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Social domination increases neuronal survival in the brain of juvenile crayfish *Procambarus clarkii*

Cha-Kyong Song*, Laurel M. Johnstone†, Manfred Schmidt, Charles D. Derby and Donald H. Edwards‡

Department of Biology, Program in Brains and Behavior, and Center for Behavioral Neuroscience, Georgia State University, Atlanta, GA 30302-4010, USA

*Present address: Laboratory of Cardiovascular Genomics, Division of Molecular Life Sciences, Ewha Womans University, Seoul, 120-750, Korea

†Present address: Arizona Research Labs, Genomic Analysis and Technology Core, University of Arizona, Tucson, AZ 85721, USA

‡Author for correspondence (e-mail: dedwards@gsu.edu)

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Summary

Olfactory cues are among the sensory inputs that crayfish use in establishing dominance hierarchies. Throughout their lives, new neurons are continuously added into brain cell clusters 9 and 10, which contain somata of olfactory local and projection interneurons, respectively. Using markers for DNA synthesis (bromodeoxyuridine) and mitosis (phospho-histone-3), we tested juvenile crayfish (*Procambarus clarkii*) to examine effects of pairwise social experience on proliferation and survival of cells in these brain regions. Proliferating and mitotic cells appeared within restricted neurogenic areas in both clusters and in 'tails' extending from them. These tails, embedded in tubulin-positive strands, are linked by a patch of cells. Neither cell proliferation nor mitotic activity was affected by social dominance. Cell survival of neuronal precursors was affected by dominance: compared to dominants, subordinates had fewer newborn cells surviving in cluster 9 after 14 days of social experience. Social experience also affected body growth rate, but the effect of social experience on neurogenesis remained when differences in body growth rate were statistically controlled. We conclude that social domination enhances survival of new olfactory interneuronal precursors compared to social subordination but not compared to social isolation.

Key words: proliferation, neurogenesis, dominance hierarchy, body growth, olfaction, Crustacea.

Introduction

Since Altman reported the occurrence of neurogenesis in the adult rat brain (Altman, 1962), many studies have demonstrated that neurogenesis continues into and throughout adulthood in two regions of the brains of vertebrates including humans: the hippocampus and the olfactory bulb (Ming and Song, 2005). Many factors regulate the level of such neurogenesis, including exercise (van Praag et al., 1999), stress (Gould et al., 1997; Gould et al., 1998; Pham et al., 2003), environmental richness (Brown et al., 2003; Kempermann et al., 1998), learning and memory formation (Shors et al., 2001; Gould et al., 1999) and enriched odor inputs (Rochefort et al., 2002). Recently, dominance hierarchy formation, i.e. a pattern of repeated agonistic interactions with an expected outcome of winner and loser, has been shown to influence neurogenesis in the hippocampus but not in the subventricular zone of the olfactory bulb (Pravosudov and Omanska, 2005; Kozorovitskiy and Gould, 2004).

Neurogenesis also persists in juvenile and adult arthropods, including in the brains of insects (Cayre et al., 2002) and crustaceans (Schmidt, 2002; Beltz and Sandeman, 2003), and is regulated by many of the same factors that influence neurogenesis in mammals. Neurogenesis in the brains of insects and crustaceans is influenced by hormones and polyamines (Cayre et al., 1994; Cayre et al., 2001), serotonin levels (Beltz et al., 2001), sensory inputs (Hansen and Schmidt, 2001; Scotto-Lomassese et al., 2002), environmental richness (Sandeman and Sandeman, 2000; Hansen and Schmidt, 2004), circadian rhythm (Goergen et al., 2002) and season (Hansen and Schmidt, 2004).

Crayfish are excellent models for examining the effect of social interactions on neurogenesis for several reasons. First, new neurons are continuously added to the populations of local and projection olfactory interneurons in the crayfish brain and olfactory receptor neurons in the sensory periphery throughout the animal's life (Sandeman et al., 1998; Schmidt and Harzsch, 1999) (for a review, see Sandeman and Sandeman, 2003). Second, crayfish form dominance hierarchies quickly and stably, leading to changes in their physiology and social and non-social behaviors (Bovbjerg, 1953; Lowe, 1956; Issa et al.,

1999; Edwards et al., 2003; Herberholz et al., 2003; Song et al., 2006). Third, crayfish use chemical signals during social interactions and in forming and maintaining dominance hierarchies (Zulandt Schneider et al., 1999; Breithaupt and Eger, 2002). Although many factors are known to influence olfactory neurogenesis in crayfish, social experience has not been examined.

In the current study, we examined the influence of social interactions on neurogenesis in the brains of juvenile crayfish, which have high rates of neurogenesis and form stable dominance hierarchies (Issa et al., 1999). We determined how the proliferation and survival of cells in the clusters containing local and projection olfactory interneurons are influenced by up to 14 days of social pairing. We show that the level of cell proliferation is similar in both dominant and subordinate animals, while the level of cell survival is higher in dominant animals than in subordinates.

Materials and methods

Animals and holding conditions

All animals used in this study hatched in the laboratory from mother crayfish, *Procambarus clarkii*, purchased from Atchafalaya Biological Supply (Raceland, LA, USA). Hatchlings were individually isolated in small cages when they became free-swimming (Fig. 1A). These cages were cylindrical (5.5 cm in diameter, 7.0–7.5 cm in height) with plastic mesh walls on which animals could climb. A lid was placed on top to prevent escape (Fig. 1B). Twenty cages were placed together in a plastic aquarium (14 l volume; 33 cm×14.5 cm×29 cm), which isolated the animals from each other physically but not chemically (Fig. 1B). The juvenile crayfish were raised in these cages for 5–9 weeks in constantly aerated water, under a 12 h:12 h light:dark cycle. They were fed twice a week in the late afternoon with a piece of turkey meat (approximately 0.1 cm^3, 0.6 mg) and a similar sized piece of carrot, and provided with water plants that are part of their normal diet (*Elodea* sp.) to eat *ad libitum*. This feeding pattern enabled the animals to grow at about the same rate so that large size disparities did not develop.

Procedures for establishing dominance hierarchies

Two groups of juvenile crayfish were tested for each experiment: paired animals and isolated animals. Paired animals differed by 10–20% in wet body mass, ranging from 0.02–0.20 g at the beginning of pairing and from 0.04–0.20 g at the end of pairing. The animal's sex is not readily distinguishable at this size (Rhodes and Holdich, 1979). Ten or more pairs of crayfish were selected for each experimental test and placed together in a small cage with a small shelter (Pair group; Fig. 1C,D). Isolated animals were in separate cages placed together in a similar, but separate plastic aquarium (Isolate group; Fig. 1C,E). All animals were fed as before once per day in the late afternoon, up from twice weekly when the animals were isolated. This higher rate helped to reduce hunger-related aggression that might lead to deaths, and it helped maintain constant daily conditions among the animals. The dominant animal was fed first to enable the subordinate to retain its food when it was given afterwards. This helped to ensure that both animals had similar opportunities and amounts to eat.

Behavioral observations

Animals were individually marked at the beginning of each experiment and after molting. The social interactions of paired animals were observed twice daily, for 5 min in the morning and in the afternoon during feeding (above), to determine their social status. 'Dominant' status was assigned to an animal when it either occupied the shelter alone or approached, chased, or attacked its opponent, causing the opponent to retreat. The opponent was designated as the 'subordinate'. Subordinate animals often clung to the mesh wall, stayed at the rim of the arena, or retreated or escaped from their dominant opponents.

