained with propidium iodide (red), a marker of nucleic acids. BrdU-labeled cells (*arrow*) can be observed within the proliferation zone in soma cluster 10, which lies adjacent to the olfactory lobe. BrdU-labeled cells can also be observed in cluster 9. The *inset* shows a higher-magnification view of BrdU-labeled cells within the cluster 10 proliferation zone. Abbreviations: AL, accessory lobe; cl 9, soma cluster 9; cl 10, soma cluster 10; HB, hemiellipsoid body; LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; OGT, olfactory globular tract; OL, olfactory lobe. Scale bars = 100 $\mu$m in (B); 20 $\mu$m in insert in (B).

Neuronal proliferation in the brains of adult decapods has been described within three different soma clusters associated with the olfactory pathway, though the specific clusters in which neurogenesis occurs varies amongst taxa (Schmidt and Harzsch, 1999; Sullivan and Beltz, 2003). However, one cluster in which proliferation occurs in adults of all decapod species thus far examined is cluster 10 (terminology from Sandeman et al., 1992), a cluster of projection neuron somata located adjacent to the olfactory lobe (OL), the primary olfactory neuropil, in the deutocerebrum. This cluster has been the focus of most of the research on adult neurogenesis in decapod crustaceans (for review, see Beltz and Sandeman, 2003). Research on the differentiation of newborn cells within this cluster has been hampered, however, by the lack of specific neuronal or neurotransmitter markers for the projection neurons whose somata reside in cluster 10.

In basal decapods, such as shrimp and prawns, projection neurons originating in cluster 10 innervate the ipsilateral OL and form the main output pathway from this neuropil to higher-order neuropils in the lateral protocerebrum (Hanström, 1931, 1947; Sullivan and

Beltz, 2004). In eureptantian decapods, such as lobsters and crayfish, cluster 10 projection neurons also innervate an additional neuropil region, known as the accessory lobe (AL), which lies adjacent to the OL (Fig. 1; Mellon et al., 1992; Wachowiak and Ache, 1994; Schmidt and Ache, 1996; Wachowiak et al., 1996; Sullivan et al., 2000). Accessory lobes appear to have arisen *de novo* in the Eureptantia (Sandeman et al., 1993; Derby et al., 2003) and are among the most prominent neuropils in the brains of crayfish and lobsters (Blaustein et al., 1988; Sandeman et al., 1993; Sandeman and Scholtz, 1995). Unlike the OL, which appears to receive only primary olfactory inputs, the AL receives higher-order multimodal inputs (Sandeman et al., 1995; Wachowiak et al., 1996). Anatomical studies in eureptantian decapods have shown that individual cluster 10 projection neurons innervate either the OL or the AL (Wachowiak and Ache, 1994; Schmidt and Ache, 1996; Wachowiak et al., 1996; Sullivan et al., 2000) and that these two neuropils have separate output pathways that project to different regions of the lateral protocerebrum [Fig. 1(A); Sullivan and Beltz, 2001, 2005]. Cluster 10 in crayfish and lobsters, therefore, is comprised of at least two groups of interneurons (OL projection neurons and AL projection neurons) that have distinct functional identities (Sullivan and Beltz, 2005).

Cell proliferation within cluster 10 occurs within a restricted region of the cluster, known as the proliferation zone [Fig. 1(B)]. Although the progenitor cells and the sequence of cell divisions in this region have been described in embryonic lobsters (Benton and Beltz, 2002), the identity of the progenitor cells in adult decapods remains unknown. Newborn cells gradually move away from the proliferation zone becoming dispersed amongst established cluster 10 neurons and have been shown to survive for at least one year (Harzsch et al., 1999; Beltz et al., 2001; Schmidt, 2001b). Schmidt (2001b) provided evidence that adult-born cells in cluster 10 of the spiny lobster, *Panulirus argus*, are contacted by the terminals of descending neurons, suggesting that these cells differentiate into neurons. In pulse-chase experiments with the proliferation marker bromodeoxyuridine (BrdU), Schmidt (2001b) also showed that most adult-born cells remain within a portion of the cluster comprised mainly of OL projection neuron somata, suggesting that most of the newborn cells differentiate into OL projection neurons. The final phenotype of adult-born cells in cluster 10 of crayfish and lobsters, however, has not yet been demonstrated definitively. It has also yet to be firmly established whether the proliferation observed in cluster 10 represents exclusively neurogenesis or whether some newborn cells also differentiate into glia (Harzsch et al., 1999).

One species that has been used extensively to study life-long proliferation in cluster 10 is the Australian freshwater crayfish *Cherax destructor* (for review, see Beltz and Sandeman, 2003). The proliferation zone within cluster 10 of *C. destructor* [Fig. 1(B)] has been characterized and the number of projection neuron somata within the cluster shown to increase linearly with the size of the animal (Sandeman et al., 1998). On hatching, the olfactory lobe of *C. destructor* is approximately three times as large as the accessory lobe (Sandeman et al., 1998). The accessory lobe subsequently experiences a period of rapid growth until the crayfish reaches a size of $\sim$1 cm carapace length at which point the accessory lobe is approximately 3.5 times larger than the olfactory lobe. Thereafter, the ratio of the volumes of the two lobes remains stable.

In the present study, we combined BrdU labeling of proliferating cells with specific neuronal (crustacean-SIFamide) and glial (glutamine synthetase, glial fibrillary acidic protein) markers, as well as neuronal tract-tracing with dextran dyes, to examine the differentiation of newborn cells in cluster 10 of *C. destructor*. Our results demonstrate that large numbers of the cells born in cluster 10 in adult crayfish differentiate into neurons expressing the recently described neuropeptide crustacean-SIFamide (Yasuda et al., 2004). No evidence was obtained, however, suggesting that the cells differentiate into glia. In addition, we used the application of dextran dyes to the OL and AL to determine the functional phenotype of the newborn neurons: OL projection neurons, AL projection neurons, or both. These studies showed that while cells born in cluster 10 during early postembryonic development differentiate primarily into AL projection neurons, neurogenesis in adult crayfish is characterized by the addition of numbers of both OL and AL projection neurons. These data demonstrate that adult-born cells differentiate into different types of neurons and that the types of neurons produced is stage-dependent.

## MATERIALS AND METHODS

### Animals

Male and female crayfish, *Cherax destructor*, were reared in the laboratory in aquaria with artificial freshwater and a light/dark cycle of 12:12 h. These animals were the offspring of adult crayfish collected from dams near Sydney, Australia. Crayfish of two size classes were used for the experiments described here: early postembryonic crayfish (carapace lengths $< 0.8$ cm) and young adult crayfish (cara-

pace lengths $\geq$ 1.4 cm). For reference, *C. destructor* has a carapace length of $\sim$ 0.3 cm on hatching.

