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Agonistic behavior enhances adult neurogenesis in male *Acheta domesticus* crickets

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SUMMARY

We examined the effect of agonistic behavior on cell proliferation and neurogenesis in the central nervous system (CNS) of adult male *Acheta domesticus* crickets. We combined 5-bromo,2′deoxyuridine (BrdU)-labeling of dividing cells with immunocytochemical detection of the neuronal marker horseradish peroxidase to examine the proliferation of progenitor cells and the survival of newborn neurons. In crickets, the mushroom bodies of the brain contain clusters of proliferative cells that divide and generate new neurons in adulthood. Pairs of male crickets were allowed to fight and establish social rank and were then injected with BrdU. Proliferation of mushroom body neurogenic cluster cells was unaffected by agonistic interactions; 24 h after a fight, the number of BrdU positive cells in fought and un-fought males did not significantly differ. However, agonistic interactions did influence cell survival. Two weeks after an agonistic interaction, fought males had more newborn neurons than males that did not fight. There was also a rank-specific effect because dominant males had significantly more new neurons than subordinates. We also report for the first time that neurogenesis in adult crickets can occur in other regions of the brain and in other CNS ganglia, including the terminal abdominal ganglion (TAG). Agonistic interactions enhanced the proliferation of these distributed precursor cells but did not increase the survival of the newborn neurons generated by these cells.

Key words: aggression, dominance, neurogenesis, proliferation, BrdU.

INTRODUCTION

It was long thought that the plasticity of adult nervous systems rested in the ability to remodel existing neural circuits. Recent evidence also supports the contribution of adult born neurons to brain plasticity. Adult neurogenesis occurs in mammals, in non-mammalian vertebrates and in invertebrates, including insects and crustaceans (Cayre et al., 2002; Taupin and Gage, 2002; Kaslin et al., 2008). This widespread occurrence of adult neurogenesis supports its evolutionary and functional importance (Lindsey and Tropepe, 2006).

Adult neurogenesis consists of cell proliferation, survival, migration and differentiation/maturation (Ming and Song, 2005). In adult mammals, clusters of stem cells are located in the subgranular zone (SGZ) of the hippocampus and subventricular zone (SVZ) of the lateral ventricles; areas designated ‘neurogenic’ regions (Kempermann, 2006). In the SGZ, adult-born neurons get functionally wired into existing hippocampal circuits (Toni et al., 2007) and participate in hippocampal-dependent learning (Snyder et al., 2005; Kee et al., 2007). In addition to these neurogenic regions, there is evidence for adult neurogenesis in regions usually considered ‘non-neurogenic’, including the neocortex, hypothalamus and substantia nigra (Gould et al., 1999; Kempermann, 2006).

As in mammals, clusters of proliferative cells are found in specific neurogenic sites in the invertebrate brain. These neurogenic regions have been well studied in adult crickets and consist of a cluster of proliferating neuroblasts located above each mushroom body (MB) (Cayre et al., 1994; Cayre et al., 1996). The mushroom bodies are the main sensory integrative centers of the insect brain (Strausfeld et al., 1998) and play a role in olfactory (Zars, 2000; Heisenberg, 2003) and spatial (Mizunami et al., 1998) learning. In adult crickets, newborn neurons produced by these proliferative cells mature into Kenyon cell interneurons (Cayre et al., 1996; Cayre et al., 2000). MB proliferative cells have been found in other insect species (Cayre et al., 1996; Gu et al., 1999; Dufour and Gadenne, 2006). However, there has been little support for adult neurogenesis within other ‘non-neurogenic’ regions of the insect brain.

Many intrinsic and extrinsic factors can modulate adult neurogenesis in vertebrates and invertebrates, including neurohormones (Gu et al., 1999; Brezun and Daszuta, 2000; Malaterre et al., 2003; Cayre et al., 2005b; Borta and Hoglinger, 2007), exercise (van Pragg et al., 1999), stress (Gould et al., 1997; Gould et al., 1998) and environmental enrichment (Kempermann et al., 1997; Sandeman and Sandeman, 2000). Many also regulate neuroblast proliferation and neurogenesis in adult crickets (Cayre et al., 2002; Cayre et al., 2007). For example, the proliferation rate of MB neurogenic cells of female crickets housed in enriched environments was greater than for females in impoverished environments (Scotto-Lomassese et al., 2000). Visual and olfactory stimuli enhance proliferation of these cells, while sensory deprivation decreases it (Scotto-Lomassese et al., 2002). When brain irradiation was used to selectively ablate MB proliferative cells of adult female crickets, olfactory learning was impaired (Scotto-Lomassese et al., 2003).

Social interactions can affect adult neurogenesis in vertebrates and invertebrates. Dominant adult male rats have more newborn hippocampal neurons than subordinate or separately caged control rats (Kozorovitskiy and Gould, 2004). However, social status did not affect proliferation of SGZ cells in these rats, indicating that dominance status enhances neurogenesis by increasing cell survival (Kozorovitskiy and Gould, 2004). Similar results have been reported for a crustacean model of neurogenesis. A persistent neurogenic niche resides in the deutocerebrum of the crustacean brain and these

proliferative cells produce new olfactory interneurons (Schmidt, 1997; Harzsch et al., 1999; Sullivan and Beltz, 1999). Dominant crayfish exhibit more new olfactory neurons than subordinates and this difference was also due to the enhanced survival of new neurons in dominants (Song et al., 2007).

In the present study, we examined the effects of agonistic interactions and social rank on neurogenesis in adult male *Acheta domesticus* crickets. Agonistic interactions of male crickets have been well characterized and consist of a series of increasingly aggressive behaviors, including antennal fencing, biting and wrestling (Alexander, 1961; Adamo and Hoy, 1995; Stevenson et al., 2000; Hofmann and Schildberger, 2001). Establishment of social rank for a pair of male crickets occurs suddenly during an agonistic encounter. At establishment, the newly dominant and newly subordinate males begin to exhibit behaviors specific to their social status. A dominant male sings aggressive song, produces jerking movements of its body and continues to approach the subordinate male, which quickly retreats. We show that the agonistic interaction itself enhanced neurogenesis because both dominant and subordinate males had more new brain neurons than males that did not fight. We also report that dominant males had significantly more new neurons than subordinates. We also show for the first time that neurogenesis is not limited to the mushroom bodies of the insect brain. Proliferative cells were found throughout the adult cricket central nervous system (CNS), in other regions of the brain as well as in other ganglia of the ventral nerve cord, and these proliferative cells gave rise to new neurons.

MATERIALS AND METHODS

Animals

Immature 7th–8th instar *Acheta domesticus* L. crickets were purchased from Flukers Cricket Farm, Port Allen, LA, USA. Upon arrival, the nymphs were maintained in groups of ~50 in large plastic boxes. Within 1–2 days of their adult molt (10th instar or D_0), males with all body parts intact were placed in individual, round, plastic containers (10 cm diameter, 8 cm high). All crickets were housed at 29°C with 12 h:12 h light:dark cycle (lights on at 06:00 h) and fed dry dog food and water *ad libitum*.

Agonistic trials

Isolated males that were either 4 days (D_4) or 9–10 days (D_{10}) in age past the adult molt were used. Each cricket was weighed one day prior to its trial. All trials took place at ~20°C during the last 6 h of the light phase (12:00 h–18:00 h). Crickets were taken to the dimly lit, quiet, recording room at least 1 h prior to the start of the trials. Each trial took place in a clear, round, Plexiglas arena (15 cm diameter, 10 cm high). Only males producing calling song were used. For each trial, each member of a pair of age- and mass-matched males was carefully placed, without direct handling, on opposite sides of an opaque Plexiglas divider in the arena center. At the same time, a mass-matched control male was placed into another arena, where it remained isolated during the trial. White paper in the bottom of each arena was replaced after each trial. Each pair acclimated to the arena for 15 min and then the barrier was removed allowing the pair to interact. Following establishment of social rank, pairs interacted for another 15 min for confirmation of each cricket's social status. All trials were recorded on VHS tape with a Videolab Flexcam camera (Minneapolis, MN, USA) and Panasonic VCR (Matsushita Consumer Electronics, Secaucus, NJ, USA).