Dominance relationships of paired animals

To test the effect of social experience and social status on neurogenesis, animals drawn from the same group of social isolates either remained isolated or were paired with another

Fig. 1. Experimental design. (A–E) Animal setup. (A) A juvenile crayfish, 2.0 cm from rostrum to telson. (B) Individual cages in an aquarium. Each cage is 5.5 cm in diameter. (C) Diagrams illustrating setup for animals in the Isolate and Pair groups. Rectangles and circles represent plastic aquaria and cages, respectively. Small ovals in each circle in the Isolate groups represent animals in isolation, while small triangles and small squares in each circle in the Pair group represent dominant and subordinate animals, respectively. Each gray object inside a circle represents a shelter. For the Pair group, 20 animals were transferred to new cages, two animals per cage, and only one shelter was provided in each cage. For the Isolate group, ten animals were individually transferred to new cages and a shelter was provided in each new cage. (D) Two animals in the Pair group (arrows) in a cage with one shelter. (E) One animal in the Isolate group (arrow) in a cage with one shelter. (F,G) Protocols for Cell Proliferation Test (F) and Cell Survival Test (G). Following the protocol in F and G, the animals in the Isolate and Pair groups were treated for cell proliferation and cell survival tests. A dotted line indicates an isolated condition; a broken line indicates a pairing condition; gray bars indicate immersion of animals in BrdU solution for 24 h. (F) Cell Proliferation Test. Animals were isolated for 6 weeks, paired or isolated for 1, 7 or 14 days, immersed in BrdU for 24 h on the last day of the pairing period, and sacrificed. (G) Cell Survival Test. All animals were isolated either for 5 or 9 weeks, paired or isolated for 7 or 14 days, immersed in BrdU for 24 h at the beginning of the pairing period, and sacrificed. A separate group of animals (age-control groups 1 and 2) was treated similarly (isolated for 5 or 9 weeks, immersed in BrdU for 24 h, and sacrificed immediately) but without any pairing. The age-control groups show the level of cell proliferation at the end of the isolation period, while the animals in the Isolate group show the level of cell survival after 7 days or 14 days of isolation. (H) Diagram of the cell division cycle. BrdU, a thymidine analog, labels cells in the S phase during which DNA replication occurs. H3P, a mitosis marker, labels cells in the M phase during which histone 3, a protein mediating the folding of DNA, is phosphorylated.

for 1, 7 or 14 days. In the 1-day test group, five of the ten pairs created were able to form and maintain stable dominance hierarchies over the morning and afternoon observation periods. In the 7- and 14-day test groups, 23 of the original 50 pairs either maintained stable dominance hierarchies (15 pairs) or experienced brief periods of status reversal (8 pairs).

Criteria for experimental inclusion and exclusion

Animals were analyzed for neurogenesis only if their social status was stable and if both of their antennular lateral flagella, the olfactory organs of the crayfish, remained intact. In the 1-day test group, the five pairs that had stable dominance relationships also had no damage to their antennular lateral flagella, and so were included in the analysis. In the 7- and 14-day test groups, six animals (two dominants and four subordinates) of the 23 pairs with stable dominance relationships were excluded from the analysis because they had suffered unilateral damage to their lateral flagella. The partners of those six animals were included in the analysis if their lateral flagella remained intact.

Cell markers

Cell proliferation was measured in two ways. *In vivo* labeling with BrdU and its subsequent detection with an anti-BrdU antibody was used to measure DNA replication. BrdU (5-bromo-2′-deoxyuridine) is a thymidine analog that is incorporated into DNA during the S phase of the cell division cycle (Fig. 1H). The monoclonal BrdU-antibody (from

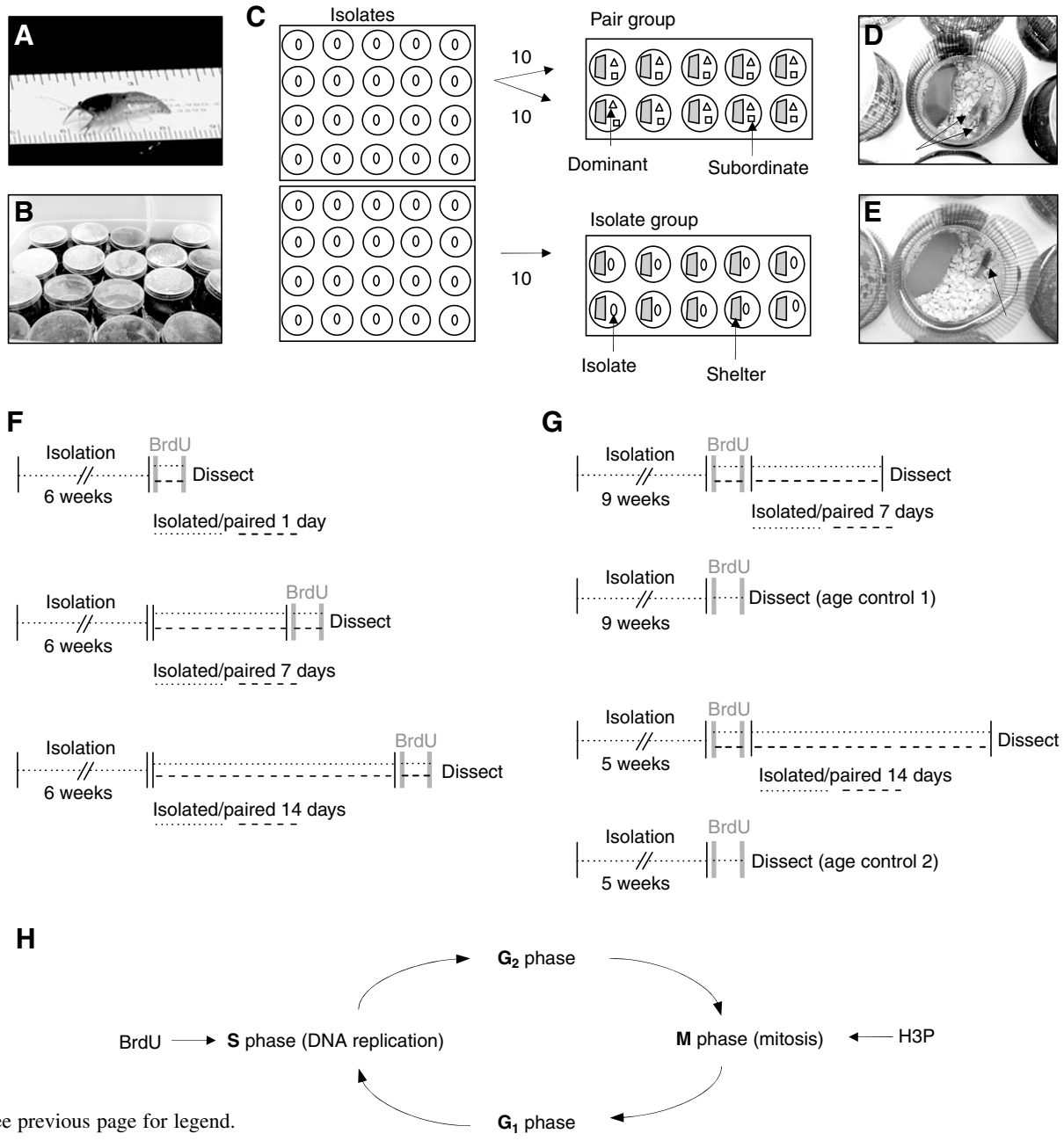

Fig. 1. See previous page for legend.

Amersham Bioscience, Bucks, UK) has been found to be a reliable and highly specific nuclear marker for proliferating cells in decapod crustaceans, including crayfish (Schmidt and Harzsch, 1999; Schmidt, 2001; Benton and Beltz, 2002; Sullivan and Beltz, 2005b). An antibody to phosphorylated histone 3 (H3P) was used as a mitosis marker to visualize cells in their M phase (Fig. 1H). H3P labels mitotic nuclei in many cell types (Hendzel et al., 1997; Wei et al., 1998; Wei et al., 1999), including crustacean neuronal precursor cells (Harzsch et al., 1999).

Tests of cell proliferation and cell survival

Neurogenesis could be affected by changes in either the proliferation or the survival of newborn cells. The paired and isolated animals were sorted into five groups to test the effect of dominance hierarchy formation on both cell proliferation (three groups) and cell survival (two groups) in the brain. To determine how experience of a social hierarchy affects proliferation in clusters 9 and 10, same-age siblings were paired for 1, 7 or 14 days, then immersed in BrdU solution (1 mg BrdU ml^{-1} water) for 24 h, and sacrificed (Fig. 1F). Corresponding groups of social isolates were exposed to BrdU and examined at the same times. After tissue preparation, BrdU+ and H3P+ nuclei in the brains of dominant, subordinate and isolate animals were counted.

To test the effect of social experience on cell survival, animals were first isolated for either 5 or 9 weeks and then exposed to BrdU for 24 h. Pairs were drawn from the 5-week isolates and placed together for 14 days, while pairs drawn from the 9-week isolates were placed together for 7 days. Other animals from the 5- and 9-week isolation groups remained isolated during the 14- and 7-day pairings, respectively. At the end of the pairing period, both paired and isolated animals in each group were sacrificed and the surviving cells counted (Fig. 1G). To provide a baseline of proliferation for each group, other animals were also isolated for 5 and 9 weeks at the same time as the experimental animals (their siblings), exposed to BrdU, and then immediately sacrificed. The count of their labeled cells provided an age control for the degree of proliferation displayed by the 5- and 9-week animals at the outset of their respective 14- and 7-day periods of pairing.

Brain histology

Animals in each group were removed from the cage and immediately placed in ice-chilled water for 10–15 min, after which the brains were dissected out in cold saline, held overnight in 4% paraformaldehyde, rinsed in 0.1 mol l^{-1} phosphate buffer (PB), and stored at 4°C in PB containing 0.1% sodium azide.