## Immunocytochemical Labeling with Neuronal and Glial-Specific Markers

In a recent study, Yasuda et al. (2004) demonstrated that large numbers of projection neurons in cluster 10 of the crayfish *Procambarus clarkii* are immunoreactive to the neuropeptide crustacean-SIFamide (SIFamide). To determine if this is also the case in *C. destructor*, brains were dissected from crayfish in cold crayfish saline (mmol $L^{-1}$: 205 NaCl, 5.4 KCl, 34.4 $CaCl_2$, 1.2 $MgCl_2$, 2.4 $NaHCO_3$, pH 7.4) and then fixed for 1 to 2 days in 4% paraformaldehyde in 0.1*M* phosphate buffer (PB; pH 7.4) at 4°C. Subsequently, preparations were rinsed for 4 h in PB, suspended in 6% Noble agar (DIFCO, Detroit, MI), and sectioned at 100 $\mu$m on a vibratome (Technical Products, St Louis, MO). Tissue sections were then rinsed in PB containing 0.3% Triton X-100 (PBTx) for 2 h and incubated overnight at 4°C in a rabbit anti-SIFamide (1:12,000; a generous gift from Dr. Akikazu Yasuda, Suntory Institute for Bioorganic Research, Osaka, Japan). Following incubation in the primary antibody, sections were rinsed for 4 h in PBTx and then incubated overnight at 4°C in a goat anti-rabbit Alexa 488 (Molecular Probes, Eugene, OR) secondary antibody diluted 1:50 in PBTx. Subsequently, sections were rinsed for 2 h in PBTx and mounted in Gelmount (Biømeda, Foster City, CA).

In order to examine the distribution of glial cells within cluster 10, brain sections were labeled using antibodies against glutamine synthetase (GS) and glial fibrillary acidic protein (GFAP). Glutamine synthetase and GFAP are both specific markers of astrocytes in the vertebrate central nervous system (Norenberg, 1979; Eng, 1985) and have also been shown to be markers of glial cells in the brains of decapod crustaceans (GS: Linser et al., 1997; GFAP: Da Silva et al., 2004). Crayfish brains were dissected, fixed, and sectioned as described above. Tissue sections were then incubated overnight at 4°C in mouse anti-GS (1:100; BD Biosciences Pharmingen, San Jose, CA) and rabbit anti-GFAP (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Subsequently, sections were rinsed for 4 h in PBTx and incubated overnight at 4°C in goat anti-mouse Alexa 488 (Molecular Probes) and goat anti-rabbit Alexa 594 (Molecular Probes) secondary antibodies diluted 1:50 in PBTx. Sections were then rinsed for 2 h in PBTx and mounted in Gelmount (Biømeda, Foster City, CA).

## BrdU Exposure Protocol

Proliferating cells within the brain of *C. destructor* were labeled using the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU). BrdU is incorporated into the DNA of replicating cells during the S-phase of mitosis and can be visualized immunocytochemically. Crayfish were placed for 10 to 12 days in solutions of BrdU (Sigma, St Louis, MO) dissolved in freshwater at a concentration of 2 mg/mL. The BrdU solution was refreshed every 2 to 3 days during this period. Subsequently, animals were rinsed for 30 min in several changes of freshwater and placed individually into aquaria measuring 28 $\times$ 17.5 $\times$ 12 cm (L $\times$ W $\times$ H). Crayfish remained within these aquaria for periods of 2 to 6 months, during which time they were fed daily with shrimp pellets.

## Projection Neuron Labeling and BrdU Immunocytochemistry

Brains were dissected from the crayfish in cold crayfish saline and desheathed in the regions surrounding the olfactory and accessory lobes. Projection neurons innervating these lobes were labeled using dextran tetramethylrhodamine 3000 MW (micro-ruby; Molecular Probes) and dextran fluorescein 3000 MW (micro-emerald; Molecular Probes). Dextrans were applied to the lobes using the technique of Utting and colleagues (2000). Briefly, a small dextran crystal was dissolved in 1 $\mu$L of distilled water and 2% bovine serum albumin (BSA) and left to dry. When water is reapplied to this mixture it develops a paste-like consistency and this was used to coat the tips of glass electrodes. The dextran-coated tips were then dipped into molten embedding wax and left to cool. The wax coating prevents the highly soluble dextrans from going into solution before they are applied to the brain neuropils. The coated electrode tips were then placed into the OL and AL and moved from side to side to dislodge the wax and bring the dextrans into contact with the neuropil. Electrodes were applied at locations distributed throughout the lobes in order to label as many projection neurons as possible.

Following the dextran applications, brains were incubated in the dark for 3 to 4 h at room temperature in L-15 medium (Sigma) altered to be iso-osmotic with crayfish saline. The brains were then fixed and sectioned as described above. Tissue sections were incubated in 2*N* HCl for 20 min and then rinsed for 1 h in several changes of PBTx. Subequently, the sections were incubated for 150 min at room temperature in a rat anti-BrdU primary antibody (1:50; Accurate Chemicals, Westbury, NY), rinsed for 1 h in PBTx, and incubated overnight at 4°C in a donkey anti-rat Cy5 secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 in PBTx. Sections were then rinsed for 2 h in PBTx and mounted in Gelmount.

Many of the preparations labeled immunocytochemically for BrdU were also subsequently processed for neuronal (SIFamide) and glial (GS, GFAP) markers, as described above. In these experiments, donkey anti-rabbit Alexa 594 (Molecular Probes) and donkey anti-mouse Cy 2 (Jackson Immunoresearch) secondary antibodies were used to label these markers.

## Confocal Microscopy and Image Processing

Specimens were viewed using a Leica TCS SP laser-scanning confocal microscope equipped with argon, krypton, and helium–neon lasers. Serial optical sections were taken

at intervals of 0.75 to 1 $\mu$m and saved as both three-dimensional stacks and two-dimensional projections.

Sections were examined for the presence of double-labeled (BrdU + neuronal or glial marker; BrdU + dextran) neurons within cluster 10. Individual neurons were considered to be double labeled when a BrdU-labeled nucleus was observed to be completely surrounded by an immunocytochemically- or dextran-labeled soma. Images were processed to adjust brightness and contrast using Adobe Photoshop 7.0 (Adobe Systems).