Each trial was scored according to Stevenson and colleagues (Stevenson et al., 2000). Most pairs made physical contact during a trial. Mutual avoidance (level 0) was only observed during interactions of D_4 adults, occurring in 8 of 35 trials. Thus, most encounters between pairs could include any, or all, of the following interactions: level 1, pre-established dominance; level 2, antennal fencing; level 3, unilateral mandible spreading; level 4, bilateral mandible spreading; level 5, mandible engagement; level 6, wrestling. Each trial was scored according to all fight levels reached, as well as maximum level reached, by each pair. Establishment of rank was scored when one male (established dominant) produced rival song and body jerks and chased the retreating male (established subordinate). For both trials, pre-established dominance (level 1) was scored when one male exhibited dominant behaviors and the other exhibited subordinate behaviors upon initial physical contact. Such encounters did not progress to higher fight levels.

All males underwent two agonistic trials. Following their first trial, each pair of males and the time-matched control male were briefly cold anesthetized. A 100 µl Hamilton syringe (Hamilton Company, Reno, NV, USA) was used to inject 10 µl of a 40 mg ml^{-1} solution of 5-bromo,2′-deoxyuridine (BrdU, Sigma-Aldrich, St Louis, MO, USA) in cricket saline into the hemolymph of the thorax. BrdU, a thymidine analog, becomes incorporated into the DNA of dividing cells during the S phase of mitosis. This is the same BrdU concentration used by Myriam Cayre and colleagues in their pioneering studies on neurogenesis in adult crickets (Cayre et al., 1996; Scotto-Lomassese et al., 2000). The solution was heated to 50°C in order for the BrdU to completely dissolve but was allowed to cool before injection. After injection, all crickets were returned to their respective containers until their second trial. All were re-matched with the same opponent from trial one. Second trials were used to confirm that each male had retained the same social status from trial one. In trial two, pairs interacted for 10 min after clear establishment of rank and were then quickly decapitated. All time-matched control males from first trials were treated the same but they did not have access to another male. Brains and terminal abdominal ganglia (TAGs) were then processed for immunocytochemical detection of BrdU-labeled cells (see below).

Light microscopy

Six groups of males were used, and males were collected as triplets (dominant, subordinate, time-matched control). To examine the effects of age on cell proliferation, pairs of D_4 males (N=35 triplets) and pairs of D_{10} males (N=46 triplets) were used (Fig. 1A). Males of each group were fought on D_4 or D_{10}, were injected with BrdU and were re-fought 24 h later, they were then killed and their brains were collected. To assess the effect of dominant and subordinate status on short-term cell survival, a third group of D_{10} males (N=28 triplets) were isolated for 48 h before second trials (Fig. 1B). The brains of all these males were processed for avidin–biotin immunocytochemistry and light microscopy.

After their second trial, the brains of all control and fought crickets were rapidly dissected under cold saline and immersed in Carnoy's fixative overnight at 4°C. TAGs were not collected. Following fixation, brains were dehydrated in ethanol, cleared in Citrisolv (Fisher Scientific, Pittsburg, PA, USA), embedded in paraffin and serially sectioned at 10 µm in the frontal plane. Sections were deparaffinized in xylene, passed through a descending ethanol series and rehydrated in distilled water. Rehydrated tissue underwent DNA hydrolysis in 2 mol l^{-1} HCl in 0.1 mol l^{-1} phosphate buffered saline (PBS) for 2 h at 20°C. Slides were incubated in PBS containing 0.3% TritonX (PBST) overnight at 4°C and blocked for 1 h at 20°C in 5% normal goat serum (NGS) in PBST. Sections were incubated overnight at 4°C in a 1:10 dilution of mouse anti-BrdU antiserum

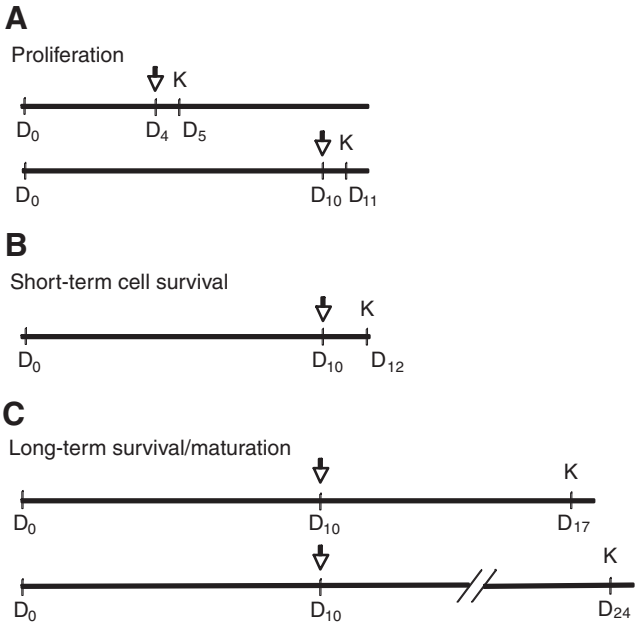

Fig. 1. Experimental design. All males were isolated at adulthood (D_0). (A) To examine the effect of age and social status on cell proliferation, pairs of males were fought on D_4 or D_{10} (4 days or 9–10 days in age past the adult molt, respectively) received a 5-bromo,2′deoxyuridine (BrdU) injection no more than 30 min after establishment of social rank (arrow) and were killed (K) 24 h later, i.e. D_5 and D_{11}, respectively. (B) To investigate the effect of social status on short-term cell survival, males were fought and injected on D_{10} and killed 48 h later on D_{12}. (C) To examine long-term cell survival, males were fought and injected on D_{10} and killed 7 (D_{17}) or 14 (D_{24}) days later. On the day that they were killed, all pairs were re-fought to confirm that social rank was maintained; only males that maintained rank were used further. Control males were treated the same, except they did not have contact with another male.

(Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) in PBST with 5% NGS. After PBST wash, sections were incubated overnight at 4°C in 1:50 biotinylated goat anti-mouse IgG secondary (Vector, Burlingame, CA, USA) in PBST. After a phosphate buffer (PB) wash, sections were incubated in Vectastain ABC solution (Vector). The antigen–antibody complex was visualized at 20°C with 1.25% 3,3′-diamino-benzidine, 0.3% H_2O_2 and 0.4% $NiCl_2$ in PB. Slides were dehydrated, cleared in xylene and mounted with permount.

Confocal microscopy

To investigate long-term cell survival, three additional groups of D_{10} males were collected (Fig. 1C). One group was fought on D_{10} and re-fought and killed 7 days later (N=7 triplets). A second group was fought on D_{10} and killed 14 days later (N=7 triplets). In order to examine cell proliferation, a third group was fought on D_{10} and killed 24 h later (Fig. 1A, bottom timeline). Brains and TAGs of all males were collected and processed for immunocytochemistry and confocal microscopy.