Immunocytochemistry

Fixed brains from cell proliferation and cell survival test groups were embedded in 14% gelatin solution (1.4 g gelatin/10 ml 0.1 mol l^{-1} PB), placed overnight in 4% paraformaldehyde at 4°C to harden the gelatin, rinsed (4×30 min in 0.1 mol l^{-1} PB), and horizontally sectioned at 70–100 μm thickness using a vibrating microtome (VT 1000 S, Leica: Wetzlar, Germany). Brain sections were incubated in 2 μmol l^{-1} HCl for 30 min at room temperature, rinsed (3×30 min in 0.1 mol l^{-1} PB), incubated for 4 h in blocking solution (5% normal goat serum, 1% bovine serum albumin, 0.1% sodium azide, and 0.3% Triton X-100 in 0.1 mol l^{-1} PB), and incubated overnight at room temperature in a mixture of two primary antibody solutions [monoclonal mouse anti-5-bromo-2′-deoxyuridine, prepared according to package instructions: RPN 202, Amersham Bioscience; and polyclonal rabbit anti-phospho-histone-3 (H3P), 1:200 dilution: Upstate, Waltham, MA, USA]. The sections were then rinsed (4×30 min in 0.1 mol l^{-1} PB), incubated in a mixture of secondary antibodies (Alexa fluor 488, goat anti-mouse IgG; Texas Red, goat anti-rabbit IgG; each diluted 1:200; Molecular Probes, Eugene, OR, USA) for 2–3 h, rinsed (4×30 min in 0.1 mol l^{-1} PB), and mounted in a 1:1 mixture of glycerol and PB. Images of brain sections were collected using a confocal microscope (LSM 510, Carl Zeiss, Thornwood, NY, USA). Stacks of individual images were reconstructed using Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA, USA) and the brightness, contrast, sharpness, and evenness of the images were adjusted for best quality.

To get a more detailed structural view of neurogenesis, another group of six 4-week old crayfish were immersed in BrdU (1 mg ml^{-1}) for 24 h. Their brains were removed and fixed overnight in 4% paraformaldehyde, then rinsed six times in 1 h in PBTX (0.1 mol l^{-1} PB with 0.25% Triton X-100). After dehydration and rehydration through an ethanol series, the brains were incubated overnight in mouse monoclonal anti-BrdU, rinsed six times in 1 h in PBTX, incubated in rat monoclonal anti-tubulin (YOL 1/34 microtubule marker, Abcam, Cambridge, MA, USA; diluted 1:200), rinsed 6× 1 h in PBTX, and then incubated in a mixture of Cy3 goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and Alexa Fluor 488 goat anti-rat (Molecular Probes, Eugene, OR, USA) each diluted 1:50. The brains were then rinsed 6× 1 h in PB, incubated in a 0.002% solution of Hoechst 33342 for nuclear staining, rinsed again in PB, dehydrated through an ethanol series, and mounted ventral side up in methyl salicylate.

Cell counting and analysis

Stacks of confocal images were imported into Image-Pro Express software (Media Cybernetics, Silver Spring, MD, USA). Unlike the vertebrate olfactory bulb, which has the somata of olfactory interneurons organized into layers within the neuropil, the crayfish brain has two large paired neuropils, the olfactory lobe and the accessory lobe, and the somata of its interneurons are positioned outside of the neuropil: paired cell cluster 9 contains the cell bodies of olfactory local interneurons, and paired cell cluster 10 contains the cell bodies of olfactory projection interneurons (Fig. 2A). All individual BrdU-labeled (BrdU+) cells in cell clusters 9 and 10 were traced and counted in a blind fashion according to the following criteria. BrdU+ cells associated with clusters 9 and 10 are typically grouped in