## RESULTS

### Labeling with Neuronal and Glial Markers in Cluster 10

Large numbers of projection neurons in cluster 10 were found to be immunoreactive to the neuropeptide SIFamide [Fig. 2(A,B)]. The axons of these neurons were labeled within the olfactory globular tract (OGT) [Fig. 2(A), inset] and could be followed to their target neuropils in the lateral protocerebrum (data not shown). SIFamide-labeled somata within cluster 10 were absent, however, in the regions in and around the proliferation zone [Fig. 2(A)], indicating that SIFamide labeling is specific to mature neurons. In order to determine whether the BrdU-labeled cells observed within the proliferation zone subsequently differentiate into neurons, adult crayfish ($n = 3$) were initially exposed to BrdU for 10 to 12 days to label large numbers of newborn cells and then left for 4 to 5 months to enable these cells to differentiate. BrdU and SIFamide labeling of the brains of these animals revealed that approximately half of the BrdU-labeled somata in cluster 10 also labeled for SIFamide [Fig. 2(B)] indicating that many of the newborn cells had differentiated into neurons.

In order to examine the possibility that newborn cells within cluster 10 could also differentiate into glial cells, the distribution of glia within the cluster was examined using the glial-specific markers GS and GFAP [Fig. 2(C,D)]. Labeling for GS revealed the presence of numerous cells with fine processes surrounding most neuropils in the brain [Fig. 2(C), inset]. Such cells, however, were never observed in or around cluster 10 [Fig. 2(C)]. GFAP-labeled cells were observed in cluster 10 in some preparations [Fig. 2(D)], though such cells were few in number. These findings are consistent with those of Harzsch and colleagues (1999) who, distinguishing between neuronal and glial soma on the basis of their size and shape in histological sections, found that cluster 10 in the lobster *Homarus americanus* is composed almost exclusively of neuronal somata with only a very small number of glial cells interspersed amongst them. It remains possible, however, that cluster 10 in *C. destructor* contains additional glial somata that are not immunoreactive to either GS or GFAP.

Double labeling for GFAP and BrdU in the brains of adult crayfish left for 4 to 6 months after exposure to BrdU did not show any evidence of double labeling ($n = 2$; data not shown). Together, these results suggest that few glial somata occur within cluster 10 in *C. destructor* and that most of the adult-born cells within this cluster that survive, differentiate into neurons.

### Spatial Arrangement of Somata within Cluster 10

Dextran labeling of projection neurons innervating the OL and AL of *C. destructor* showed that the somata of these neurons have contrasting spatial distributions within cluster 10 [Fig. 3(A,B)]. The somata of OL projection neurons (red) occur medially within the cluster and are found in greater numbers rostrally than caudally. In contrast, AL projection neuron somata (green) are found primarily laterally within cluster 10 and are more concentrated in its caudal than its rostral half. This spatial arrangement was observed in crayfish of all sizes examined.

Dextran-labeled somata were not observed in and around the cluster 10 proliferation zone [Fig. 3(C)]. Similarly, double-labeled (BrdU + dextran) somata were never observed in cluster 10 in animals examined 24 h after exposure to BrdU [Fig. 3(C)]. These results demonstrate that the BrdU-labeled somata within the proliferation zone belong to newborn cells that do not yet have processes projecting to the OL and AL.

### Neuronal Differentiation of Newborn Cells in Cluster 10 of Early Postembryonic Crayfish

In order to determine whether newborn cells in cluster 10 of early postembryonic crayfish differentiate into OL projection neurons or AL projection neurons, or both, crayfish with carapace lengths less than 0.8 cm ($n = 6$) were exposed to BrdU for 10 to 12 days and then left for periods of 2 to 6 months. The brains of these animals were subsequently labeled with dextrans and immunocytochemically for BrdU. They were then examined for evidence of double-labeled (BrdU + dextran) somata within cluster 10.

Several months after exposing early postembryonic crayfish to BrdU, the somata of the cells that incorporated BrdU had been displaced caudally away from the proliferation zone (adjacent to the OL) to a position adjacent to the AL [Fig. 4(A)]. The location of these cells within the cluster suggests that many had differ-