Standard procedures for confocal microscopy were employed. All brains (frontal plane) and TAGs (cross section) were cut at 10 µm. Sectioned tissue underwent deparaffinization, rehydration and DNA hydrolysis as described above. After blocking for 1 h in 5% NGS in PBST, sections were incubated for 24 h at 4°C in 1:200 rat anti-BrdU (Abcam, Cambridge, MA, USA) and 1:200 rabbit anti-HRP (horseradish peroxidase) (Jackson ImmunoResearch, West Grove, PA, USA) in PBST. In insects, HRP is an endogenous membrane surface protein associated with the cell bodies, dendrites and axons of neurons (Jan and Jan, 1982), and anti-HRP antibodies can be used to label nerve cells (Loesel et al., 2006). After thorough washing in PBST, sections were incubated for 2 h at 20°C in 1:200 dilutions of 488 AlexaFluor goat anti-rat and 555 AlexaFluor goat anti-rabbit secondaries (Invitrogen-Molecular Probes, Carlsbad, CA, USA). Slides were washed in 0.1 mol l^{-1} PBS, rinsed in water and covered with Vectashield mounting media (Vector) and a coverslip.

Cell counts

For light microscopy, brain sections were viewed with an Olympus BX52 compound microscope equipped with a camera lucida (Olympus, Center Valley, PA, USA). BrdU positive (BrdU+) cells were drawn from each section and counted. The experimenter was blind to the status of each sample, i.e. whether it came from a control, dominant or subordinate male. To account for the overestimation associated with counting the same nuclei in two adjacent sections, the formula of Abercrombie (Abercrombie, 1946) was used to calculate the number of BrdU+ cells in the MB neuroblast clusters: $N=[(n\times t)/(t+d)]$, where n is total number of BrdU+ nuclei counted for all sections, d is mean nuclear diameter of 10 labeled cells and t is section thickness (see Scotto-Lomassese et al., 2003). BrdU+ nuclei were also found scattered in other brain regions and in the TAG. As these BrdU+ cells were few in number and not located in clusters, their identification from section to section was easily accomplished.

For confocal microscopy, sectioned brains and TAGs were viewed with an Olympus F500 Fluoview confocal microscope equipped with Argon Ion (488 nm) and Green Helium–Neon (543–546 nm) lasers (Olympus). Double labeling of a cell with BrdU and HRP confirmed its identity as an adult-born neuron. The experimenter was blind to each sample's status. For brain neurogenic clusters, a Z-series of images was taken at 0.9 µm for each brain section containing cluster cells (8–10 sections), and cells were counted and summed from both halves of the brain. The formula of Abercrombie (Abercrombie, 1946), as described above, was applied to the labeled nuclei counted from all sections. BrdU+ cells in non-neurogenic brain regions and in TAGs were counted directly from each section. Cells co-labeled with BrdU and HRP (BrdU+/HRP+) were counted and identified as newborn neurons.

Statistical analyses

Comparisons of mean maximum fight level scores between groups were analyzed using Proc Generalized Linear Model (GLM) in SAS 9.1 (SAS Institute, Cary, NC, USA). Comparison of mean maximum fight level scores for first and second trials within a group were analyzed with Student's t-test (SAS 9.1). The proportion of D_4 and D_{10} pairs that exhibited agonistic behavior was compared with Fisher's Exact Test (InStat 3.06, GraphPad Software, San Diego, CA, USA). All other behavioral analyses were performed with a χ^2 (SAS 9.1). Analysis of variance (ANOVA) with Bonferroni *post-hoc* test (InStat 3.06) was used for all cell count comparisons. Values were reported as means ± s.e.m. and $P<0.05$ was considered statistically significant.

RESULTS

A cluster of labeled proliferative cells was detected above each MB after an injection of BrdU into adult male crickets (Fig. 2A). These results agree with those reported for adult female *A. domesticus* crickets by Cayre and colleagues (Cayre et al., 1994;

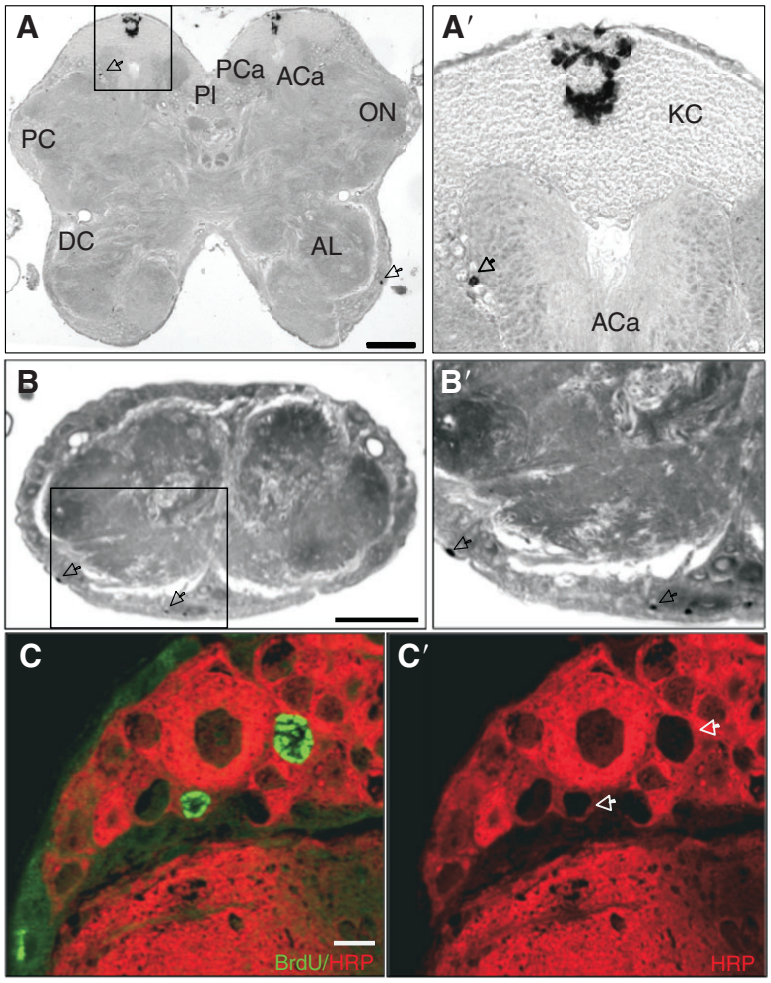

Fig. 2. 5-bromo,2′deoxyuridine (BrdU)-labeled proliferative cells in the adult cricket brain and terminal abdominal ganglion (TAG). (A,A′) 10 μm frontal section of the brain of a D_{11} male killed 24 h after BrdU injection and processed with the avidin–biotin peroxidase procedure. Clusters of BrdU+ nuclei (black) located above the calyx of each mushroom body are surrounded by unlabeled Kenyon cell (KC) interneurons. Two BrdU+ distributed cluster cells (DPCs), adjacent to the calyx (top arrow) and in the deutocerebrum (DC, bottom arrow) are visible in this section. Boxed area in (A) is enlarged in (A′). (B,B′) 10 μm cross section of a D_{10} control male TAG showing several BrdU+ cells (arrows). Boxed area is enlarged in (B′). (C,C′) Confocal images of two BrdU+/HRP+ cells (arrows) in the TAG of a control male that was killed 14 days after BrdU injection. These cells are located in rostral TAG, near the connectives. The elongated nucleus of a glial cell in the perineurial sheath (asterisk, cell is out of plane of focus) is BrdU+/HRP–. ACa=anterior calyx; AL=antennal lobe; ON=optic nerve; PC=protocerebrum; PCa=posterior calyx; PI=pars intercerebralis. Scale bars: (A,B) 100 μm, (A′,B′) 25 μm, (C,C′) 10 μm. The scale bars for A′, B′ and C′ correspond to those in A, B and C, respectively.