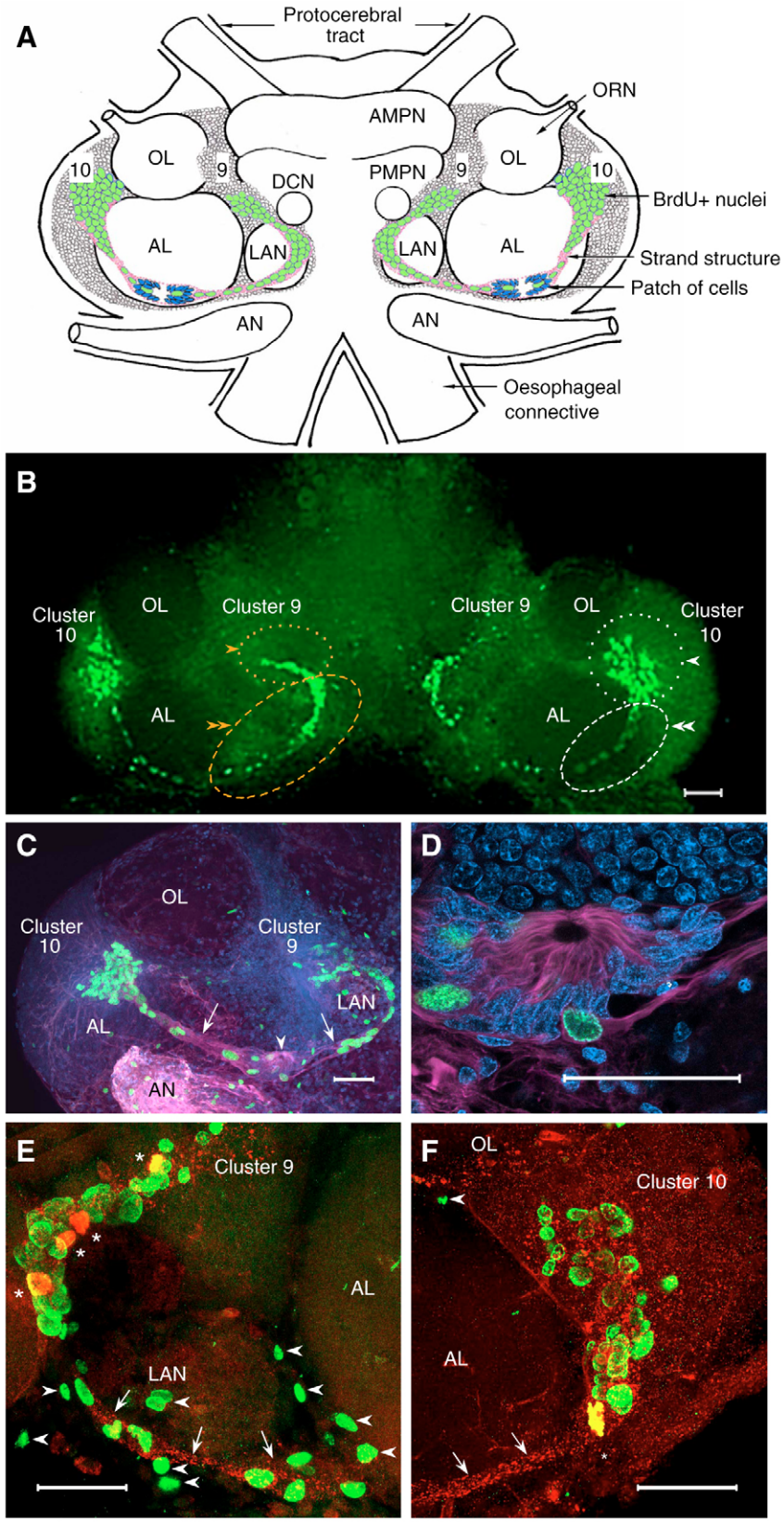

Fig. 2. 'Comma-shaped' neurogenic areas in the juvenile crayfish brain. (A) Diagram of the juvenile crayfish brain identifying the neuropils and cell clusters [modified from Sandeman et al. (Sandeman et al., 1992)] as well as the neurogenic areas in the deutocerebrum (this study). Primary sensory inputs from olfactory receptor neurons (ORN) in olfactory organs enter the olfactory lobe (OL). The cell bodies of the olfactory local and projection interneurons comprise cell clusters 9 and 10, respectively. Groups of BrdU+ cells (green) appear in cell clusters 9 and 10 and lines of BrdU+ cells, surrounded with a strand-like structure (violet), meet at a patch of cells (blue) near the posterior accessory lobe (AL). 9, cell cluster 9; 10, cell cluster 10; AMPN, anterior median protocerebral neuropil; AN, antennal neuropil; DCN, deutocerebral commissure neuropil; LAN, lateral antennular neuropil; PMPN, posterior median protocerebral neuropil. (B) Juvenile crayfish brain showing robust cell proliferation in cell clusters 9 and 10. The image is taken with the camera attached to the epifluorescence microscope. Each dotted area (orange for cluster 9 head and white for cluster 10 head) indicates regions where a ball of BrdU+ cells is present within the cluster boundary. Each broken area indicates a region around the LAN (orange for cluster 9 tail) or around the posterior AL (white for cluster 10 tail) where 'tails' of BrdU+ cells are located. Scale bar, 50 μm. (C–F) Collapsed stacks of confocal images taken at 1.0 μm intervals in (C) and 0.5 μm intervals in (D–F). The entire stack for each image was approximately 60 μm. (C) A strand labeled with anti-tubulin (violet, arrows), surrounds lines of BrdU+ cells (green) from clusters 9 and 10, and a patch of cells (arrowhead). Scale bar, 50 μm. (D) High-resolution image of the patch of cells (green, anti-BrdU; blue, Hoechst, nuclear staining; violet, anti-tubulin) indicated by an arrowhead in (C). The patch of cells and a few BrdU+ cells are surrounded by a tubulin+ strand. Scale bar, 50 μm. (E) Lines of BrdU+ cells (green) in the tail of cluster 9 are surrounded by a strand faintly labeled with anti-H3P (red, arrows). BrdU+ cells outside the strand (arrowheads) were not included in our counts of labeled cells. Asterisks indicate H3P+ (mitotic) cells. Scale bar, 50 μm. (F) Lines of BrdU+ cells (green) in the tail of cluster 10 are surrounded by a strand faintly labeled with anti-H3P (red, arrows). A BrdU+ cell on the border of AL (arrowhead) was not included in our counts of labeled cell. Asterisk indicates H3P+ (mitotic) cells. Scale bar, 50 μm.

a 'comma' shape, consisting of a 'head' and 'tail' surrounded by a 'strand', as we describe in the Results (Fig. 2B). We counted BrdU+ cells separately in the head and tail of cell clusters 9 and 10. For the head, we counted BrdU+ cells within the boundaries of cell clusters 9 and 10, excluding BrdU+ cells with lenticular shaped nuclei (see dotted circles in Fig. 2B). We

excluded these nuclei to reduce the possibility of including putative glial cells or glial precursors, which occur scattered throughout the brain with no obvious correlation to neurogenic areas. For the tail, which includes a patch of cells near the posterior AL, we counted BrdU+ cells within the strand, excluding BrdU+ cells with lenticular nuclei (see broken circles in Fig. 2B–D). We did not count BrdU+ cells that were outside cell clusters 9 and 10 and the strands (arrowheads in Fig. 2E,F). In this way, we counted all individual BrdU+ nuclei appearing within the comma-shaped neurogenic areas for clusters 9 and 10, and thus did not need to apply stereological estimation. H3P-labeled (H3P+) cells in cell clusters 9 and 10 were counted in the same manner as described above.

In three preparations, damage to one side of the brain occurred during handling and processing. The number of BrdU+ cells in the intact side of the brain tissues was counted and its number was doubled assuming a symmetry of neurogenesis in both sides of the brain. This is justifiable because of a high correlation between numbers of BrdU+ cells in the left and right sides of brains of undamaged animals in our study (linear regression, $R_{(\text{cell cluster 9})}$=0.945 and $R_{(\text{cell cluster 10})}$=0.794 when R=1.0 represents symmetry).

Statistical analysis

The numbers of BrdU+ cells and H3P+ cells in isolate, dominant, and subordinate animals were subjected to non-parametric one-way ANOVA (Kruskal–Wallis test), treating all three social classes independently. These non-parametric data were reported as median ± interquartile range. When this analysis revealed a statistically significant difference, non-parametric multiple comparisons tests were used to determine which groups differed (Siegel and Castellan, 1988). Separate analyses were run for counts of the entire neurogenic area of BrdU+ cells, the comma head and the comma tail, for both cluster 9 and cluster 10.

To determine if the social status of an animal affected the number of BrdU+ cells above and beyond possible effects of body growth rate, analysis of covariance (ANCOVA) was used to control for differences in the body growth rates of the animals over the experimental period. For this analysis, the univariate General Linear Model procedure of SPSS 12.0 for Windows (SPSS, Chicago, IL, USA) was used.

Results

Neurogenic areas in the juvenile crayfish brain

The brain of the crayfish is dominated by the deutocerebrum with its antennular and olfactory processing regions: the olfactory lobes (OLs), accessory lobes (ALs), lateral antennular neuropils (LANs), and deutocerebral commissure neuropils (DCNs) (Fig. 2A). The OL and AL contain olfactory local interneurons (OLNs) and olfactory projection interneurons (OPNs) whose cell bodies are in cell clusters 9 and 10, respectively (Fig. 2A). In juvenile crayfish, cell cluster 9 extends posteriorly much farther in the ventral regions than in more dorsal regions; it is medial to the AL and OL and its most posterior region is ventral to the LAN. Cell cluster 10 is lateral to the OL and AL throughout the ventral–dorsal plane (Fig. 2A).

After BrdU exposure, we found that numerous BrdU+ cells were present in clusters 9 and 10 (Fig. 2A,B), as has been reported previously for juvenile crayfish (Sandeman and Sandeman, 2000; Sandeman and Sandeman, 2003). The sets of BrdU+ cells associated with clusters 9 and 10 form a ‘comma’ shape, consisting of a ‘head’ and a ‘tail’ that emerges from the head (Fig. 2A,B) (Song et al., 2005). The tail of the BrdU+ cells extends from the head beyond the cluster boundary, and the two tails from cell clusters 9 and 10 meet at a patch of cells near the posterior AL (Fig. 2A,C,D).

The tail of BrdU+ cells is delineated by a filamentous ‘strand’ that is strongly labeled with anti-tubulin (Fig. 2C,D), and faintly labeled with anti-H3P (Fig. 2E,F) (Song et al., 2005). This structure also occurs in adults, where it has been labeled with anti-glutamine-synthetase (Sullivan et al., 2006). We used the strand as a location marker for counting BrdU+ cells. Although we do not know whether BrdU+ cells within the strand are functionally related to BrdU+ cells within the boundaries of cell clusters 9 and 10, our images suggest their structural connection (Fig. 2C). In cell cluster 9, the tail of BrdU+ cells curves around the LAN and extends toward the posterior AL (orange broken circle in Fig. 2B). In cell cluster 10, the tail of BrdU+ cells extends medially and ventrally across the ventral surface of the AL (white broken circle in Fig. 2B). The mitosis marker, anti-phospho-histone-3 (H3P), also labeled cells in clusters 9 and 10 within the comma-shaped neurogenic areas. The comma-shaped arrangement of BrdU+ and mitotic (H3P+) cells was present in clusters 9 and 10, together with the strand and the patch of cells, regardless of the social status of an animal (e.g. Fig. 3, Table 1). BrdU+ labeling was much more common than H3P+ labeling, with the number of H3P+ cells being only ca. 5% of the number of BrdU+ cells (e.g. Fig. 3, Table 2).

Significantly more BrdU+ cells were found in the head than in the tail of the comma. The effects of social experience on labeled cell numbers were generally similar for the head and tail regions; thus in the figures and tables representing these data, we use the counts from the entire comma (i.e. the sum of the head and tail) and describe in the text any differences in effects of social experience on cell counts in head *vs* tail regions. This is also true for the H3P+ cells.

In addition to the mostly round BrdU+ nuclei in the comma and the round nuclei of unlabeled mature neurons in clusters 9 and 10, a few lenticular nuclei were scattered in clusters 9 and 10, and on the borders of neuropils and along neurite tracts throughout the crayfish brain. Morphological evidence suggests that these lenticular nuclei represent the nuclei of glial cells (Linser et al., 1997; Schmidt, 1997; Harzsch et al., 1999).

