Environmental Enrichment Influences Neuronal Stem Cells in the Adult Crayfish Brain

Neishay Ayub, Jeanne L. Benton, Yi Zhang, Barbara S. Beltz

Neuroscience Program, Wellesley College, Wellesley, MA 02481

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ABSTRACT: **New neurons are incorporated throughout life into the brains of many vertebrate and nonvertebrate species. This process of adult neurogenesis is regulated by a variety of external and endogenous factors, including environmental enrichment, which increases the production of neurons in juvenile mice and crayfish. The primary goal of this study was to exploit the spatial separation of the neuronal precursor cell lineage in crayfish to determine which generation(s) of precursors is altered by environmental conditions. Further, in crayfish, an intimate relationship between the first-generation neuronal precursors (stem cells) and cells circulating in the hemolymph has been proposed (Zhang et al., 2009). Therefore, a second goal was to assess whether environmental enrichment alters the numbers or types of cells circulating in the hemolymph. We find that neurogenesis in the brains of sexually differentiated procambarid crayfish is enhanced by environmental enrichment as previously demonstrated by Sandeman and Sandeman (2000) in young, sexually undifferentiated *Cherax destructor*. We also show that environmental enrichment increases the cell cycle rate of neuronal stem cells. Although there was no effect of environment on the overall numbers of cells circulating in the hemolymph, enrichment resulted in increased expression of glutamine synthetase (GS), a marker of the neuronal stem cells, in a small percentage of circulating cells; there was little or no GS immunoreactivity in hemolymph cells extracted from deprived animals. Thus, environmental enrichment influences the rate of neuronal stem cell division in adult crayfish, as well as the composition of cells circulating in the hemolymph.** Develop Neurobiol 71: 351–361, 2011

Keywords: **adult neurogenesis; hematopoietic system; olfactory pathway; self-renewal; neurogenic niche**

INTRODUCTION

Neurogenesis persists throughout life in many vertebrate and invertebrate species. In mammals, including humans, adult-born neurons are incorporated into olfactory bulb and hippocampal circuits (Gage et al., 2008). In the crustacean brain, adult neurogenesis produces neurons that are integrated into primary olfactory and higher-order processing centers, the olfactory and accessory lobes [Fig. 1(A)] (Schmidt, 1997; Schmidt and Harzsch, 1999).

Enriched environmental conditions increase the survival of adult-born hippocampal neurons in young mice (Kempermann et al., 1997), and increase proliferation and survival of newly born hippocampal neurons in old mice, reversing age-related decreases in neuronal proliferation (Kempermann et al., 2002). The number of adult-born olfactory neurons is not, however, altered by these conditions (Brown et al., 2003). Environmental enrichment of crayfish beginning at 3 days after the molt to the initial and still sexually undifferentiated adult stage (ADI_3) stimulates the proliferation of precursor cells and survival of interneurons that innervate the olfactory and accessory

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

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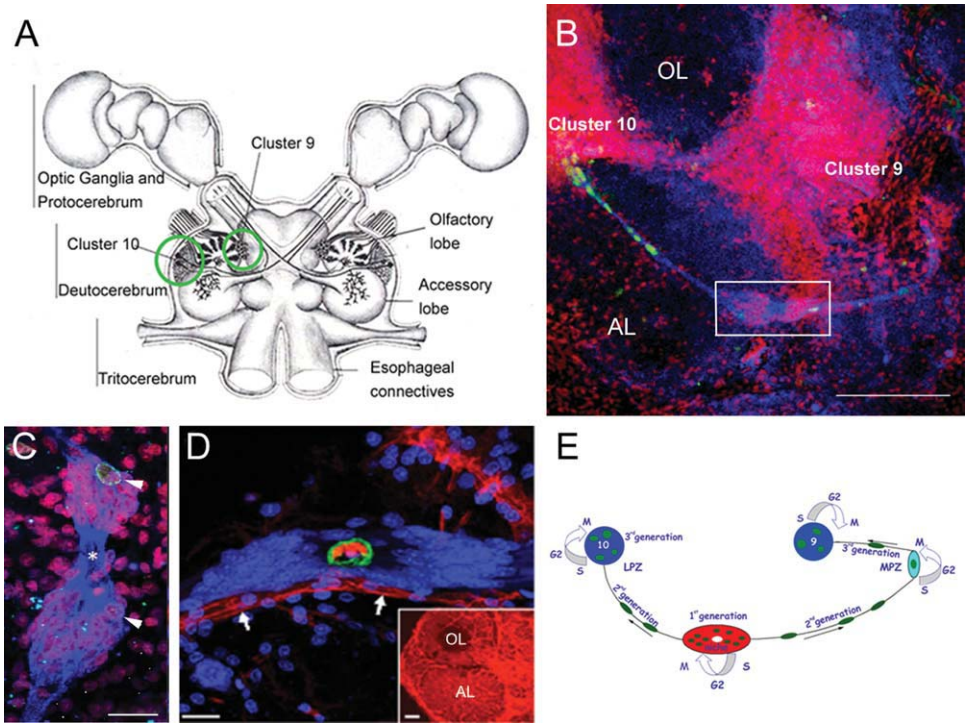