Cayre et al., 1996), who identified these cells as neuroblasts and ganglion mother cells (GMCs). In insects, proliferative neuroblasts divide asymmetrically to produce a neuroblast and a GMC; each GMC then divides symmetrically to produce two daughter neurons (Doe and Skeath, 1996; Corley and Lavine, 2006; Egger et al., 2008). We also detected BrdU+ cells in several other brain regions, including the perineurial sheath, deutocerebrum, protocerebrum, pars intercerebralis, optic nerve, central body and an area lateral to the MB calyxes (Fig. 2A′, arrow). BrdU+ cells were found within all ganglia of the ventral nerve cord, including the TAG (Fig. 2B,B′). Throughout this paper, we refer to proliferative cells associated with the MBs as neurogenic cluster cells (NCCs) whereas proliferative cells not associated with the mushroom bodies are distributed precursor cells (DPCs). Unlike NCCs, the BrdU+ DPCs in the brains (Fig. 2A) and ventral ganglia (Fig. 2B) of males killed 24 h after BrdU injection were widely scattered and located singly.

Agonistic interactions produce lasting behavioral changes

Our goal was to investigate the effect of both agonistic interaction and social status on the proliferation and survival of newborn cells in adult male crickets. Because age can influence the proliferative capacity of MB NCCs in female crickets (Scotto-Lomassese et al., 2000), we used both young (D_4) and mature (D_{10}) males in our initial study. Significantly fewer D_4 pairs (77%) engaged in agonistic behavior than D_{10} pairs (100%, P=0.009), indicating that young males have a reduced motivation to fight. However, the mean fight level of D_4 pairs engaging in agonistic behavior (N=27 of 35 pairs) was not significantly different from D_{10} pairs (Fig. 3A). After 24 h, pairs that had established rank were re-fought and most D_4 (86%, N=23 of 27 pairs) and D_{10} (91%, N=42 of 46 pairs) males retained the same social rank acquired during their first trial.

We examined the maximum fight level reached during the first and second trials of pairs separated for 24 h (Fig. 3A). Second trials of D_4 (P<0.0001) and D_{10} (P=0.0008) pairs were significantly less aggressive than first trials (Fig. 3A), due to a significant increase in D_4 (P=0.0008) and D_{10} (P<0.0001) pairs that showed pre-establishment during second trials (Fig. 3B). The relative change in level of aggression of the two trials and the proportion of pairs that exhibited pre-establishment for each trial was not significantly different for the two age groups. Thus, age did not affect the aggressiveness of a trial or the likelihood that a trial would be resolved with pre-establishment.

We also examined the agonistic behavior of D_{10} pairs that underwent two trials separated by 48 h (Fig. 3). When compared with D_{10} males separated for 24 h, fewer of these pairs retained the same rank for both trials (75%, N=21 of 28 pairs), although this difference was not significant (P=0.58). As with 24 h D_4 and D_{10} pairs, second trials of 48 h D_{10} pairs were less aggressive than first trials (P=0.01; Fig. 3A), because more 48 h D_{10} pairs exhibited pre-establishment during second trials (P=0.007; Fig. 3B). Again, these results indicate that the second agonistic encounters of male crickets are less aggressive, even after 48 h.

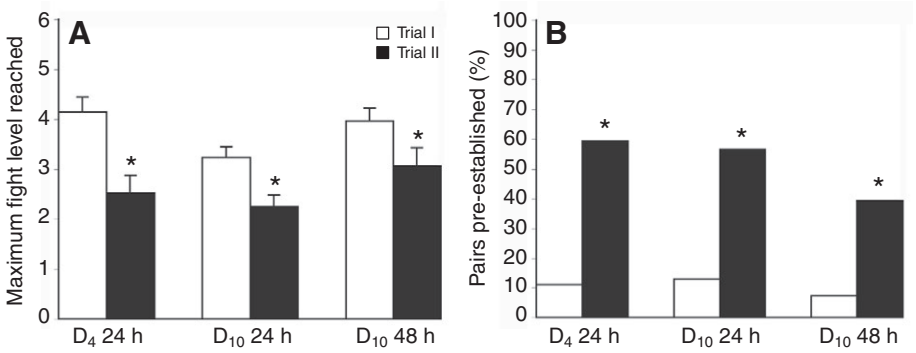

Fig. 3. Both D_4 and D_{10} males are less aggressive and show more pre-establishment of social rank during second agonistic trials. Each male pair was allowed to interact twice, with each trial separated by a span of 24 h for D_4 and D_{10} males or 48 h for a second group of D_{10} males. (A) The maximum fight level (means ± s.e.m.) was significantly lower during second trials. (B) The percentage of pairs exhibiting pre-establishment was increased during second trials. *N*=27 for D_4 males; *N*=46 for D_{10} males separated for 24 h; *N*=28 for D_{10} males separated for 48 h.

Agonistic interactions and adult proliferation

The effect of agonistic interactions on cell proliferation in the brains of D_4 and D_{10} adult male crickets killed 24 h after social rank formation was examined. The brains of males that maintained the same social rank for both trials were processed for BrdU immunocytochemistry using the avidin–biotin peroxidase procedure (see Fig. 2A). At 24 h, ~350 MB NCCs were labeled with BrdU in D_4 (Fig. 4A) and D_{10} (Fig. 4B) males. Neither agonistic behavior nor social rank influenced MB NCC proliferation because the number of BrdU+ cells in control, dominant and subordinate males did not differ significantly for either age group. The number of labeled cells for D_4 *vs* D_{10} controls, D_4 *vs* D_{10} dominants and D_4 *vs* D_{10} subordinates did not significantly differ, indicating that age did not influence the proliferative capacity of NCCs.

We compared the number of BrdU+ DPCs in the brains of these same males. At 24 h, ~50–100 DPCs were BrdU+ in both age groups (Fig. 4A′,B′). BrdU+ DPCs were always unpaired in males killed at 24 h. Agonistic behavior increased DPC proliferation because both