Cell proliferation tests

BrdU+ cells in Cluster 9

After 1 day of social experience, the number of BrdU+ cells in cluster 9 did not differ among isolates, dominants and subordinates (overall difference P=0.87; Fig. 4A). After 7 days,

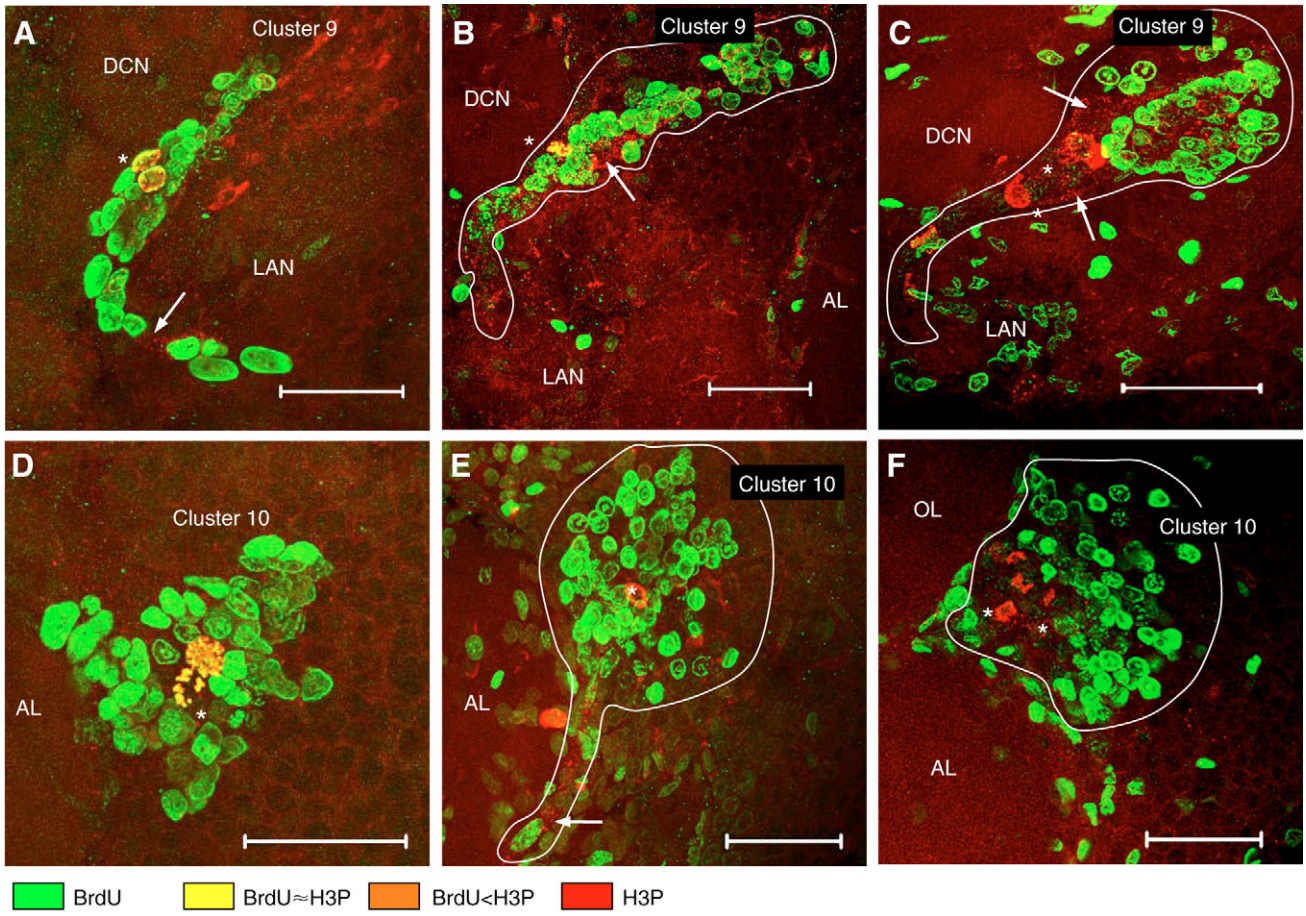

Fig. 3. Cell proliferation and cell survival in 'comma-shaped' neurogenic areas in cell clusters 9 (A–C) and 10 (D–F) after 1, 7 and 14 days of social interaction. BrdU+ cells are shown in green and H3P+ cells are shown in yellow or in orange, depending on the amount of BrdU/H3P colocalization (asterisks). Arrows indicate strand surrounding BrdU+ cells. For clarity, counted BrdU+ cells are outlined in white in B,C and E,F. Collapsed stacks of confocal images were taken at 0.5 μm intervals. The entire stack for each image was approximately 60–80 μm. Scale bar, 50 μm in all images. (A,B) Lines of BrdU+ cells are present in the ventral posterior part of cell cluster 9 and around the lateral antennular neuropil (LAN) after 1 day (A) and 7 days (B) of social interaction. BrdU+ cells were present in the same location in all cell proliferation tests irrespective of the length of social interaction. (C) After 14 days of survival time, a ball of BrdU+ cells was present in the posterior part of cell cluster 9 with a faint line of BrdU+ cells around the anterior edge of the LAN. (D–F) A ball of BrdU+ cells was present in cell cluster 10 near the olfactory lobe (OL) and accessory lobe (AL) after 1 day (D), 7 days (E) and 14 days (F) of social interaction. Regardless of the length of social interaction, all cell proliferation tests produced a ball of BrdU+ cells in the same location of cell cluster 10. As the survival time increased, the center of the ball of BrdU+ cells was fainter than the outer ring of BrdU+ cells, indicating the dilution of BrdU due to cell divisions. AL, accessory lobe; DCN, deutocerebral commissure neuropil; LAN, lateral antennular neuropil; OL, olfactory lobe.

an overall difference appeared among these groups (P=0.02), in which isolates had more BrdU+ cells than the subordinates (P<0.05; Fig. 4B). However, no significant difference was found in the number of BrdU+ cells of isolates *vs* dominants or of dominants *vs* subordinates (P>0.05; Fig. 4B). After 14 days of social experience, there was no overall difference in BrdU+ cells (P=0.64; Fig. 4C). We note that only one subordinate animal out of ten pairs retained two fully intact lateral flagella (Fig. 4C), so we were unable to determine the effect of social status on these animals. Similar results were obtained when BrdU+ cells in the head and tail of the cluster 9 comma were counted separately.

In addition, differences in the numbers of BrdU+ cells also developed among the isolates across the groups (P=0.01). This overall difference resulted from significantly more BrdU+ cells in the 7-day isolates than in the 1-day isolates (P=0.002). No other differences were significant.

BrdU+ cells in Cluster 10

A similar pattern emerged for cluster 10. After 1 day of social experience, the number of BrdU+ cells did not differ across the groups (overall difference P=0.32; Fig. 4D; Table 1). After 7 days, an overall difference appeared among the groups in cluster 10 (P=0.03; Fig. 4E). A multiple comparisons test failed to identify any pairwise significant differences, although the differences between isolates *vs* dominants and isolates *vs* subordinates approached significance (0.05<P<0.10) (Fig. 4E). After 14 days, the numbers of BrdU+ cells were not different

Table 1. *Number of BrdU+ cells in cell clusters 9 and 10*

Time of social interaction	Isolate	Dominant	Subordinate	Age control
Cell Proliferation Test				
1 day				
Cluster 9	82, **137**, 242	93, **166**, 223	114, **173**, 212	NA
Cluster 10	261, **371**, 548	387, **411**, 551	394, **448**, 481	
N	7	5	5	
7 days				
Cluster 9	252, **255**, 318	146, **186**, 260	144, **186**, 196 [140]	NA
Cluster 10	432, **550**, 566	307, **394**, 460	366, **427**, 474 [381]	
N	5	6	5 [1]	
14 days				
Cluster 9	152, **178**, 291	155, **184**, 220 [155]	158 [129, 150, 163]	NA
Cluster 10	320, **405**, 439	291, **414**, 452 [372]	398 [314, 342, 392]	
N	6	4 [1]	1 [3]	
Cell Survival Test				
7 days				
Cluster 9	272, **398**, 409	375, **430**, 461 [278]	354, **384**, 531	204, **215**, 248
Cluster 10	270, **447**, 624	276, **369**, 550 [233]	307, **375**, 603	387, **443**, 472
N	6	5 [1]	6	5
14 days				
Cluster 9	578, **783**, 1217	902, **1039**, 1143	441, **727**, 822	182, **222**, 227
Cluster 10	457, **808**, 1479	603, **893**, 1200	520, **798**, 1213	380, **512**, 561
N	6	6	6	6

Values are numbers of BrdU+ cells (minimum, **median**, maximum) in each cluster.

Numbers in brackets represent the number of BrdU+ cells from brains of animals having partial damage to one antennular lateral flagellum. *N*, number of brains examined.

across groups (overall difference P=0.91; Fig. 4F). Results for the cells in the head and tail regions of the comma-shaped group of BrdU+ cells were similar to those of the entire group.

As in cluster 9, differences in BrdU+ labeling also developed in cluster 10 of the isolates (overall difference across isolates: P=0.02). Both the 1-day isolates (P=0.02) and the 14-day isolates (P=0.02) had fewer BrdU+ cells in cluster 10 than did the 7-day isolates. No such differences appeared with time among the other social groups.