Figure 1 **A:** Diagram of a crayfish brain. Cell Clusters 9 and 10, which are composed of interneurons in the olfactory pathway, are the sites where adult-born neurons are integrated. **B:** Olfactory lobe, accessory lobe, neurogenic niche and streams in the *P. clarkii* brain labeled for 5-bromo-2-deoxyuridine (BrdU) (green, S-phase marker), GS (blue, glial marker), and propidium iodide (red, nucleic acid marker). The neurogenic niche is outlined by a white box. **C:** The neurogenic niche, labeled for GS (blue), propidium iodide (red), and BrdU (green). The asterisk marks the vascular cavity in the niche that is confluent with the circulation (Sullivan et al., 2007a; Sandeman et al., 2009). Arrowheads point to BrdU-labeled cells. **D:** Dextran Micro-Ruby (red) injection into the dorsal artery fills blood vessels in the brain (inset) and vascular cavity of the niche (outlined by anti –Elav [green; a neuronal RNA binding protein marker] and propidium iodide [blue]). The niche lies on a blood vessel, also filled with dextran (arrows). **E:** Model illustrating our current hypothesis regarding the generations of precursors responsible for the production of adult-born neurons. The first-generation (stem) cells reside in the niche. They divide symmetrically and their daughters (second-generation cells) migrate along a fibrous tract created by the niche cells, to either cell Cluster 9 or 10. At least one more division occurs in the proliferation zone before the descendants (third and later generations) differentiate into neurons. AL, accessory lobe; LPZ, lateral proliferation zone; MPZ, medial proliferation zone; OL, olfactory lobe; S, G2 and M-labeled arrows indicate cells that are actively in the cell cycle. Scale Bars: 200 μm in B; 25 μm in C; 25 μm in D; 100 μm in inset. [Fig. 1(D) from Sullivan et al., 2007a; E from Zhang et al., 2010].

lobes (Sandeman and Sandeman, 2000). It is not known whether environmental enrichment is an effective regulator of neuronal production in crayfish after the initial adult stage (ADI).

Our understanding of environmental regulation of neurogenesis is incomplete without a knowledge of which generations in the neuronal precursor lineage are influenced by enriched conditions. Changes in the numbers of first-generation precursors (stem cells) or their cell cycle rate have a much greater potential to alter neuronal proliferation than influences exerted on later generations in the lineage. However, this knowledge is not available in either mammals or crustaceans. In mammals, defining the influence of environment on specific neuronal precursor generations is particularly challenging, because several types of progenitor cells coexist in the neurogenic niches producing adult-born neurons (García-Verdugo et al., 1998; Seri et al., 2004; Zhao et al., 2008), and the lineage relationships among these cell types have not been directly demonstrated (Kan et al., 2010). Therefore, to detect changes in the mitotic index in specific classes of precursors, multiple markers that characterize stages in this lineage must

be assessed in conjunction with cell cycle indicators (Kuhn and Peterson, 2008).

In contrast, adult neurogenesis in the crayfish brain involves generations of precursor cells that are spatially segregated from each other, except for a small transitional region between each compartment [Fig. 1(B,E)] (Sullivan et al., 2007a,b). The neuronal stem cells (first-generation precursors) comprise a vascularized niche [Fig. 1(B–D)]; these bipolar cells also provide a tract along which their progeny (the second-generation precursors) migrate (Sullivan et al., 2005, 2007a,b). These migratory precursors move toward proliferation zones in cell Clusters 9 and 10 [Fig. 1(A)] (terminology of Sandeman et al., 1992), where they divide at least once more. Their progeny differentiate into Clusters 9 (local) and 10 (projection) interneurons that innervate the primary olfactory processing areas (the olfactory lobes) and higher-order processing centers (the accessory lobes) (Sullivan and Beltz, 2005). Because of the spatial segregation of the first, second, and third and later generations of neuronal precursors in crayfish, influences of environmental enrichment on different parts of the lineage are easily assessed.

To define the influence of environmental enrichment on adult neurogenesis in crayfish, Sandeman and Sandeman (2000) gauged the incorporation of 5-bromo-2-deoxyuridine (BrdU) into the third-generation neuronal precursors and their descendants in the proliferation zones of Clusters 9 and 10, where they found increased cell proliferation and survival. The neuronal precursor cell lineage in crayfish [see Fig. 1(E)] had not yet been discovered, and so influences on the first and second-generation precursors were not determined. The primary goal this study, therefore, was to exploit the spatial separation of this lineage to examine the influence of environmental enrichment on these precursors. The second goal was to test whether environmental enrichment alters adult neurogenesis in older crayfish, or whether these effects are confined to very young (ADI) crayfish.

Finally, experiments in crayfish have indicated that the neuronal stem cells are not a self-renewing population (Zhang et al., 2009; Benton et al., 2010). Given the close relationship between the vasculature and the niche where these first-generation precursors reside, we have hypothesized that the hematopoietic system is a potential source of neuronal stem cells in crayfish (Zhang et al., 2009). If hematopoietic stem cells indeed contribute to the first-generation precursor pool, environmental conditions may also affect their numbers or properties. The final goal of the present studies, therefore, was to ask if living conditions influence the numbers or specific composition of cells circulating in the hemolymph, which would be products of the hematopoietic system.