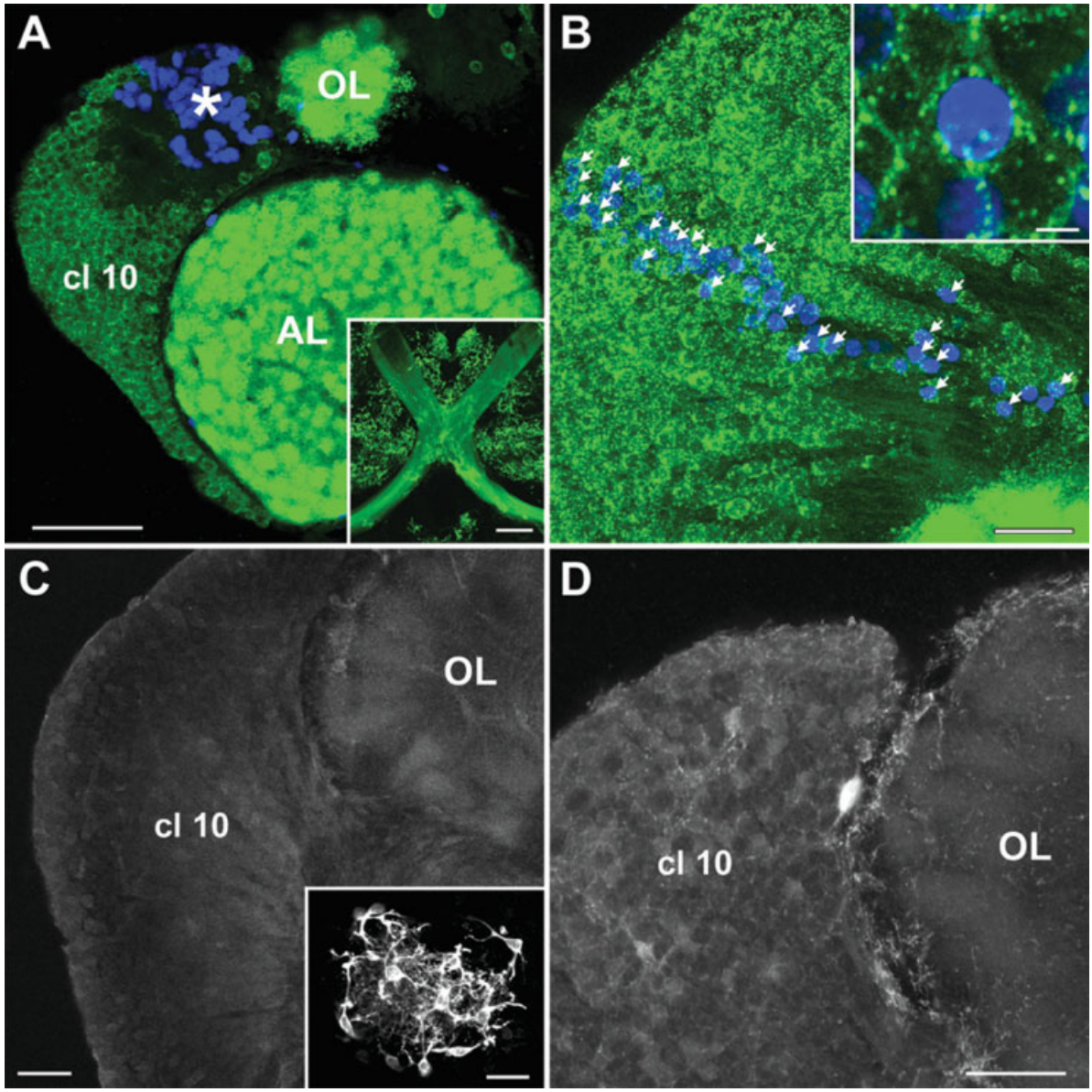


**Figure 2** Labeling of cluster 10 of *C. destructor* with specific neuronal and glial markers. Stacked confocal images of horizontal sections through the deutocerebrum. (A) BrdU (blue) and SIFamide (green) immunolabeling in cluster 10 of an adult crayfish exposed to BrdU for 24 h prior to dissection and fixation of the brain. Note the absence of SIFamide-immunoreactive cells in and around the proliferation zone of cluster 10 (asterisk). The inset shows SIFamide-labeled projection neuron axons within the OGT, which is composed exclusively of the axons of OL and AL projection neurons. (B) BrdU (blue) and SIFamide (green) immunolabeling in cluster 10 of an adult crayfish exposed to BrdU for 12 days and then left for 5 months before dissection and fixation of the brain. The arrows identify neurons double-labeled for BrdU and SIFamide. The inset shows a higher-magnification of the soma of a double-labeled neuron. (C) Immunocytochemical labeling of the deutocerebrum with an antibody against glutamine synthetase. Note the lack of immunolabeled cells within cluster 10. Inset shows glutamine synthetase-immunoreactive glial cells surrounding the ventral portion of the lateral antennular neuropil, which lies adjacent to the accessory lobe (Sandeman et al., 1992). (D) Immunocytochemical labeling with an antibody against GFAP. A small number of immunolabeled cells were observed within cluster 10. Abbreviations: AL, accessory lobe; cl 10, soma cluster 10; OL, olfactory lobe. Scale bars = 100 $\mu$m in (A); 80 $\mu$m in insert in (A); 50 $\mu$m in (B); 5 $\mu$m in insert in (B); 50 $\mu$m in (C); 40 $\mu$m in insert in (C); 50 $\mu$m in (D).

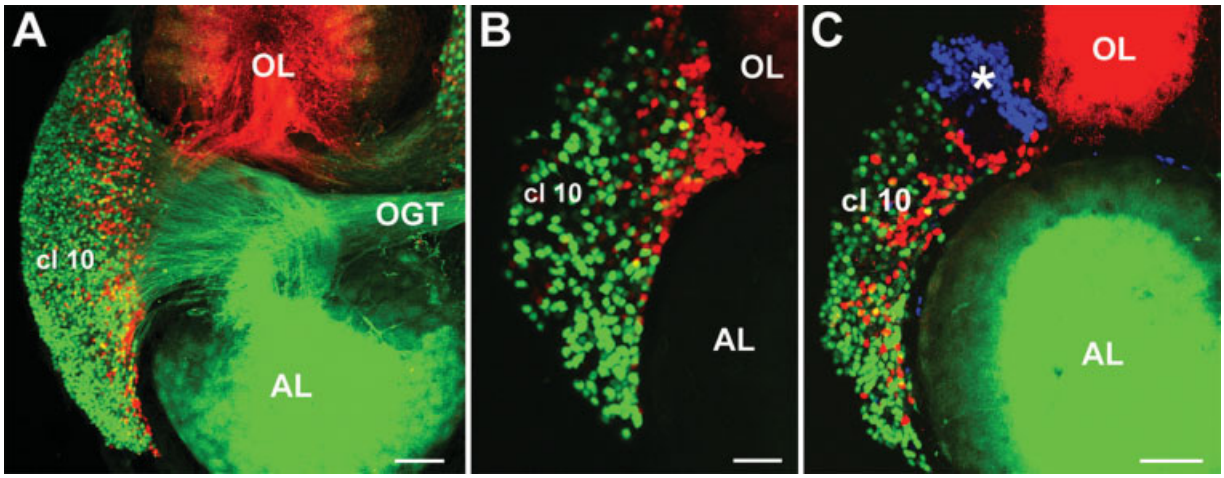


**Figure 3** Dextran labeling of the olfactory and accessory lobe projection neuron populations of *C. destructor*. Horizontal sections through brains in which micro-ruby dextran (red) was applied to the olfactory lobe and micro-emerald dextran (green) was applied to the accessory lobe. (A, B) Sections showing the different spatial distributions of olfactory and accessory lobe projection neuron somata within cluster 10. The section shown in (A) is dorsal to that shown in (B). (C) BrdU (blue) and dextran labeling of the brain of a crayfish exposed to BrdU for 24 h before the dextran applications. BrdU-labeled cells within the cluster 10 proliferation zone (asterisk) were unlabeled by the dextrans, indicating that these cells do not possess processes that project to either of the deutocerebral lobes. Abbreviations: AL, accessory lobe; cl 10, soma cluster 10; OGT, olfactory globular tract; OL, olfactory lobe. Scale bars = 100 $\mu$m in (A); 50 $\mu$m in (B); 80 $\mu$m in (C).

entiated into AL projection neurons, as this region of cluster 10 is tightly packed with these neurons (Fig. 3). Dextran-labeling of the AL and OL projection neuron populations, combined with BrdU immunocytochemistry, confirmed that this is, in fact, the case [Fig. 4(B,C)]. Large numbers of double-labeled AL projection neurons were observed in these animals [Fig. 4(B)] and the axons of many of these neurons could be clearly resolved [Fig. 4(C)]. Double-labeled OL projection neurons were also encountered [Fig. 4(D)], although infrequently, indicating that small numbers of cells also differentiate into OL projection neurons. Significant numbers of double-labeled projection neurons were only observed in crayfish examined 4 to 6 months after exposure to BrdU, indicating that these neurons require at least 4 months to differentiate. Together, these results indicate that the majority of newborn cells in early postembryonic crayfish differentiate into AL projection neurons, and relatively few into OL projection neurons, and that this differentiation occurs over at least 4 months.

## Neuronal Differentiation of Newborn Cells in Adult Crayfish

While the early postembryonic development of *C. destructor* is characterized by the rapid growth of the AL relative to the OL, the ratio of the volumes of the two lobes remains fixed once animals have attained a carapace length of ~0.8 cm (Sandeman et al., 1998). In order to determine whether the disproportionate addition of AL projection neurons is restricted to early postembryonic development or whether it is also characteristic of adult neurogenesis, we examined the differentiation of newborn cells in adult crayfish (carapace lengths $\geq$ 1.4 cm). Like the early postembryonic crayfish, these crayfish ($n = 4$) were incubated in BrdU for 10 to 12 days and then left for periods of 4 to 6 months to allow cells that incorporated BrdU to differentiate.