Cell survival tests

BrdU+ cells in Cluster 9

The number of BrdU+ cells in cluster 9 that survived after 7 days of social interaction was not influenced by the social status

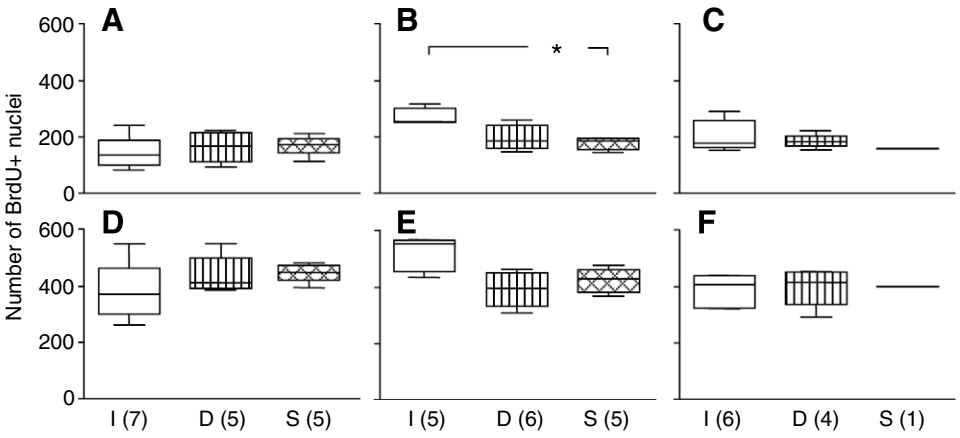

Fig. 4. Cell proliferation test for cell clusters 9 and 10. Number of BrdU+ cells in cluster 9 (A–C) and in cluster 10 (D–F) after 1 (A,D), 7 (B,E) and 14 (C,F) days of social interaction. Values are the sum of labeled cells from the two bilateral cell clusters 9 or 10 of a brain, and expressed as median ± interquartile range. (A,D) After 1 day, the number of BrdU+ cells did not differ for dominant (D), subordinate (S) and isolate (I) animals (Kruskal–Wallis test for overall difference: $P_{cluster9}$=0.87; $P_{cluster10}$=0.32). (B,E) After 7 days, isolated animals had significantly more BrdU+ cells in cluster 9 than subordinate animals but not more than dominant animals (Kruskal–Wallis test for overall difference: *P<0.05 (P=0.02); multiple comparison tests: P_{IvsS}<0.05; P_{IvsD} and P_{DvsS} >0.05). There was a non-significant trend for the isolates to have more BrdU+ cells in cluster 10 than subordinate and dominant animals (Kruskal–Wallis test for overall difference: P=0.03; multiple comparisons test: P_{IvsD} is >0.05 and P_{IvsS} <0.10; P_{DvsS}>0.10. (C,F) After 14 days, the number of BrdU+ cells did not differ among the groups (Kruskal–Wallis test for overall difference: $P_{cluster9}$=0.64; $P_{cluster10}$=0.91). (Numbers in parentheses indicate the number of animals examined.)

Table 2. *Number of H3P+ cells in cell clusters 9 and 10*

Time of social interaction	Isolate	Dominant	Subordinate	Age control
Cell Proliferation Test				
1 day				
Cluster 9	0, **1**, 5	1, **3**, 223	0, **2**, 11	NA
Cluster 10	0, **8**, 13	3, **9**, 13	0, **4**, 16	
N	7	5	5	
7 days				
Cluster 9	2, **6**, 14	2, **6**, 9	4, **5**, 8 [0]	NA
Cluster 10	11, 17, 28	6, **9**, 12	9, **11**, 12 [6]	
N	5	6	5 [1]	
14 days				
Cluster 9	0, **4**, 8	2, **3**, 5 [2]	3 [0, 2, 6]	NA
Cluster 10	5, **11**, 14	61, **10**, 14 [13]	9 [5, 6, 6]	
N	6	4 [1]	1 [3]	
Cell Survival Test				
7 days				
Cluster 9	0, **3**, 8	1, **2**, 5 [0]	0, **4**, 6	3, **4**, 10
Cluster 10	0, **2**, 4	0, **2**, 4 [2]	0, **3**, 8	5, **9**, 13
N	6	5 [1]	6	5
14 days				
Cluster 9	0, **6**, 13	7, **11**, 16	1, **7**, 10	1, **2**, 10
Cluster 10	8, **11**, 18	11, **14**, 18	7, **13**, 19	2, **7**, 17
N	6	6	6	6

Values are numbers of H3P+ cells (minimum, **median**, maximum) in each cluster.

Numbers in [brackets] represent the number of H3P+ cells from brains of animals having partial damage to one antennular lateral flagellum.

N, number of brains examined.

of an animal (overall difference P=0.28; Fig. 5A, Table 1). However, after 14 days of social experience, differences did develop (overall difference P=0.03). Dominant animals had significantly more surviving BrdU+ cells than subordinate animals (P<0.05), but their numbers were not significantly different from those of the animals that remained isolated for 14 days (P>0.05; Fig. 5B). These status-related differences appeared only in the head and not in the tail of the BrdU+ group in 14-day animals.

BrdU+ cells in Cluster 10

Unlike in cluster 9, the numbers of BrdU+ cells in cluster 10 that survived 7 or 14 days after pairing were not influenced by the social status of an animal (7 day: P=0.98, Fig. 5C; 14 day: P=0.93, Fig. 5D). This effect was similar for the head and tail of the BrdU+ cell group.

Mitotic activity in cell clusters 9 and 10

Mitotic activity at the time of the animal's sacrifice was revealed by H3P label. The number of H3P+ cells (i.e. mitotic cells) in clusters 9 and 10 was less than 5% of the number of BrdU+ cells (see Table 2). The number of H3P+ cells in clusters 9 and 10 was not different in isolate, dominant, and subordinate animals over the 14 days of the experiment.

Social status, body growth rate and neurogenesis

Because it was possible that neurogenesis could be affected by body growth, we measured each animal's wet mass at the beginning and end of the experimental period, and calculated the average body growth rate as the difference between these divided by the time difference between the measurements. In the 7-day and the 14-day cell proliferation test groups and in the 7-day cell survival test group, no significant differences in the body growth rate of the animals were found, although the values in the 7-day proliferation group approached statistical significance (overall difference $P_{7day,proliferation}$=0.052, $P_{14day,proliferation}$=0.36, $P_{7day,survival}$=0.72; Fig. 6A,C). However, in the 14-day cell survival test group, significant differences were found in the body growth rate of the dominant and subordinate animals (overall difference $P_{14day,survival}$=0.03, P_{DvsS}=0.015, P_{IvsD}=0.39, P_{IvsS}=0.065; Fig. 6D). These differences in body growth rates for animals of different social status occurred despite having provided all the animals with the same amount of protein-rich food.

In the 14-day cell survival test group, where dominant and subordinate animals differed in body growth rate, the body growth rates correlated positively with the number of BrdU+ cells in cell cluster 9 (Pearson correlation R_{cl9}=0.80, P_{cl9}=0.002). These results suggest that the effect of social status on neurogenesis might be secondary to the effect on body growth. Alternatively, social status may affect neurogenesis directly and in addition to any effect of body growth. To determine whether cell proliferation and cell survival were influenced by the social status of an animal independent of the effect of body growth, we subjected our data to analysis of covariance (ANCOVA; univariate, general linear model) to