These studies show that neurogenesis in the brains of sexually differentiated (but not yet sexually active) mid-life crayfish (*Procambarus spp.*) is enhanced by environmental enrichment as previously demonstrated in young ADI_3 *Cherax destructor* (Sandeman and Sandeman, 2000). In addition, we show that environmental enrichment increases the rate of BrdU incorporation into the first-generation neuronal precursors, indicating that the cell cycle time of these stem cells decreases. In small adult crayfish (carapace length 4 mm, CL4), enrichment also increases the number of first-generation precursor cells residing in the niche, although this effect was not seen in larger animals (carapace length 10 mm, CL10). Although there was no effect of environment on the overall numbers of cells circulating in the hemolymph, enrichment resulted in immunoreactivity for glutamine synthetase (GS), a marker of the neuronal stem cells residing in the niche [Fig. 1(B,C)], in a small percentage of circulating cells; there was little or no expression of this enzyme in hemolymph cells extracted from animals maintained in the deprived environment. Thus, enrichment influences the first-generation neuronal precursors (stem cells) as well as the composition of cells circulating in the hemolymph in adult crayfish. These findings have been previously published in abstract form (Ayub et al., 2010).

MATERIALS AND METHODS

Animals

Experiments were conducted using two closely related sympatric species of freshwater crayfish (*Procambarus clarkii* and *Procambarus acutus*) obtained from a commercial supplier (Carolina Biological Supply Company, Burlington, NC). Crayfish were housed and maintained in the Wellesley College Animal Care Facility, where they were kept at room temperature on a 12/12 light/dark cycle. Two sizes of crayfish with starting carapace lengths (CL) of 4 mm and 10 mm ($\sim$ADII and ADVII, respectively) were used in these experiments. CL4 crayfish are sexually undifferentiated, whereas the CL10 crayfish are sexually differentiated but not yet sexually active. Mixed groups of male and female crayfish of both sizes were therefore used in these studies.

Enriched and Deprived Environments

Four groups of crayfish ($n = 10$ per group) were maintained in a 20-gallon aquarium with recirculating, filtered artificial pond water: (1) enriched CL4, (2) enriched CL10, (3)

deprived CL4, and (4) deprived CL10. Each group consisted of 10 crayfish at the beginning of the experiment, a number small enough that they could establish territories but be freely active. The enriched environment consisted of group housing in a 20-gallon tank (30″ × 12.5″ × 12.5″) filled with gravel, foliage, tunnels, a tree-like structure, and a rock; CL4 and CL10 crayfish were separated by a large mesh divider, which divided the tank in half. In the deprived environments, crayfish were individually housed in containers that floated on top of the tank. Ice cube trays in which the bottom was replaced with nylon mesh housed the CL10 crayfish; CL4 animals were maintained in conical tubes that were cut down and covered with nylon mesh. For both groups, the deprived crayfish had 1.8 cm^3 space/mm carapace length. Water flowed freely through the mesh bottoms in these containers, insuring that the deprived animals shared the same water as the enriched animals. To increase the sample size and to replicate initial findings, two sequential trials were conducted; as there were no statistically significant differences in the results from the two trials, data were combined for the final results.

Hemolymph Analysis

After ~3 weeks exposed to these environmental conditions, hemolymph samples were taken between 11:00 and 13:00 h from four CL10 animals from both enriched and deprived groups. This time frame was chosen based on the need for a minimum of 2 weeks to observe the influence of environmental enrichment in ADI crayfish (Sandeman and Sandeman, 2000). Hemolymph was drawn from the dorsal sinus of the animal with a 1 mL syringe and 25 5/8 gauge needle, and mixed 1:2 with anticoagulant buffer (Söderhäll et al., 2003). This mixture was combined with Trypan blue (Sigma) (1:1), and 10 μL of this solution placed on the counting grid of a hemocytometer for quantification of hemolymph cells. Average counts were calculated from 2 to 3 separate samples taken from each blood draw. Hemolymph from CL4 crayfish was not evaluated because the blood volume is too small to provide adequate samples for analysis. One factor that may influence hemolymph cell counts is time of day; hemolymph counts in the mole-crab (*Emerita asiatica*) were found to be higher in the afternoon than in the morning (Ravindranath, 1977). In the present studies, to control for time of day, counts were conducted in a random order and they were completed within 2 h on the same day. Therefore, circadian factors are not likely to have influenced the results of the present studies.