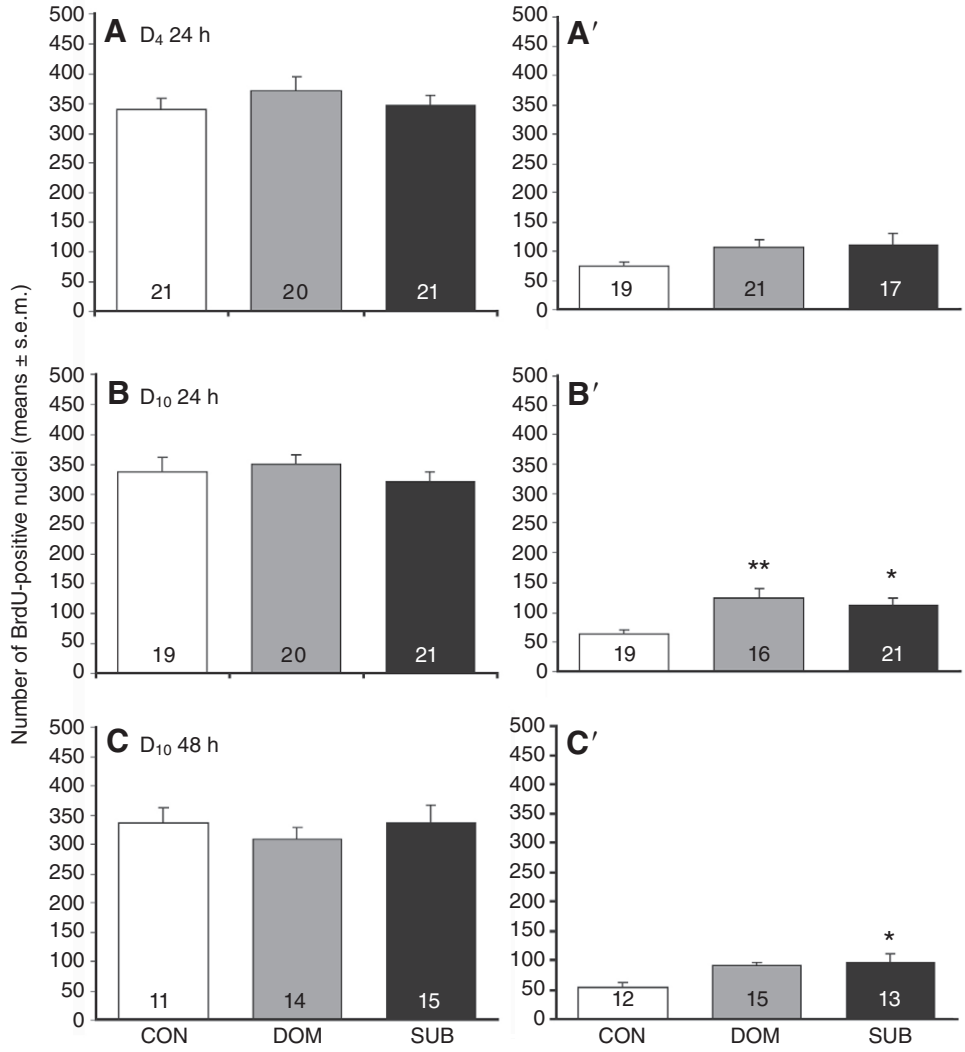

Fig. 4. Agonistic interactions increased the proliferation of distributed precursor cells but not mushroom body (MB) neurogenic cluster cells (NCCs) in the brain. Males were killed 24 or 48 h after establishment of social rank. Number of 5-bromo,2′deoxyuridine positive (BrdU+) NCCs in (A) D_4 and (B) D_{10} males killed at 24 h and (C) D_{10} males killed at 48 h. (A′,B′,C′) Number of BrdU+ distributed precursor cells (DPCs) for the same three groups of males. Asterisks indicate significance at **P*<0.05, ***P*<0.01 relative to controls. The number of BrdU+ DPCs did not differ significantly for dominant and subordinate males. CON=control; DOM=dominant; SUB=subordinate. Values are means ± s.e.m. Sample sizes as indicated.

dominants and subordinates had more BrdU+ DPCs than controls (Fig. 4A′,B′), although this difference was only significant for D_{10} males (D_{10}, P=0.0014; D_4, P=0.12) (Fig. 4B′). Thus, a single agonistic interaction can increase the proliferation of brain DPCs and this fight-induced enhancement of proliferation may be influenced by age but not social rank.

We asked if this increase in proliferation for fought males could be attributed to the enhanced proliferation of DPCs located in specific brain regions. BrdU+ DPCs lateral to the MB calyxes, and in the central body, deutocerebrum, protocerebrum and pars intercerebralis of D_{10} control, dominant and subordinate males killed 24 h after BrdU injection were counted and compared (Table 1). BrdU+ cells were also located within the optic nerves. However, because many optic nerves were lost during tissue processing, we only counted BrdU+ cells located at the nerve base. A small number of BrdU+ nuclei were found within the brain perineurial sheath in 66% of D_{10} males (N=37 of 56 males). These sheath cells were never HRP+ (see below) and were thus considered glial cells. Sheath cells were not included in our overall counts of BrdU+ DPCs.

Dominant D_{10} males had significantly more BrdU+ DPCs than control males in the lateral MB calyx region (P=0.01) and deutocerebrum (P=0.03) whereas subordinates had more labeled DPCs than controls in the protocerebrum (P=0.0043; Table 1). The number of BrdU+ cells in these six brain regions was similar for D_4 males killed 24 h after BrdU injection (data not shown). There was a trend for fought D_4 males to have more BrdU+ cells in the lateral MB calyx region than controls (CON: 18.4±1.7; DOM: 30.7±3.3; SUB: 38.5±11.2), although this difference was not statistically significant (P=0.08).

Agonistic interactions and short-term cell survival

We assessed the effect of both agonistic interaction and social status on short-term cell survival by counting the number of BrdU+ cells in D_{10} males killed 48 h after social rank formation. As at 24 h, there was no significant difference in the number of BrdU+ NCCs for control, dominant and subordinate males (P=0.65; Fig. 4C). The number of BrdU+ mushroom body NCCs in each of the three groups of 48 h D_{10} males was also not significantly different from each of the corresponding groups of D_{10} males killed at 24 h (compare Fig. 4B with Fig. 4C). By contrast, agonistic interaction did increase the number of BrdU+ DPCs in the 48 h D_{10} males but only subordinates had significantly more BrdU+ cells than controls (P=002, Fig. 4C′). These results suggest that the DPCs of subordinate males may exhibit greater survival. However, when we compared the total number of BrdU+ DPCs of 48 h control, dominant and subordinate males with their behavioral counterparts killed at 24 h (compare Fig. 4B′ with Fig. 4C′), no significant differences were found. Pair-wise comparisons of 48 h and 24 h D_{10} males revealed no significant differences in labeled DPCs within each of the six specific brain regions.

Agonistic behavior enhances neurogenesis in the brain

We used confocal microscopy and immunocytochemistry for BrdU and HRP to examine the survival of newborn adult brain cells in fought male crickets. Similar to our results with light microscopy (Fig. 2), BrdU+ proliferating cells were found in the MB neurogenic cluster of male crickets killed 24 h after BrdU injection (Fig. 5A). Older Kenyon cells were HRP+ but their nuclei lacked BrdU (Fig. 5A,A′) whereas the proliferating cells within the MB NCCs were never HRP+ (Fig. 5A′,B′). By 7 days or 14 days, only a few proliferative cells retained BrdU (Fig. 5B, arrowhead). Most BrdU+ cells were now located outside the cluster, in the surrounding population of Kenyon cells, where they co-expressed HRP (Fig. 5B,B′, arrows). Similarly, none of the BrdU+ DPCs in other brain regions were HRP+ at 24 h (not shown) but some were HRP+ in males killed at 7 days or 14 days (Fig. 5C,C′), indicating that these cells could also acquire a neuronal phenotype and have the potential to become functionally incorporated into existing neural circuits.

We used confocal microscopy to determine if both agonistic behavior and social status could affect the survival of newborn adult brain cells. NCCs and DPCs that were BrdU+ (Fig. 6) or both BrdU+ and HRP+ (Fig. 7) were counted from the brains of D_{10} controls, dominants and subordinates killed 24 h, 7 days or 14 days after an agonistic trial. As in our previous study, only pairs that retained the same social rank from trial one were used and included 79% of 24 h pairs (N=14), 71% of 7 days pairs (N=14) and 75% of 14 days pairs (N=16). Males killed at 24 h had between 200–300 BrdU+ NCCs, and no significant differences were found in the number of labeled cells for control, dominant and subordinate males (P=0.16; Fig. 6A). There was also no significant difference in the total number of BrdU+ NCCs in controls, dominants and subordinates killed 7 days after social rank formation (P=0.07; Fig. 6B). However, when we compared the number of BrdU+/HRP+ NCCs in these males, dominants had more new neurons than controls (P=0.01; Fig. 7A). At 7 days, subordinates also tended to have more BrdU+/HRP+ cells than controls (Fig. 7A) but this difference was not significant.