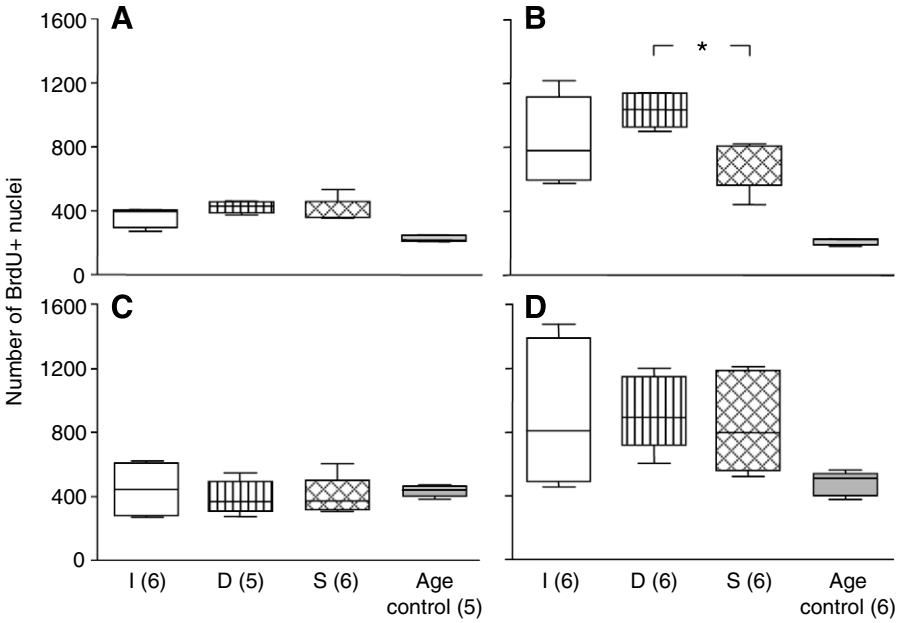

Fig. 5. Cell survival test for cell clusters 9 and 10. Number of BrdU+ cells in cluster 9 (A,B) and in cluster 10 (C,D) after 7 days and 14 days of social interaction. Values are the sum of labeled cells from the two bilateral cell clusters 9 or 10 of a brain, and expressed as median ± interquartile range. (A) 7 days of social interaction did not affect the number of surviving BrdU+ cells in cluster 9 (Kruskal–Wallis test for overall difference: P=0.28). The number of BrdU+ cells in the three experimental groups doubled relative to that of the age-control animals. (B) After 14 days of social interaction, dominant animals (D) had significantly more surviving BrdU+ cells in cluster 9 than subordinate animals (S) but were not different from isolate animals (I) (Kruskal–Wallis test for overall difference: *P<0.05 (P=0.029); multiple comparison tests: P_{DvsS}<0.05; P_{IvsD} and P_{IvsS}>0.10). Over 14 days of survival time, the number of BrdU+ cells in the three experimental groups approximately doubled twice relative to that of the age-control animals. (C,D) The number of cluster 10 BrdU+ cells did not differ among social groups after surviving 7 days (C) or 14 days (D) of social interaction (Kruskal–Wallis test for overall difference: P_{7days}=0.98, P_{14days}=0.93). The number of BrdU+ cells in the 7-day survival groups was similar to that of the age-control animals (C), while the number of BrdU+ cells in the 14-day survival groups approximately doubled (D). (Numbers in parentheses indicate the number of animals examined.)

measure the portion of the variance in cell proliferation and cell survival that was uniquely associated with social status. We found that body growth rate of an animal significantly influenced cell survival in cluster 9 after 14 days of social interaction (P_{cl9}=0.001, estimates of effect size being 0.74); however, after removing the effect of body growth, the effects of social status on cell survival (P_{cl9}=0.036, estimates of effect size being 0.404) were still significant. Thus, social experience significantly influenced neurogenesis in crayfish, independent of body growth.

Body growth results from molting, and molting could affect proliferation independent of body growth. However, we found that the six dominant and six subordinate (cluster 9 survival) animals experienced nearly the same total number of molts (10 and 9, respectively) over the 14 day experimental period, whereas the six isolates experienced somewhat fewer (7). Although there are no published studies of the effect of molting on neurogenesis in crayfish, one unpublished study in *Cherax* found that the rate of neuronal proliferation decreased briefly around the time of the molt and recovered afterwards (Beltz and Sandeman, 2003), and our own preliminary study found no effect of molt stage on proliferation. We conclude that it is

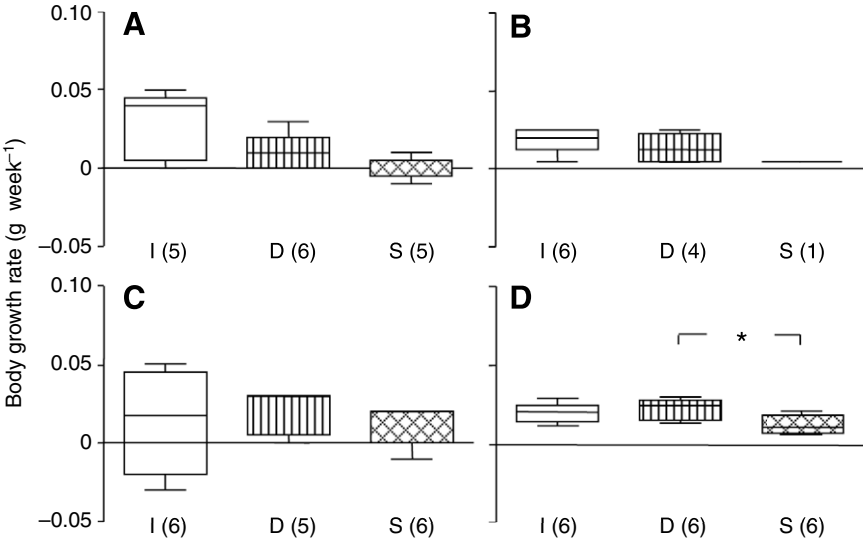

Fig. 6. Effect of social status on body growth rate. Values are median ± interquartile range of the average body growth rate from the beginning to the end of the experimental period, expressed as the increase in wet body mass per week in the experiment (g week^{-1}). (A,B) Body growth rate in the Cell Proliferation Test groups. Body growth rate of animals was not influenced by 7 days (A) or 14 days (B) of social interaction (Kruskal–Wallis test for overall difference: P_{7days}=0.052, P_{14days}=0.36). (C,D) Body growth rate in the Cell Survival Test groups. (C) 7 days of social interaction did not alter the body growth rate of animals (Kruskal–Wallis test for overall difference: P=0.72). (D) After 14 days of social interaction, body growth rate was significantly higher for dominant than subordinate animals (Kruskal–Wallis test for overall difference: *P<0.05 (P=0.03); multiple comparisons test: P_{IvsD}=0.39, P_{IvsS}=0.065; P_{DvsS}=0.02).

unlikely that the small difference in the molt experience of dominants and subordinates in the 14-day survival animals accounts for the growth-independent difference in survival of cluster 9 neurons. Instead, we attribute the difference in survival to the difference in the animals' social status.

Dynamics of cell division

To understand the dynamics of cell division in cell clusters 9 and 10 of the crayfish brain, we examined age-control animals for each of the two-cell survival test groups of isolated animals. The age-control groups allowed us to compare the number of proliferating (BrdU+) cells that were present at a given time point (the incubation of the animal in BrdU) with the number of cells originating from them and surviving over time periods of 7 and 14 days. Since the number of BrdU+ cells after 7 and 14 days must be the net result of division of BrdU+ cells and programmed cell death (Harzsch et al., 1999; Schmidt, 2001; Sandeman and Sandeman, 2003), these comparisons are a prerequisite for developing models for the dynamics of cell divisions in olfactory neurogenesis in the brain of juvenile crayfish. The number of BrdU+ cells that survived in cell cluster 9 of isolate animals approximately doubled once after 7 days and twice after 14 days compared to the number in the age-control animals (median for age control$_{7days}$=215, isolate$_{7days}$=398; age control$_{14days}$=222, isolate$_{14days}$=783; Table 1). The number of BrdU+ cells that survived in cell cluster 10 increased, but by less than twofold, after 14 days compared to the number of BrdU+ cells in the age-control animals (median for age control$_{14days}$=512, isolate$_{14days}$=808; Table 1). These data show that the dynamics of cell divisions differ between clusters 9 and 10. For cluster 10, the simplest model to explain the almost twofold higher number of BrdU+ cells after a 14-day survival period is that all BrdU+ cells undergo one round of cell division within this time period (with some possible losses due to programmed cell death). An approximate doubling of the number of BrdU+ cells after survival times of one to several weeks has also been observed in cluster 10 of embryonic lobsters *Homarus americanus* (Benton and Beltz, 2002), adult shore crabs *Carcinus maenas* (Schmidt, 1997), and adult spiny lobsters *Panulirus argus* (Schmidt, 2001), suggesting a common principle for cell division in this cluster. Accordingly, for cluster 9, the simplest model to explain our data is that all BrdU+ cells undergo two rounds of cell divisions, one that is complete after a survival time of 1 week and a second one that is complete after a survival time of 2 weeks.

Discussion

We found that continuous pairing of juvenile crayfish leads to an increase in cell survival in socially dominant animals relative to social subordinates after 2 weeks. These results are consistent with earlier findings that neurogenesis in crustaceans is plastic, and that it varies with living conditions (Sandeman and Sandeman, 2000) and according to circadian and seasonal rhythms (Goergen et al., 2002; Hansen and Schmidt, 2004). This first report that neurogenesis in crustaceans depends on social status is also consistent with similar reports of the dependence of neurogenesis on social status and social stress in rats (Gould et al., 1997; Kozorovitskiy and Gould, 2004) and birds (Pravosudov and Omanska, 2005).

Comparison with the effects of communal living vs isolation in Cherax

Our findings differ in some detail from those reported earlier by the Sandemans (Sandeman and Sandeman, 2000) on juvenile crayfish of a different species, *Cherax destructor*. They compared rates of neurogenesis in juvenile crayfish living communally in a large aquarium with rates of other crayfish in the same aquarium that were newly isolated both physically and socially. The communally housed animals had opportunities to interact socially with others and to explore the large living space, while the isolated crayfish were confined to a relatively small space at the water surface. The Sandemans found no difference in proliferation between the communal ('enriched') and isolated ('impoverished') animals after 1 week, but proliferation was reduced in the isolated animals after 2 weeks and 5 weeks. This differs from our result, which was that proliferation tended to be greater in isolates than in some socially paired animals on day 7, but not earlier (day 1) or later (day 14) (Fig. 4).