BrdU Exposure and Immunohistochemistry

Beginning at 19:00 h on the day hemolymph samples were taken, crayfish were incubated in the S-phase marker BrdU (Sigma; 2 mg/mL of pond water). Crayfish from the enriched environment were incubated in BrdU together, while those from the deprived environments were incubated individually, thus replicating the social environment of their living conditions. After 18 h in BrdU, they were sacrificed. Brains were dissected in cold crayfish saline and fixed overnight in 4% paraformaldehyde in 0.1*M* phosphate buffer (PB) and processed immunohistochemically as in prior studies (Zhang et al., 2009). Briefly, brains were rinsed multiple times in PB containing 0.3% Triton X-100 (PBTx), then incubated in 0.2 N HCl and rinsed in PBTx. Brains were incubated with rat anti-BrdU (Accurate Chemical and Scientific, Westbury, NY; 1:50 dilution) at room temperature for 3 h. Subsequently, brains were rinsed and incubated for 12 h at 4°C with mouse anti-GS (Becton Dickinson; 1:100), rinsed again and incubated with secondary antibodies (Cy2-conjugated goat anti-rat IgG and Cy5-conjugated goat anti-mouse IgG; Jackson Immunoresearch; 1:100; 12 h at 4°C). Brains were rinsed in PBTx, incubated in the nuclear marker propidium iodide (10 μg/mL in PB; Sigma) for 10 min, and rinsed again in PB. The brains were mounted on slides with Gel/Mount™ (Electron Microscopy Sciences, Hatfield, PA).

At the time of dissection, hemolymph was taken from four CL10 crayfish from each environmental condition and smeared on slides prepared with poly-L-lysine to promote cell adhesion. Hemolymph cells were fixed with 4% paraformaldehyde for 15 min, rinsed for 1 h with PBTx, and incubated with donkey anti-mouse IgG FAB (Jackson Immunoresearch; 1:10) and donkey anti-rabbit IgG (Jackson Immunoresearch; 1:100); samples were fixed again with 4% paraformaldehyde. These treatments were done to prevent nonspecific binding of subsequent antibodies. Hemolymph smears were then rinsed for another hour, and all but one of the samples from the deprived animals was incubated overnight at 4°C with mouse anti-GS; the slide without this antibody served as a no-primary control to test the specificity of the secondary antibody. Slides were rinsed for 1 h with PBTx, incubated with donkey anti-mouse IgG Cy5 (1:500) for 30 min at room temperature, and rinsed again with PBTx. Finally, the hemolymph smears were incubated with propidium iodide for 5 min at room temperature, rinsed with PB and mounted with Gel/Mount™.

Confocal Microscopy

Brains and hemolymph smears were imaged with a Leica TCS SP5 laser scanning confocal microscope using argon, krypton, and helium-neon lasers. Serial optical sections were taken at 1–2 μm intervals and saved as stacks.

Data Analysis and Statistics

GS labeling of neuronal precursors provided a means of visualizing the neurogenic niche and migratory streams, and BrdU allowed quantification of the number of S-phase cells in these regions. Propidium iodide, a nucleic acid marker that labels all nuclei, permitted quantification of total numbers of niche cells as well as the numbers of blood cells in smears. The numbers of BrdU-labeled cells in the niche, streams and Clusters 9 and 10 were used as a measure of the

proliferation rate of neuronal precursors, and hence an indication of the cell cycle time. Labeled neuronal precursors and cells in the hemolymph were counted by projecting each image in the stack onto a computer screen, and tracing the labeled cells onto a transparent plastic sheet. The traced cells were counted, and independent samples *t*-tests and analysis of variance (ANOVA) with a *post hoc* Tukey were run with JMP7 (SAS, Cary, NC).

RESULTS

Environmental Enrichment Influences Third-Generation Neuronal Precursors and Their Descendants in Clusters 9 and 10 in CL4 and CL10 Crayfish

The findings of Sandeman and Sandeman (2000) demonstrated that environmental enrichment enhances cell proliferation of third-generation precursors and their descendants in Clusters 9 and 10 in the brains of ADI crayfish. Our studies tested whether environment has a similar influence in crayfish at later stages of development. An independent samples *t*-test assessed the effect of environmental enrichment and deprivation on the mean number of BrdU cells in Clusters 9 and 10 for crayfish of CL4 and CL10. For the younger/smaller CL4 animals, enrichment significantly enhanced cell proliferation in Cluster 9 ($t(18) = -3.504$, $p = 0.003$) [Fig. 2(A)] and Cluster 10 ($t(17.104) = -8.217$, $p < 0.001$) [Fig. 2(B)]. Similar effects were observed in the older/larger crayfish (CL10): Cluster 9 ($t(39.911) = -3.710$, $p = 0.001$), and Cluster 10 ($t(43.999) = -4.868$, $p < 0.001$). Mean numbers of BrdU-labeled cells in Clusters 9 and 10 of all groups also were compared using ANOVA and *post hoc* Tukey tests to ask whether the degree of environmental influence on neurogenesis was size/age-dependent. The ANOVA analysis confirmed the effects of environmental conditions on neurogenesis in these regions and showed that size did not influence the magnitude of these effects, over the range tested. The influence of environment on neurogenesis is therefore not restricted to ADI animals ($\sim$CL2) (Sandeman and Sandeman, 2000), but persists in larger/older sexually differentiated crayfish (CL4–CL10).

Influence of Environmental Enrichment on the First-Generation Precursors (Neuronal Stem Cells)

The primary goal of this study was to determine whether the first- and second-generation neuronal precursors are also influenced by environmental conditions. In both CL4 and CL10 crayfish, environmental enrichment resulted in higher numbers of BrdU-labeled cells in the niche relative to crayfish maintained in the deprived environment [CL4: ($t(18) = 3.432$, $p = 0.003$); CL10: ($t(21.159) = -7.210$, $p < 0.001$)] [Fig. 3(A)]; this indicates an increase in the cell cycle rate of the neuronal stem cells in the enriched relative to deprived conditions. The ANOVA and *post hoc* analyses confirmed the results of the *t*-tests, and further suggested a trend such that the effect of environment on BrdU incorporation tends to be greater in the larger crayfish ($F(1) = 4.016$, $p = 0.051$).