Newborn cells in adult crayfish, as in early postembryonic crayfish, are displaced away from the proliferation zone over time [Fig. 5(A)]. While the populations of BrdU-labeled cells could be observed to have moved dorsally [Fig. 5(A)] and caudally [Fig. 5(A), upper inset] away from the proliferation zone they did not exhibit the extensive displacements characteristic of cells born during early postembryonic development. The BrdU-labeled somata remain clustered together and in some preparations they formed linear arrays [Fig. 5(A), lower inset]. Dextran labeling of the OL and AL projection neuron populations, combined with BrdU immunocytochemistry, revealed the presence of many double-labeled

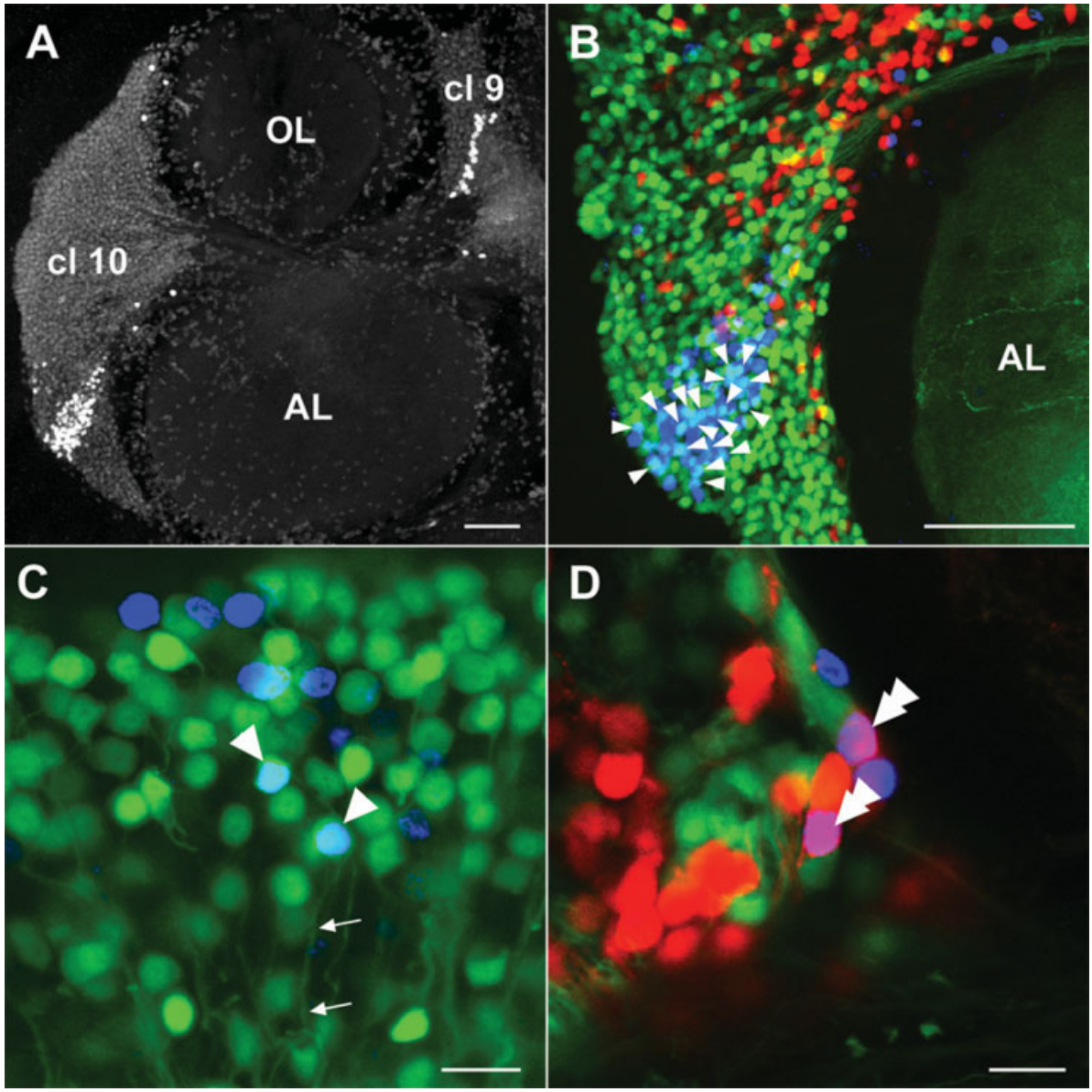


**Figure 4** BrdU and dextran labeling in the brains of crayfish exposed to BrdU during early postembryonic development and then left for 4 to 6 months. Horizontal sections. (A) BrdU labeling shows that cells which incorporated BrdU in the cluster 10 proliferation zone (adjacent to the OL) have been displaced caudally in the cluster and are now located adjacent to the AL. (B-D) Sections through brains in which micro-ruby dextran (red) was applied to the OL, micro-emerald dextran (green) was applied to the AL, and BrdU (blue) was labeled immunocytochemically. (B, C) Many double-labeled AL projection neuron somata (cyan) could be observed in cluster 10 (arrowheads) and the axons [arrows in (C)] of many of these cells could be clearly distinguished. (D) A small number of double-labeled OL projection neuron somata (purple; double arrowheads) were also observed in these preparations. Abbreviations: cl 9, soma cluster 9; cl 10, soma cluster 10; AL, accessory lobe; OL, olfactory lobe. Scale bars = 100 $\mu$m in (A) and (B); 20 $\mu$m in (C) and (D).

neuronal somata within cluster 10 [Fig. 5(B–D)]. Unlike in crayfish exposed to BrdU during early postembryonic development, however, double-labeled OL (purple) and AL (cyan) projection neuron somata were both frequently encountered in these animals [Fig. 5(B)], with lateral regions of cluster 10 containing numerous double-labeled AL projection neurons [Fig. 5(C)] and more medial regions containing num-