Two factors may account for these differences. First, all of our animals experienced a change in conditions at the beginning of the experimental period that included a move to a new cage and aquarium, addition of a shelter, and an increase in the rate of feeding (see Materials and methods). These changes appear to have prompted a transient increase in proliferation that became apparent by day 7 but was absent at day 14, perhaps because the isolates had habituated to the new conditions by that time. Second, the paired animals had previously been isolated and were placed together in similar small cages at the onset of the experiment, so that each paired animal lost individual living space and gained a social partner. These possibly stressful changes in the paired animals' lives appear to have prevented the transient increase in proliferation experienced by the isolates (Fig. 4). These changes are consistent with the reduction in proliferation seen in the newly confined animals in the Sandemans' experiments, and in the decreased proliferation seen upon bringing crustaceans into laboratory aquaria from the wild (Sandeman and Sandeman, 2000; Hansen and Schmidt, 2004).

Other differences in proliferation seen in the two sets of experiments are less easy to account for. In *Procambarus,* the rates of proliferation in cluster 9 were similar to those in cluster 10 in both isolates and paired animals (Fig. 4), whereas in *Cherax* proliferation in cluster 10 was nearly 3 times greater than in cluster 9 for both isolated and communal animals (Sandeman and Sandeman, 2000). Moreover, the peak counts were much higher in *Cherax* (approx. 800/24 h) compared to *Procambarus* (approx. 300/24 h). These differences may reflect the difference in species or the difference in age, as the *Procambarus* animals were likely a few weeks older than

Cherax animals. Neurogenesis in crustaceans has been found to slow down as an animal's age and size increases (Sandeman et al., 1998; Schmidt and Demuth, 1998).

We found that after 2 weeks of pairing (but not earlier), survival of new neurons in cluster 9 was greater in dominants than in subordinates. Survival in cluster 9 of isolates was between that of dominants and subordinates, but not significantly different from either. Survival in cluster 10 was similar in all three groups. In *Cherax*, the Sandemans found that cell survival in either cluster did not differ between the communal and isolated animals, even after 5 weeks of treatment. Survival did differ in these animals for cluster 10 (but not cluster 9) cells born after 2 weeks of treatment and examined after 4 more weeks in each treatment. In that instance, fewer cells survived in the isolated animals than in the communal animals. Moreover, the variance in cell survival was much greater in the communal cluster 10 cells than in the isolates, suggesting that survival among the communal animals may be affected by social factors, including status. Given our results with *Procambarus*, it is interesting to speculate that this variance may reflect a greater cell survival among dominant members of the communal group of *Cherax*. However, this difference, if it existed, developed in cluster 10, not cluster 9 as in *Procambarus*. These differences highlight the complexity of the mechanisms that regulate neuronal survival in these animals, which may differ with species, age and experimental treatment.

The adaptive significance of our result is unclear because we do not fully understand the role played by cluster 9 and 10 neurons in sensory processing. Cluster 9 neurons are local interneurons that receive inputs from the olfactory afferents within the subcap region of the olfactory lobe (OL) and project to the accessory lobe (AL). They branch nearly exclusively within the cortex of the AL, with few projections into the terminal medulla (Sullivan and Beltz, 2005a). Cluster 10 neurons are projection interneurons that originate in the AL and terminate in the hemiellipsoid body of the lateral protocerebrum, or originate in the OL and terminate in the terminal medulla (Sullivan and Beltz, 2001). It is apparent then, that neurogenesis affecting clusters 9 and 10 acts on distinct elements in the olfactory and multimodal sensory pathway (Beltz et al., 2003; Beltz and Sandeman, 2003). While the specific meaning of our results in the context of sensory processing is unclear, they do suggest that in the longer term, socially dominant crayfish may be better able to process chemosensory signals than subordinates.

The dominant *vs* subordinate difference in neuronal survival described here for crayfish has also been seen in mammals. An increased survival of newborn neuronal precursors occurs in the hippocampus of dominant rats (Kozorovitskiy and Gould, 2004). This common effect is likely to be the result of the common conditions of dominant and subordinate animals across species. Whereas dominants are largely free to move about, explore their environment, and have first access to available resources, subordinates are restricted to spaces, resources, and access to items that the dominants do not want (Song et al., 2006; Herberholz et al., 2003). This leads to behavior patterns that share many features with the behavior engendered by 'learned helplessness' and, in humans, clinical depression: withdrawal, excessive caution and avoidance of new experience (Malatynska and Knapp, 2005). During 2 weeks of social interaction, subordinate crayfish may experience a greater suppressive effect of stress on cell survival than dominants as the subordinates avoid their dominants and defend against attacks when they cannot. Dominant animals, which move freely about regardless of the presence of their subordinate partners, may be resistant to this suppressive effect (Kozorovitskiy and Gould, 2004).

The effect of body growth

Juvenile crayfish given a surfeit of food, as was done here, normally grow rapidly and molt frequently. We found that body growth rates were greater in dominant than in subordinate animals in the 14-day cell-survival test group (Fig. 6) even though both dominant and subordinate animals had access to the same amount of food and had a similar rate of molting, once or twice during 14 days. This is consistent with the earlier finding that persistent contact between large (probably dominant) and small (probably subordinate) crayfish reduces the body growth rate of the smaller animal by 50% even when food is freely available (Karplus and Barki, 2004).

These dominant and subordinate animals were the same that developed the differences in cell survival. Our statistical analysis showed that some of the enhancement in survival of newborn neuronal precursors in dominant animals would result from their relatively greater body growth, independent of the direct effects of social status differences on growth. A similar relationship between size and neurogenesis was observed in *Cherax*, where the faster growing communal animals displayed greater neurogenesis than the slower growing isolated animals (Sandeman and Sandeman, 2000).

These results raise the question of whether the effect of status (or living experience) on neurogenesis is direct or is instead an indirect effect of differential body growth and molting. Our statistical analysis of the effects of body growth and social status on survival of cluster 9 interneurons showed that the effect of status remained once the effect of body growth was considered. Moreover, dominants and subordinates experienced nearly the same number of molts, so that molting cannot account for the difference in neuronal survival.

Comma-shaped neurogenic area in the crayfish brain

We found that newborn neuronal precursors in the brains of juvenile crayfish are located in comma-shaped neurogenic areas. The comma 'heads' occupy the previously identified neurogenic areas of cell clusters 9 and 10 (Sandeman and Sandeman, 2000; Sandeman and Sandeman, 2003), and the comma 'tails', which are surrounded by a tubulin+ and H3P+ strand, extend from the clusters and join at a patch of cells located near the posterior region of the accessory lobe.

The nuclear labeling that we obtained with anti-H3P serum is consistent with this notion since the H3P+ nuclei were a

small sub-population of the BrdU-positive nuclei and usually were of irregular shape, as typical for nuclei in various phases of mitosis. The weaker but still selective labeling of the strand structure with anti-H3P serum could either be due to non-specific binding of the antibody to a related epitope or could instead be caused by specific binding to extranuclear located histone-3 or a molecule derived from it. Immunocytochemical labeling with anti-tubulin, to our knowledge, has not been performed in the brain of decapod crustaceans until now. It is, however, well established that multiple isoforms of α- and β-tubulin exist in decapod crustaceans (Demers et al., 1996; Varadaraj et al., 1997), that α-tubulin is expressed in the brain (Mykles et al., 2000), and that microtubules are common structures within neurons and glial cells of the CNS (Warren and Rubin, 1978; Warren, 1984). In our immunostainings of the crayfish brain, anti-tubulin labeled filamentous structures within neuronal and non-neuronal cells that closely resemble the reported morphological arrangement of microtubules. We thus conclude that the labeling we obtained with anti-tubulin likely reflects the presence of one or several tubulin isoforms.

In general, neurogenesis generates neurons that provide new elements for neural circuits and new substrates for synaptic changes during learning and memory. For example, neurogenesis in rat hippocampus is linked to trace memory formation (Shors et al., 2001), and neurogenesis in olfactory brain regions affects responses to and learning of odors in mammals (Rochefort et al., 2002; Lledo and Saghatelyan, 2005) and insects (Scotto-Lomassese et al., 2003). Olfaction, mediated by the olfactory lobes where primary sensory neurons and the two types of olfactory interneurons communicate, plays a prominent role in crayfish and other crustaceans, including discrimination of social odors (Derby and Steullet, 2001; Horner et al., 2004; Johnson and Atema, 2005). The precise roles of newborn olfactory interneurons are uncertain; however, the changing nature of these olfactory cues and the constant necessity of learning new cues may provide a likely reason for their continuous production and turnover.

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