In addition, environmental deprivation *increased* the total numbers of first-generation precursor cells in

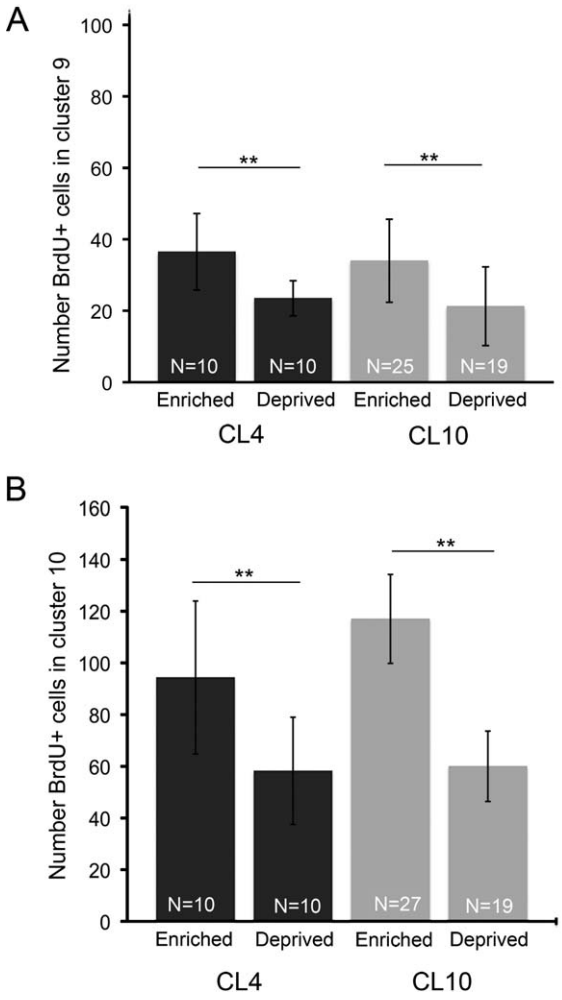

Figure 2 Environmental enrichment has a significant effect on cell proliferation in Cluster 9 (A) and Cluster 10 (B), as indicated by increased incorporation of BrdU in crayfish housed in enriched compared to deprived conditions. Histograms represent mean values for each group of crayfish, with standard deviations indicated. N = number of cell clusters. Asterisks represent *t*-test significance values: ** indicates a p value <0.005.

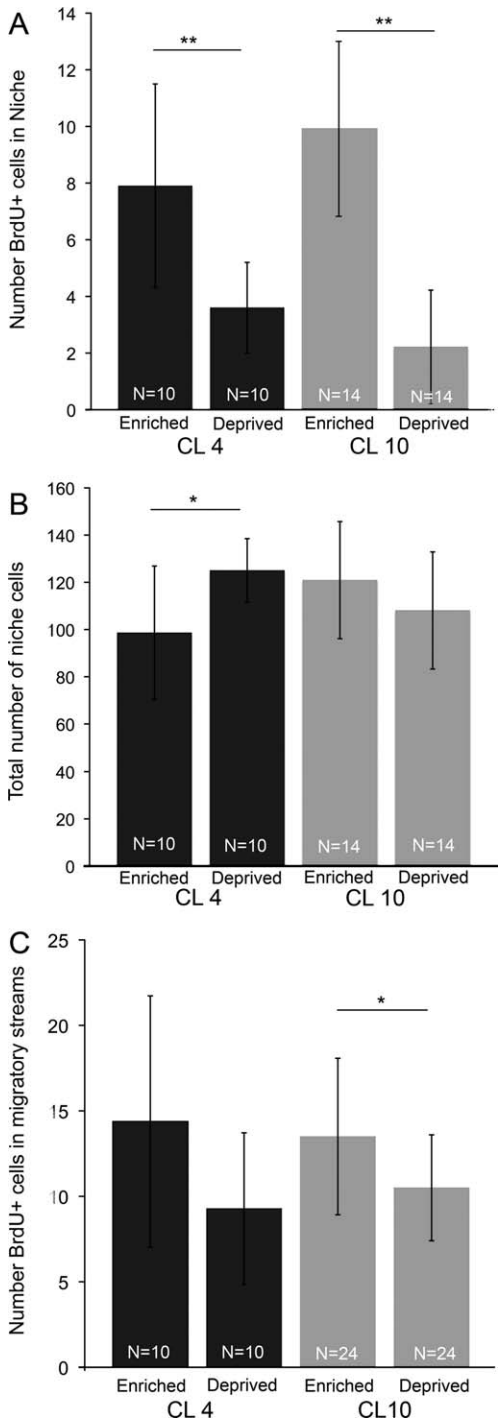

Figure 3 Environmental enrichment has a significant effect on the cell cycle rate of first-generation neuronal precursors in the niche (A) and second-generation precursors in the migratory streams (C), as indicated by the mean numbers (±S.D.) of BrdU-labeled cells in these areas. The mean numbers of cells in the niche of crayfish housed in enriched conditions is increased relative to crayfish maintained in deprived conditions (B). N = number of niches (A, B) or streams (C), and asterisks represent *t*-test significance values: ** signifies a p value <0.005, and * signifies a p value of <0.05.