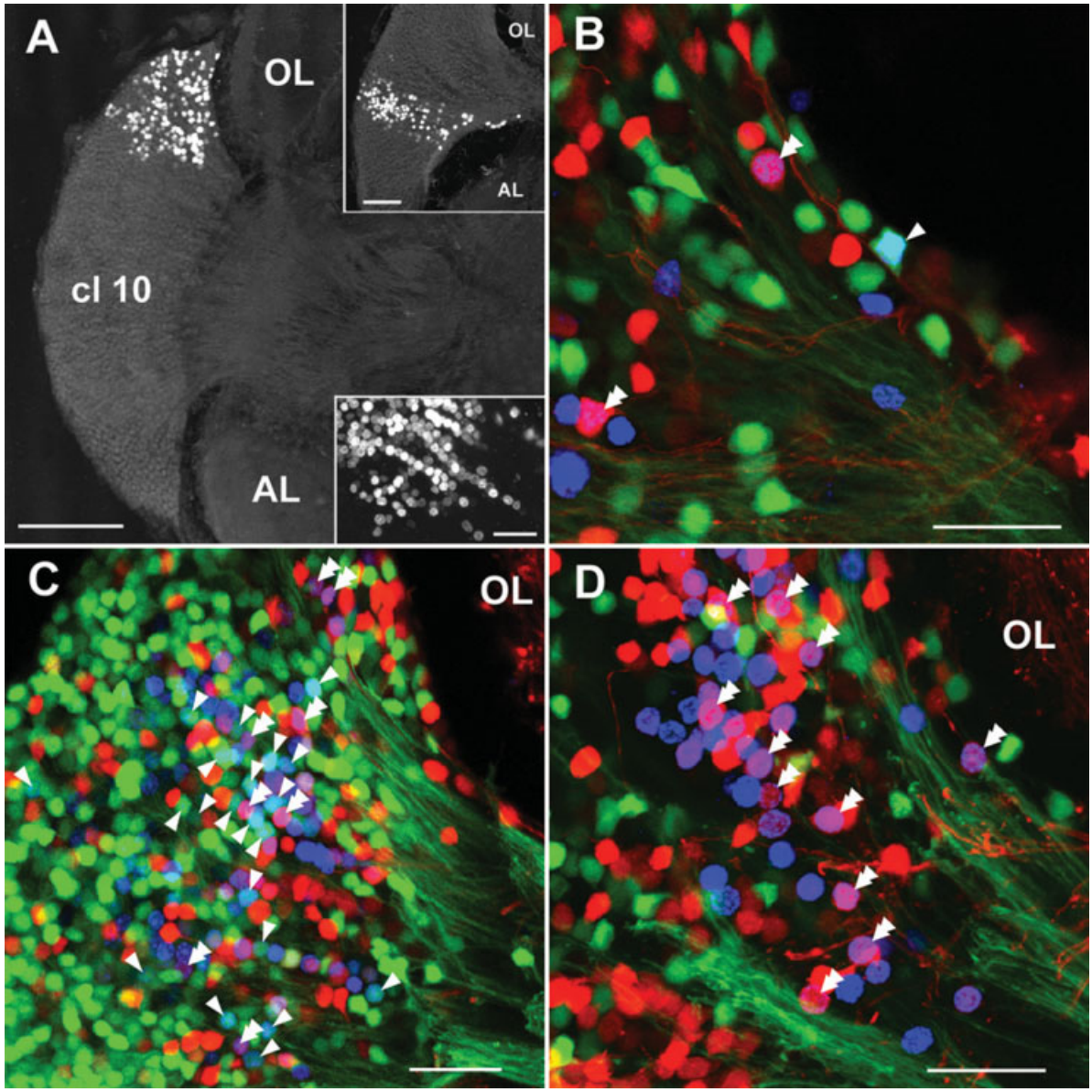


**Figure 5** BrdU and dextran labeling in the brains of adult crayfish exposed several months earlier to BrdU for 10 to 12 days. Horizontal sections. (A) BrdU labeling shows that the cells that incorporated the substitute nucleoside have moved dorsally and caudally away from the proliferation zone. The upper inset shows a section dorsal to that shown in (A). The lower inset shows that some BrdU-labeled somata form linear arrays. (B–D) Sections through brains in which micro-ruby dextran (red) was applied to the OL, micro-emerald dextran (green) was applied to the AL, and BrdU (blue) was labeled immunocytochemically. Double-labeled OL (purple; double arrowheads) and AL (cyan; arrowheads) projection neuron somata were both frequently observed in cluster 10 (B) with more lateral regions containing numerous double-labeled AL projection neuron somata (C) and more medial regions containing numbers of double-labeled OL projection neuron somata (D). Abbreviations: cl 10, soma cluster 10; AL, accessory lobe; OL, olfactory lobe. Scale bars = 200 $\mu$m in (A); 100 $\mu$m in upper inset in (A); 50 $\mu$m in lower inset in (A); 40 $\mu$m in (B); 50 $\mu$m in (C) and (D).

bers of double-labeled OL projection neuron somata [Fig. 5(D)]. These data demonstrate, therefore, that neurogenesis in adult crayfish is characterized by the differentiation of substantial numbers of newborn cells into both OL and AL projection neurons. In addition, they also provide evidence for a marked shift during early postembryonic development in the phenotypes of neurons differentiating in cluster 10.

### Neuronal Proliferation and Differentiation within Soma Clusters Associated with the Optic Neuropils of *C. destructor*

In the course of our studies of neurogenesis within the deutocerebrum of *C. destructor* we observed that significant numbers of BrdU-labeled cells were also present within soma clusters associated with the optic neuropils [medulla interna, medulla externa, lamina ganglionaris; Fig. 1(A)]. In both early postembryonic and adult crayfish, groups of proliferating cells were observed adjacent to the medulla externa and interna [Fig. 6(A)]. Proliferating cells were also observed in long, thin arrays both rostral and caudal to the ventral surfaces of the medulla externa and the lamina ganglionaris [Fig. 6(A)]. In order to determine whether some of these newborn cells differentiate into neurons, adult crayfish were exposed to BrdU for 10 to 12 days and then left for periods of 4 to 6 months. Subsequently, different dextran dyes were applied to the optic neuropils of these animals ($n = 6$) and the tissues processed immunocytochemically for BrdU. Double-labeled (BrdU + dextran) somata were observed in preparations in which dextrans were applied to the medulla externa [Fig. 6(B)]. We found no double-labeled cells innervating either the lamina ganglionaris or the medulla interna (data not shown). Immunocytochemical labelling of the optic lobes of these animals for both BrdU and SIFamide ($n = 2$) revealed the presence of double-labeled somata [Fig. 6(C)]. Together, these results demonstrate that some of the newborn cells within the optic lobes of adult crayfish differentiate into neurons.

In some preparations, tissues were also labeled immunocytochemically for serotonin, a neurotransmitter that is known to be expressed by numerous neurons innervating the optic ganglia of *C. destructor* (Sandeman et al., 1988). Although double-labeled somata were not observed in adult crayfish (data not shown), they were observed in crayfish exposed to BrdU during early postembryonic development indicating that some of the serotonergic cells in the optic ganglia are born postembryonically.