The effects of both agonistic interaction and social rank on MB neurogenesis were evident in males killed at 14 days. In these males, there was a significant difference in total number of BrdU+ NCCs for controls, dominants and subordinates (P<0.0001; Fig. 6C). There was also an effect of social rank because dominants had more BrdU+ cells than both controls (P<0.001) and subordinates (P<0.01). The agonistic interaction itself had an effect on neurogenesis because subordinate males also had more BrdU+ NCCs than controls (P<0.01). Most BrdU+ NCCs co-expressed HRP at 14 days (see Fig. 5B,B′), and agonistic behavior significantly increased neurogenesis in males killed at this time point (Fig. 7B). At 14 days, dominants had significantly more BrdU+/HRP+ cells than controls (P<0.001) and subordinates (P<0.01) whereas subordinates had significantly more new neurons than controls (P<0.01).

Table 1. Fought D_{10} male *Acheta domesticus* crickets show increased proliferation of distributed precursor cells within specific brain regions

Brain region	CON (N=19)	DOM (N=16)	SUB (N=21)
Calyx region	20.21±2.27[a] (5–44)	38.38±5.85[b] (12–110)	34.52±4.55[a,b] (6–77)
Central body	3.37±0.69 (0–10)	2.94±0.69 (0–10)	2.52±0.50 (0–9)
Deutocerebrum	23.58±2.43[a] (4–41)	52.06±10.17[b] (18–177)	42.76±8.22[a,b] (5–164)
Protocerebrum	9.00±1.14[a] (1–18)	14.69±1.77[a,b] (4–29)	18.10±2.41[b] (2–42)
pars intercerebralis	6.47±1.02 (0–17)	13.88±5.31 (1–86)	7.67±1.47 (0–31)
Optic nerve	1.37±0.37 (0–6)	2.19±0.88 (0–11)	1.29±0.35 (0–6)

Mean ± s.e.m. number of 5-bromo,2′deoxyuridine (BrdU) positive cells in each brain region 24 h after BrdU administration. Numbers in parentheses indicate range. Different letters indicate significant difference at P<0.05. CON, control males; DOM, dominant males; SUB, subordinate males.

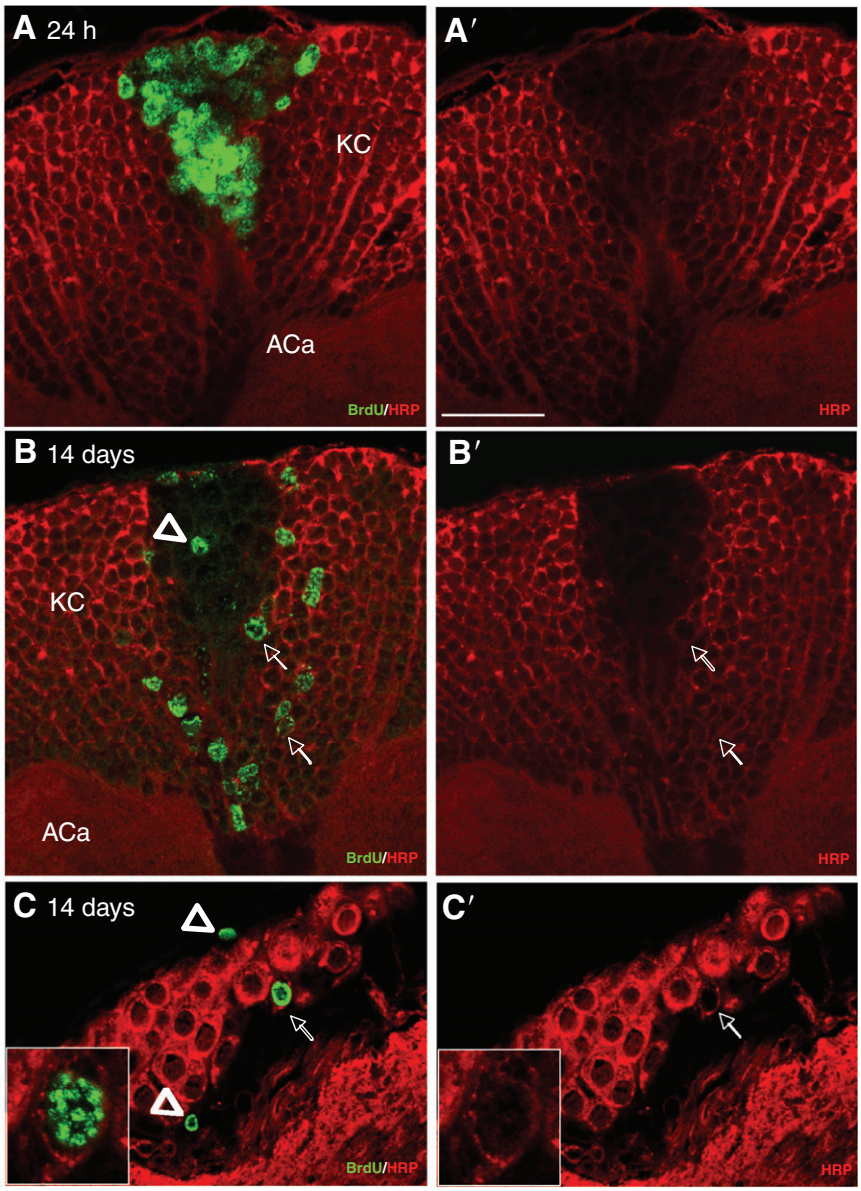

Fig. 5. Many 5-bromo,2′deoxyuridine positive (BrdU+) cells expressed the neuron specific marker horseradish peroxidase (HRP). (A) In this confocal image from a control male brain, only proliferating neuroblasts and ganglion mother cells are immunoreactive for BrdU (green) 24 h after BrdU injection. HRP (red) could be localized to Kenyon cell (KC) cell bodies and to calyx neuropil. Nuclei of mature KCs were unstained. (B) At 14 days, most BrdU+ cells near the neurogenic clusters co-expressed HRP (red). Two such cells are indicated by arrows. A BrdU+ proliferative cell (arrowhead) is still visible in this brain section from a dominant male. (C) BrdU+ cell (arrow) that is HRP+ in the protocerebrum of a subordinate male killed at 14 days. Arrowheads indicate two BrdU+ cells that were HRP–. Inset: magnified view of the cell indicated by arrow (image taken in a slightly different plane of focus). (A′,B′,C′) Confocal images produced with the laser that excites the Alexa Fluor 555 secondary antibody for HRP. Note lack of HRP immunoreactivity in the mushroom body (MB) neurogenic cluster cells. ACa=anterior calyx. Scale bar (shown in A′): (A–C) 50 μm, (inset) 25 μm.

We compared the number of BrdU+ NCCs in controls, dominants and subordinates across the three time points (Fig. 6A–C). There was a significant effect of the time the animals were killed on the number of BrdU+ NCCs for control (P=0.002) and subordinate (P=0.002) but not dominant (P=0.06) males. Control and subordinate males had fewer BrdU+ NCCs at 14 days than at 7 days (P<0.01) and 24 h (P<0.01); the number of cells at 24 h and 7 days was not significantly different. At 14 days, most BrdU+ cells in the MBs were HRP+ and could thus be considered to be new Kenyon cell interneurons (compare Fig. 6C with Fig. 7B). Both controls (P=0.01) and subordinates (P=0.002) had fewer BrdU+/HRP+ cells at 14 days than at 7 days whereas these counts were not significantly different for dominants (compare Fig. 7A with Fig. 7B). These results support the conclusion that agonistic behavior increases neurogenesis in the MBs of adult male crickets by promoting the long-term survival of new neurons, and that this effect was greatest in socially dominant males.