the younger animals (CL4), revealed by propidium iodide labeling of nuclei in the younger animals ($t(17) = -2.649$, $p < 0.02$) [Fig. 3(B)]. This effect was not, however, significant in the older crayfish (CL10) ($t(25.306) = -1.481$, $p > 0.1$). An underlying assumption here is that all of the niche cells have the potential to become neuronal precursors, and three lines of previously published evidence support this view. First, all niche cells label with the G1-phase marker MCM2-7, except when they are progressing through S to M phase (Sullivan et al., 2007a). This indicates that the niche cells are actively in the cell cycle although resting in G1, suggesting that these are not terminally differentiated cells. Second, all niche cells, including those in M phase, label immunocytochemically for GS, a glial marker; this indicates common molecular properties shared among all the niche cells (Sullivan et al., 2007a). Third, examination of semi-thin sections shows that the vast majority of niche cells have identical cellular characteristics (Zhang et al., 2009). Finally, the results of the present study [Fig. 3(A)] demonstrate that the number of S-phase precursors in the niche is regulated by environmental enrichment, suggesting that there is a quiescent pool of precursor cells that can be recruited into S-phase in response to local conditions.

Influence of Environmental Enrichment on the Migratory Second-Generation Cells

The mean number of BrdU-labeled cells in the migratory streams is not statistically different in the enriched and deprived groups in CL4 crayfish ($t(19.289) = -1.8522$, $p = 0.084$), but is significantly larger in CL10 crayfish maintained in enriched, relative to deprived, conditions ($t(43) = -2.761$, $p < 0.010$) [Fig. 3(C)]. The variability in these results was greater in the CL 4 crayfish than in the CL 10 animals, and the "n" was lower, which may have contributed to the lack of statistical significance in the smaller animals. These data, overall, may nevertheless indicate a tendency towards a higher cell cycle rate in the second-generation precursor cells in the enriched condition, relative to the deprived condition.

Influence of Environmental Enrichment on Hemolymph Cells

In the blood system, there is no impact of environment on counts of live cells ($F(1,6) = 0.397$, $p > 0.5$), dead cells ($F(1,6) = 7.574$, $p > 0.05$), or total

cells ($F(1,6) = 3.576$, $p > 0.05$) circulating in the hemolymph. As reported in the literature (Ravindranath, 1977; Owens and O'Neill, 1997), total counts of circulating cells are highly variable in decapod crustaceans, both between animals and for an individual animal on different days. In our studies, total hemocyte counts for individual animals ranged from 0.6 to 13.8 $\times$ 10^6 cells per milliliter of hemolymph. In spite of this variability, however, there is a significantly greater expression of the niche precursor cell marker GS in circulating cells from animals maintained in an enriched environment, relative to the deprived environment ($t(10) = 2.228$, $p < 0.001$). In the environmentally deprived animals, GS is expressed in 1.1% of circulating hemolymph cells; 3.7% of circulating cells in crayfish housed in enriched conditions expressed GS (Fig. 4). These percentages were calculated based on counting a total of 12,851 hemocytes from 27 blood samples taken from enriched animals, and 7,148 hemocytes from 21 blood samples from deprived crayfish. These data indicate that enrichment enhances the expression of this enzyme in cells circulating in the hemolymph, which are derived from hematopoietic tissues.

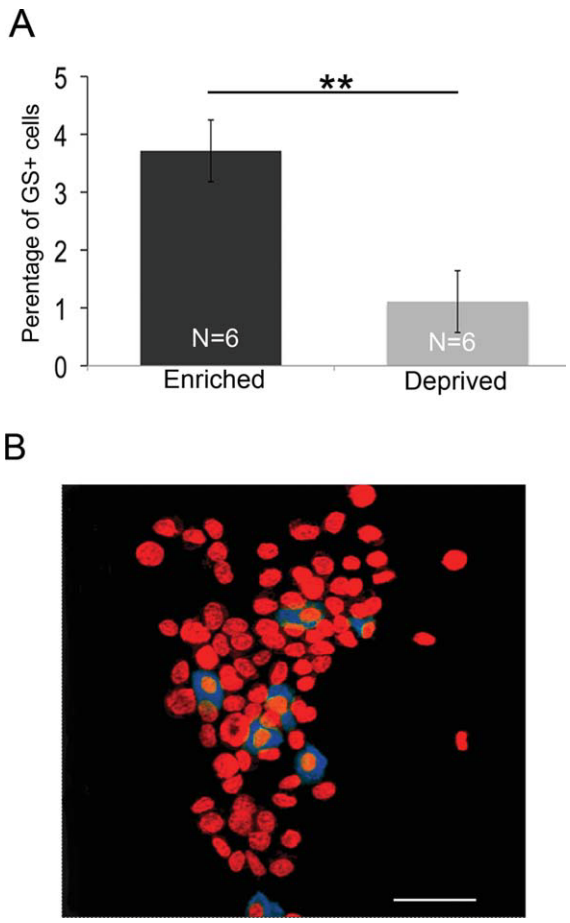

Figure 4 Enrichment has a significant effect on the percentage of GS-labeled cells in the hemolymph. In the environmentally deprived animals, GS is expressed in 1.1% of circulating hemolymph cells; 3.7% of circulating cells in crayfish housed in enriched conditions expressed GS (A). B: A small percentage of all circulating cells (PI, red) from enriched CL10 crayfish label with GS (blue). N = number of animals from which hemolymph was sampled. Asterisks represent *t*-test values: ** signifies a *p* value <0.005. Scale bar = 20 μm.