## DISCUSSION

Although life-long proliferation within cluster 10 has been shown to be a characteristic feature of the brains of all decapod crustacean taxa studied, examination of the differentiation of these cells has been impeded by the lack of specific crustacean neuronal markers and by the fact that the identities of the neurotransmitters used by mature OL and AL projection neurons were, until recently, unknown. In the absence of specific immunocytochemical markers to label these neurons, it was only possible to demonstrate that adult-born cells become dispersed throughout cluster 10 and that they survive for at least a year (Harzsch et al., 1999; Beltz et al., 2001; Schmidt, 2001b). It has not been possible to demonstrate directly, however, whether these newborn cells do, in fact, differentiate into neurons and what the eventual phenotypes of these neurons might be. In the present study, we used an antibody against the newly described crustacean neuropeptide SIFamide, as well as specific glial markers, to examine the differentiation of newborn cells in cluster 10 of the crayfish, *C. destructor*. Large numbers of adult-generated cells were found to differentiate into mature SIFamide-expressing neurons, demonstrating definitively that neurogenesis occurs within cluster 10 in adult crayfish. In contrast, no evidence was obtained for the differentiation of newborn cells within this cluster into glia.

In addition to demonstrating the neuronal fate of adult-generated cells, we used the application of dextran dyes to the OL and AL of *C. destructor* to determine the final phenotypes of these neurons: OL or AL projection neurons, or both. The application of these dyes to the brains of crayfish exposed several months earlier to BrdU demonstrated that during the early postembryonic development of *C. destructor* large numbers of AL projection neurons are added to cluster 10, with a comparatively small number of newborn cells differentiating into OL projection neurons. This addition of AL projection neurons occurs during a developmental period when the AL increases rapidly in size relative to the OL (Helluy et al., 1993; Sandeman et al., 1998). The accelerated growth of the AL relative to the OL appears to be accomplished, at least in part, therefore, by the selective addition of AL projection neurons. The accelerated growth of the AL relative to the OL ceases once crayfish have attained a carapace length of $\sim$0.8 cm and thereafter the ratio of the volumes of the two lobes remains stable (Sandeman et al., 1998). In crayfish with carapace lengths $\geq$ 1.4 cm we observed that substantial numbers of both OL and AL projection neurons are added to cluster 10. The changes in the growth patterns of the deutocerebral lobes appear therefore to be accompanied by changes in the final phenotypes of neurons differentiating in cluster 10. Together, these results indicate that the disproportionate addition of AL projection neurons is restricted to the early postembryonic development of *C. destructor* and that the continuous proliferation observed in cluster 10 of