We examined the proliferation and survival of DPCs in these males. At 24 h, both dominants and subordinates had significantly more BrdU+ DPCs than controls (P<0.0001; Fig. 6A′), indicating enhanced proliferation of DPCs in fought males. At 7 days, only subordinates had significantly more BrdU+ DPCs than controls (P=0.005; Fig. 6B′). At 7 days, about half of the DPCs were HRP+ (compare Fig. 6B′ with Fig. 7A′), and subordinates had significantly more BrdU+/HRP+ DPCs than controls (P<0.01) and dominants (P<0.05). However, at 14 days, the number of BrdU+ DPCs (P=0.09; Fig. 6C′) and BrdU+/HRP+ DPCs (P=0.12; Fig. 7B′) in the three groups of males did not significantly differ. There was also a significant effect of the time the animals were killed on the number of BrdU+ DPCs in fought males (Fig. 6A′–C′). In controls, the number of BrdU+ DPCs was not significantly different at 24 h, 7 days and 14 days (P=0.08). However, dominants (P<0.0001) and subordinates (P=0.007) showed a significant decrease in BrdU+ DPCs over time. Similarly, the number of BrdU+/HRP+ cells was not significantly different for 7 day and 14 day control males (P=0.62) but both dominants (P=0.01) and subordinates (P=0.005) had significantly fewer new neurons at 14 days than at 7 days (Fig. 7A′,B′). These results thus show that, in contrast to MB NCCs, the proliferative capacity of brain DPCs can be increased by agonistic behavior.

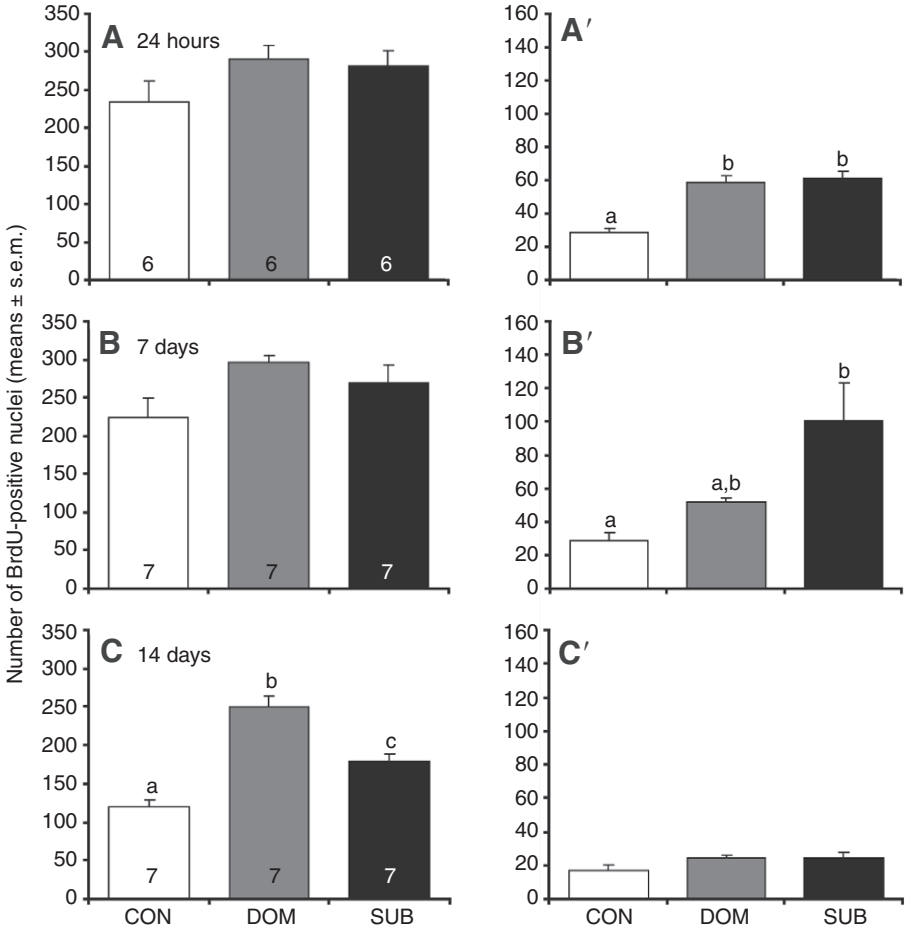

Fig. 6. Two weeks after establishment of social rank, dominant male crickets had more 5-bromo,2′deoxyuridine positive (BrdU+) neurogenic cluster cells than subordinate and control males. The number of BrdU+ cells was counted for control (CON), dominant (DOM) and subordinate (SUB) males killed at (A) 24 h, (B) 7 days or (C) 14 days after BrdU injection. (A′,B′,C′) Number of BrdU+ distributed precursor cells (DPCs) in these same males. Dominant and subordinate males killed at 24 h had enhanced proliferation of DPCs relative to controls but this difference was not evident in males killed at 14 days. Different letters indicate significant differences at $P<0.05$ (ANOVA). Sample sizes as indicated. Values are means ± s.e.m.

However, neither agonistic interactions nor social status increase the long-term survival of BrdU+ DPCs cells.

Agonistic behavior enhances neurogenesis in the terminal abdominal ganglion

BrdU+ DPCs were found in all ganglia of the CNS, including the TAG (Fig. 2B) and some were HRP+ (Fig. 2C,C′), indicating that neurogenesis is not restricted to the adult cricket brain. As for brain DPCs, none of the 20–70 BrdU+ DPCs in the TAGS of males killed at 24 h were HRP+ (Fig. 8A); however, BrdU+/HRP+ neurons were evident in males killed at 7 days (Fig. 8B′) and 14 days (Fig. 8C′). BrdU+ DPCs were found within the outer rind of TAG neurons and in the ganglionic sheath (Fig. 2C,C′). As in the brain, these sheath cells had elongated nuclei characteristic of glial cells and none co-expressed HRP, even in males killed at 14 days (Fig. 2C,C′). These putative glial cells were not included in our counts of BrdU+ DPCs.

Agonistic interaction had a significant effect on the proliferation of TAG DPCs (Fig. 8A). At 24 h, subordinates had significantly more BrdU+ DPCs than controls ($P<0.001$) or dominants ($P<0.01$). At 14 days (Fig. 8C), fought males had significantly more BrdU+ cells than control males ($P=0.004$) but there was no rank-specific effect. Approximately half of BrdU+ DPCs were also HRP+ in males killed at 7 days (Fig. 8B′) and 14 days (Fig. 8C′). Agonistic interactions had a significant effect on the number of BrdU+/HRP+ DPCs at 14 days; both dominants ($P<0.001$) and subordinates ($P<0.01$) had significantly more new neurons than controls (Fig. 8C′), and there was again no social rank specific effect.

We compared the number of BrdU+ TAG DPCs in control, dominant and subordinate males across the three time points (Fig. 8A–C). Control ($P=0.0004$), dominant ($P=0.001$) and subordinate ($P=0.007$) males all showed a significant decrease in the number of BrdU+ cells when the time till the animals were killed was increased. If we postulate that this decrease is associated with cell death, then the greatest loss of new cells was between 24 h and 7 days for controls and dominants and between 7 days and 14 days for subordinates (Fig. 8A–C), with all changes statistically significant (controls, $P<0.01$; dominants, $P<0.05$; subordinates, $P<0.05$). By 7 days, about a third of control male cells, half of dominant male cells and a third of subordinate male cells were HRP+ (Fig. 8B′), and only subordinate males showed a significant decrease in new neurons between 7 days and 14 days ($P=0.01$, compare Fig. 8B′ with Fig. 8C′). We conclude that neurogenesis can occur in what have previously been considered non-neurogenic regions of the adult insect nervous system. Agonistic behavior can significantly increase the proliferation but not the survival of DPCs in the TAG, an effect similar to that observed for brain DPCs (see Fig. 6A′–C′).