DISCUSSION

The current experiments exploited the spatial separation of the first-, second-, and third-generations of neuronal precursor cells (Sullivan et al., 2007a, b) in the crayfish brain, to determine which of these may be altered by environmental conditions. Two sizes of crayfish were utilized in the present studies, to ask whether the influence of environment is size/age-dependent. Finally, the numbers and types of circulating cells were assessed in the CL10 crayfish, to test whether environmental enrichment alters the blood cell count or composition of the hemolymph.

Influences of Environmental Enrichment on the Precursor Cell Lineage that Produces Adult-Born Neurons

The most compelling finding of this study is that environmental factors influence the cell cycle rate of first-generation neuronal precursors in the neurogenic niche. In both CL4 and CL10 crayfish, significantly more BrdU-labeled first-generation cells were detected in the brains of animals maintained in enriched, relative to deprived, environments. This is the first demonstration that the proliferative potential of the neuronal stem cells can be manipulated by altering the quality of the environment in which the animals live. In contrast, serotonin does not influence the cell cycle rate of first or early second-generation precursor cells in *P. clarkii*. Effects on BrdU incorporation are confined to the late second and later generations of cells in the proliferation zones of Clusters 9 and 10, influences that are correlated with the expression of serotonin receptors in these late precursor generations (Zhang et al., 2011). The more extensive influence of environment on this lineage likely reflects the many physiological changes that occur in response to living conditions, where many variables are introduced.

Kempermann et al. (1997) investigated the effect of environmental enrichment on neurogenesis and neuronal progenitor cells in young mice (~postnatal day 21). The enriched environment consisted of mice living communally in a large cage with a set of tunnels, running wheels, and toys to increase the complexity of living conditions; these environments were rearranged daily, to create novelty. The control environment included three mice living in a standard cage. After the 40-day environmental exposure, levels of BrdU incorporation in hippocampal cells were compared in short- and long-survival time studies, to determine differences in cell proliferation and survival, respectively. Contrary to the findings presented here, neuronal proliferation was unaffected by environmental enrichment, but cell survival (measured 4 weeks after BrdU administration) increased. However, in older mice long-term exposure (10 months) to enrichment did result in increased precursor proliferation *and* neuronal survival (Kempermann et al., 2002). Our results and those of Sandeman and Sandeman (2000) showing an influence of short-term environmental exposure on both proliferation and survival in crayfish may represent species differences in the regulation of neurogenesis by environment, or could be a reflection of the very distinct environments to which the crayfish were exposed. The control environment in the mouse studies was equivalent to standard group housing for mice. However, in the current series of experiments in crayfish, the deprived condition included space limitations that limited physical activity, social isolation and a barren landscape, replicating the conditions of the Sandeman and Sandeman (2000) study.

Previous studies in mice and crustaceans have attempted to determine the individual contributions of the various environmental components to the regulation of neurogenesis. For example, voluntary physical activity and exercise have been shown to increase neurogenesis in mice as well as to reverse the decline in neurogenesis experienced by older animals (Van Praag et al., 1999; 2005). The enriched conditions used in the current studies provided a complex environment that included social interaction, a large space with interesting features such as plants and burrows, and the opportunity for exploration and physical activity. Our study did not clarify which of these elements may mediate the beneficial effects of enrichment. To establish the enriched and deprived conditions, however, several other environmental arrangements were tested. We found that the space limitations were a critical aspect of the deprived condition, and that use of a larger deprived environment eliminated the overall influence on proliferation, measured by BrdU incorporation, between the two groups of animals (data not shown). Space restrictions have been shown to inhibit animals' growth (Rugh, 1934), and it is therefore possible that limitations in space and physical activity may be primary determinants of neurogenic potential in crayfish in these studies. Indeed, locomotory studies in large crayfish do show a weak correlation between the level of physical activity and the survival of adult-born neurons in large crayfish (CL > 25 mm), but no effect on proliferation (Sandeman, unpublished results). Locomotion therefore may be one critical element that differed between the two conditions in our study. Isolation versus social interaction did not appear to play a role in determining levels of neurogenesis in our study, as crayfish isolated in larger environments while developing the environmental conditions for these experiments did not have reduced levels of neurogenesis compared to those maintained in groups in the enriched environments (data not shown). Song et al. (2007) also studied the relationship between social interactions and neurogenesis in young adult crayfish, and found no effect of social dominance or social isolation on cell proliferation in Clusters 9 and 10, although social domination enhanced the survival of BrdU-labeled cells when compared to social subordination. Additional experiments are necessary to define which specific components of the enriched environment are crucial for the influences observed in the present study.