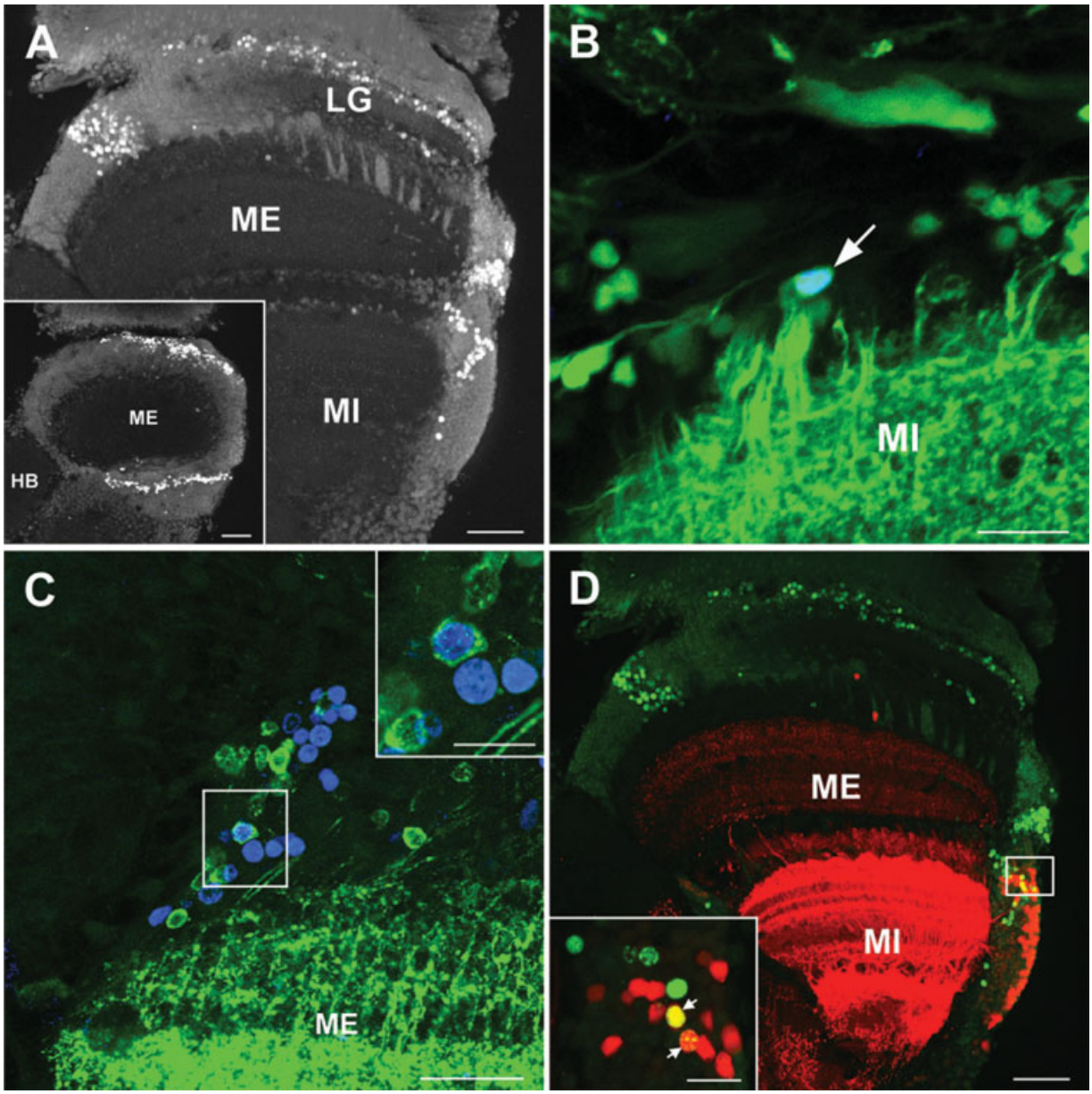


**Figure 6** Cell proliferation within soma clusters associated with the optic neuropils of *C. destructor*. Horizontal sections through the optic lobes of early postembryonic (A, D) and adult (B, C) crayfish exposed to BrdU for 10 to 12 days and then left for 4 to 6 months. (A) Groups of BrdU-labeled cells can be observed adjacent to the medulla interna and the medulla externa. Lines of labeled cells can also be observed rostral and caudal to the lamina ganglionaris. The inset shows that lines of BrdU-labeled cells also occur rostral and caudal to the medulla externa at its ventral surface. (B) Dextran (green) and BrdU (blue) labeling in an optic lobe of an adult crayfish in which micro-emerald dextran was applied to the medulla externa and the tissue subsequently processed immunocytochemically for BrdU. Note the presence of a double-labeled (cyan) soma (arrow) adjacent to the medulla interna. (C) Immunocytochemical labeling for the neuropeptide SIFamide (green) and BrdU (blue) in an optic lobe of an adult crayfish. The square highlights a double-labeled neuronal soma shown at higher-magnification in the inset. (D) Immunocytochemical labeling for serotonin (red) and BrdU (green) in the optic lobe of a crayfish exposed to BrdU during early postembryonic development. The square highlights a region of a soma cluster adjacent to the medulla interna in which double-labeled (yellow) somata were observed. This region is shown in higher magnification in the inset in which two double-labeled (arrows) can be observed. Double-labeled somata were not observed in the optic lobes of crayfish exposed to BrdU as adults. Abbreviations: cl 10, soma cluster 10; AL, accessory lobe; OL, olfactory lobe. Scale bars = 100 $\mu$m in (A) and in inset in (A); 25 $\mu$m in (B); 50 $\mu$m in (C); 20 $\mu$m in inset in (C); 100 $\mu$m in (D); 25 $\mu$m in inset in (D).

adult crayfish represents the addition of both OL and AL projection neurons.

Dextran labeling of the OL and AL projection neurons of *C. destructor* also showed that the somata of these two populations have different spatial distributions within cluster 10. A spatial segregation of OL and AL projection neuron somata has also been described in cluster 10 of the lobster, *Panulirus argus* with OL projection neuron somata occurring predominantly in the lateral half of cluster 10 and AL projection neuron somata occupying the medial half (Wachowiak and Ache, 1994; Schmidt and Ache, 1996; Wachowiak et al., 1996). BrdU labeling studies have shown that the proliferation zone in cluster 10 of adult *P. argus* is located laterally within the cluster (Schmidt and Harzsch, 1999; Schmidt, 2001b) and that even after 14 months nearly all BrdU-labeled cells remain within the lateral half of the cluster (Schmidt, 2001b), suggesting that most adult-born cells differentiate into OL projection neurons. In contrast, the results of the present study indicate that neurogenesis in adult *C. destructor* is characterized by the addition of both OL and AL projection neurons. While the functional importance of life-long neurogenesis in the olfactory pathways of decapod crustacean remains unknown, the present results suggest that it plays a role in the functioning of both the OL and AL in adult *C. destructor*. Furthermore, they suggest that the AL of *C. destructor* may possess a functional plasticity which *P. argus* lacks.

Dextran applications to the OL and AL of *C. destructor* reliably label large numbers of OL and AL projection neurons. Although care was taken in each preparation to apply the dextrans at points distributed throughout both lobes so as to label as many projection neurons as possible, it is difficult to determine the proportions of the projection neuron populations labeled in each preparation and also how these might vary between preparations. This technique cannot be used, therefore, for a precise quantitative assessment of the numbers of adult-generated cells differentiating into OL and AL projection neurons. The fact, however, that double-labeled OL and AL projection neurons were encountered consistently with similar frequencies suggests that approximately equal numbers of OL and AL projection neurons are added to cluster 10 in adult crayfish. Additional, specific markers of these neuronal types will be required, however, to determine the exact numerical proportions of newborn OL and AL projection neurons.

Little is currently known about the identities of the progenitor cells present in cluster 10 of adult decapod crustaceans (Schmidt, 2001b; Beltz and Sandeman, 2003). It is also unclear, therefore, whether cells that differentiate into OL or AL projection neurons are the progeny of different progenitors or whether all newborn cells have the capacity to differentiate into either neuronal type. In the mammalian olfactory bulb, where adult-generated cells differentiate into both granule and periglomerular cells (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Winner et al., 2002), a subset of progenitor cells has been shown to give rise to periglomerular, but not granule, cells (Beech et al., 2004). These results suggest that distinct progenitor cells may give rise to these two adult-generated neuronal populations. If this is also the case in *C. destructor*, the marked change in the pattern of neuronal differentiation observed in cluster 10 during early postembryonic development may be indicative of changes in the progenitor cell population within the cluster. Further information about the precursor cells in cluster 10 is crucial, therefore, for determining the extent to which the final phenotype of the adult-generated neurons in these animals depends upon intrinsic programming or extrinsic factors.

Unlike the olfactory lobe, which is involved in the processing of primary olfactory inputs, the accessory lobes of crayfish and lobsters receive higher-order multimodal inputs (Sandeman et al., 1995; Wachowiak et al., 1996). We recently reported evidence of important functional differences between the two subregions (cortex and medulla) of the accessory lobe of *C. destructor*, suggesting that the accessory lobe cortex is involved primarily in the processing of olfactory inputs whilst the medulla is involved in the processing of multimodal (including olfactory) inputs (Sullivan and Beltz, 2005). These two accessory lobe subregions were also found to have separate output pathways to the two lobes of the hemiellipsoid body. There appear, therefore, to be at least two functionally and anatomically distinct populations of AL projection neurons, with one group being involved primarily in the processing of olfactory inputs and the other in the processing of multimodal inputs. Preliminary experiments in the present study in which dextran dyes were applied to the hemiellipsoid body lobes failed to label AL projection neuron somata because of the long distances between cluster 10 and the hemiellipsoid body in adult crayfish (Jeremy Sullivan, unpublished observation). We were unable to determine, therefore, whether adult-generated AL projection neurons innervate the accessory lobe cortex or medulla, or both. Resolution of this question will be an important step in understanding the functional importance of adult neurogenesis in the crustacean brain.

In addition to the proliferation zone present in cluster 10 of decapod crustaceans, proliferation zones within other soma clusters associated with the olfac-

tory pathway have also been described in certain decapod taxa. In crabs, for example, life-long neurogenesis has been described amongst interneurons innervating the lateral protocerebrum, the target neuropil of the OL projection neurons (Schmidt, 1997; Schmidt and Harzsch, 1999; Hansen and Schmidt, 2001). The current study is the first, however, to present evidence of postembryonic neurogenesis amongst interneuronal populations innervating the optic neuropils of a decapod crustacean. Clusters of proliferating cells were observed adjacent to all three of the optic neuropils of *C. destructor* and some of these newborn cells in adult crayfish were shown to differentiate into neurons innervating the medulla externa. Proliferation zones within these soma clusters also occur in adult spider crabs, *Libinia emarginata* (Jeremy Sullivan, unpublished observation) suggesting that adult neurogenesis within this region of the brain may be characteristic of other decapod species, as well. Life-long neurogenesis may therefore be a functionally important characteristic of both the olfactory *and* optic pathways of decapod crustaceans.

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