Concomitant with the increase in BrdU incorporation into first-generation precursors in CL4 crayfish, there was a decrease in the total numbers of niche cells in the enriched animals compared with deprived; these data were acquired by counting the numbers of propidium iodide labeled nuclei in the niche. These findings are logical in the sense that an increased cell cycle rate among the niche precursors would result in more cells migrating away from the niche; this might result in fewer total numbers of niche precursors remaining in the niche. If the cell cycle rate slows, as in the deprived environment, niche precursors will be retained in the niche, causing their numbers to be larger relative to the enriched environment. Although these relationships are potentially interesting, this result was not replicated in the older CL10 crayfish, and so this effect may be specific to younger animals. Further, in CL10 animals, enrichment enhanced BrdU incorporation into the migratory second-generation neuronal precursors; a trend in this direction was also seen in CL4 crayfish, but this result was not statistically significant. Finally, our results confirm the influence of environment on BrdU incorporation among the third-generation precursors and their descendants in Clusters 9 and 10 previously reported by Sandeman

and Sandeman (2000) in ADI *Cherax destructor*. The present studies utilized larger/older crayfish of two species, *P. clarkii* and *P. acutus*, demonstrating that environmental effects on adult neurogenesis in crayfish persist beyond the first adult (ADI) stage.

Effects of Environmental Enrichment on Cells Circulating in the Hemolymph

The results of Zhang et al. (2009) indicated that niche precursor cells are not a self-renewing pool of cells, a suggestion that has now been directly demonstrated using double nucleoside labeling (Benton et al., 2010). It has been hypothesized that the hematopoietic system may be a source of first-generation neuronal progenitors (neuronal stem cells); the vasculature and the niche are closely associated physically, and the niche precursors and cells in nearby blood vessels have similar morphologies (Zhang et al., 2009). Another goal of this study, therefore, was to determine if environmental conditions influence the numbers or types of circulating cells. Counts of cells in hemolymph samples from enriched and deprived CL10 crayfish showed no differences in the numbers of circulating live, dead or total cells in the two environmental conditions.

GS is an enzyme that converts glutamate to glutamine, and is a marker of astrocytes and early stem cells (Wen et al., 2007; 2009). Antibodies generated against GS label the neuronal stem cells in crustacean brain [e.g., see Fig. 1(B–D)], as well as a small percentage of hemolymph cells (Benton et al., 2010). GS expression was investigated in circulating cells of crayfish (CL10) maintained in enriched and deprived environments, to determine if the presence of GS in circulating cells in the hemolymph is altered by environmental conditions. The results indicate that although percentages of GS-labeled cells in hemolymph samples are variable, on average 3.7% of the circulating cells in enriched crayfish express GS; an average of 1.1% of circulating cells in crayfish maintained in deprived conditions expressed this niche cell marker. In some of the environmentally deprived animals no GS-labeled cells were observed in blood samples, a situation that was never encountered in the environmentally enriched crayfish. These results indicate that environmental enrichment enhances GS expression in circulating hemolymph cells of crayfish. These findings are consistent with a relationship between the hematopoietic and neurogenic systems, and this possibility is being carefully examined.

The fact that antibodies generated against GS bind to both the neuronal stem cells and to a small proportion of circulating cells is intriguing. A characteristic feature of neurogenic niches in both vertebrate and invertebrate organisms is vascularization (Tavazoie et al., 2008). Supportive (e.g., nutrients) and instructive (e.g., hormones, cytokines and other circulating factors) roles for the vascular input have been widely acknowledged in the vertebrates (Riquelme et al., 2008; Kan et al., 2010). There are also suggestions that the vascular contribution may be even more central to the adult neurogenesis story. Cells derived from mammalian bone marrow have a tendency to migrate to the brain when infused into a host animal (Eglitis and Mezey, 1997; Kopen et al., 1999; Brazelton et al., 2000), including into areas undergoing active postnatal neurogenesis. The descendants of these cells express a variety of glial and neuronal markers (Eglitis and Mezey, 1997; Mezey et al., 2000; Chen et al., 2001), and in one study these developed the characteristics of astrocytes (Kopen et al, 1999). *In vitro*, bone marrow cells have been induced by various means to form neurons (Sanchez-Ramos et al., 2000, 2001, 2002; Kohyama et al., 2001), and in one case the bone marrow-derived neurons responded to depolarizing stimuli, showing a rapid and reversible calcium increase in response to acetylcholine, a response characteristic of neurons (Kohyama et al., 2001). Other studies, however, suggest that cell fusion may account for the acquisition of such broad properties by stem cells (Morshead et al., 2002; Wagers et al., 2002; Wells, 2002; Coyne et al., 2006), casting doubt on the significance of prior studies. Nevertheless, the idea that cells derived from bone marrow or umbilical cord blood can transdifferentiate into neurons and glia in response to signals in the brain persists in the literature. Direct tests of the potential relationships between cells circulating in the vasculature and mechanisms of adult neurogenesis are possible in the crayfish, and these experiments are currently underway in our laboratory.

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