DISCUSSION

As first shown by Cayre and colleagues, a cluster of proliferative cells is located above each MB in the adult cricket brain (Cayre et al., 1994; Cayre et al., 1996). In the present study, we combined BrdU-labeling of proliferating cells with immunocytochemical detection of HRP to examine the proliferation and survival of adult-born neurons. In insects, HRP is a neuron-specific protein (Jan and Jan, 1982) that labels cell bodies, dendrites and axons of nerve cells

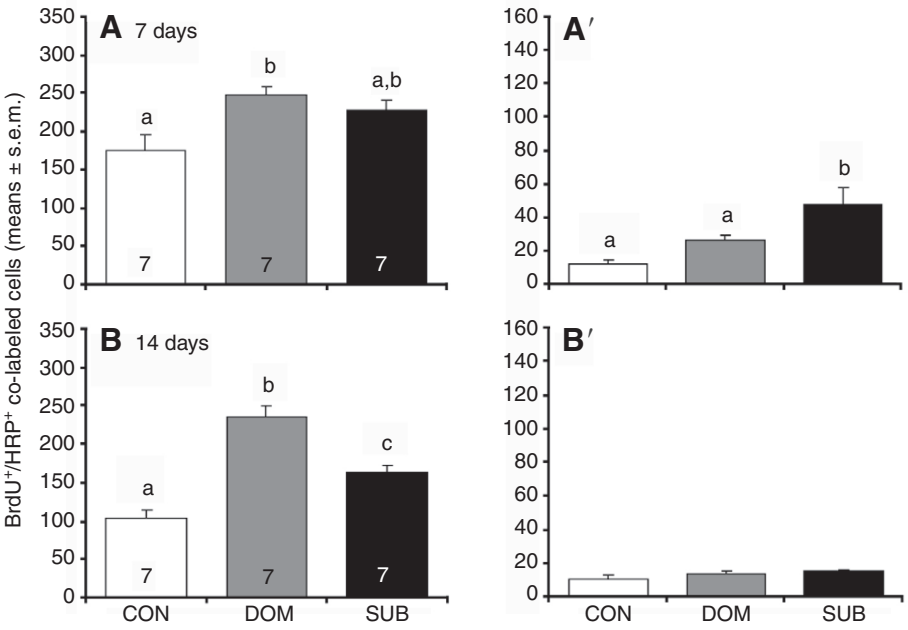

Fig. 7. Dominant males had more newborn Kenyon cell interneurons than subordinate and control males at 14 days. The number of mushroom body cells that were 5-bromo,2′deoxyuridine positive/horseradish peroxidase positive (BrdU+/ HRP+) were counted for control (CON), dominant (DOM) and subordinate (SUB) male crickets killed (A) 7 days or (B) 14 days after an agonistic interaction. These are the same males as in Fig. 6. No data is available for males killed at 24 h because no cells expressed both markers at this time point. (A′,B′) BrdU+/HRP+ distributed precursor cells (DPCs) in the same groups of males. Subordinate males killed at 7 days had more BrdU+/HRP+ DPCs than control and dominant males but this difference was not evident in males killed at 14 days. Different letters indicate significant differences at $P<0.05$ (ANOVA). Sample sizes as indicated. Values are means ± s.e.m.

(Loesel et al., 2006). We determined that the neuroblasts and GMCs within the MB proliferative clusters (see Cayre et al., 1996) have no detectable HRP label. By 7 days, few proliferative cells retain BrdU, presumably due to its dilution by repeated cell divisions. Also by 7 days (and as early as 3 days, K.G., M.G. and K.A.K., unpublished), BrdU+ cells that are also HRP+ are located just outside the proliferative clusters, indicating that progeny of these proliferative cells had differentiated into Kenyon cells. These new neurons do not divide and should thus lose BrdU only through cell death. In addition, some BrdU+ distributed precursor cells located in other parts of the brain and in the TAG never express HRP, even 14 days after BrdU administration. We thus propose that CNS cells that are BrdU+ but do not express HRP can include neuroblasts, GMCs, glioblasts and adult-born glia whereas cells that are

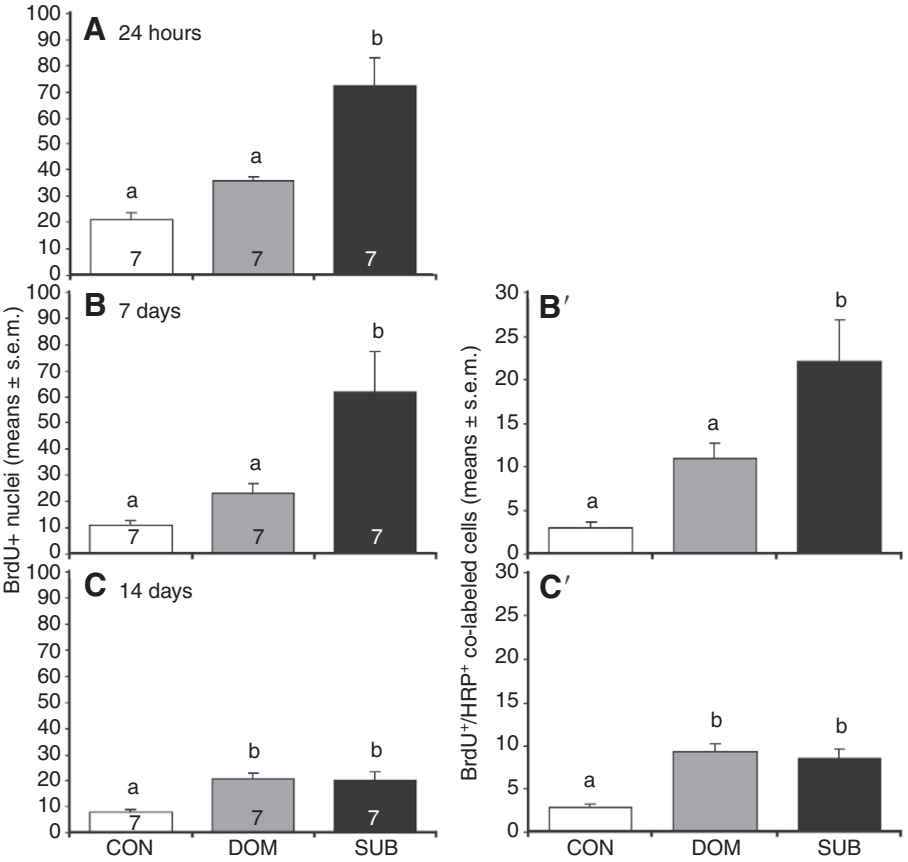

Fig. 8. A single agonistic interaction can increase the proliferation of distributed precursor cells in the terminal abdominal ganglion (TAG). (A,B,C) Total number of 5-bromo,2′deoxyuridine positive (BrdU+) cells in the TAGs of control (CON), dominant (DOM) and subordinate (SUB) male crickets killed (A) 24 h, (B) 7 days or (C) 14 days after a single BrdU injection. Two weeks after establishment of social rank, fought males had more BrdU+ cells in the TAG than controls. These are the same males as in Figs 6 and 7. (B′,C′) Number of BrdU+/HRP+ (horseradish peroxidase positive) cells in the TAGs of these same males. At 24 h, all BrdU+ TAG cells were HRP–. Different letters indicate significant differences at $P<0.05$ (ANOVA). Sample sizes as indicated. Values are means ± s.e.m.

BrdU+/HRP+ are adult-born neurons. Future studies are needed to determine if these neurons become functionally wired into existing brain circuits.

We have shown that a single agonistic interaction can increase neurogenesis in the mushroom bodies of adult male crickets; both dominant and subordinate males had more new neurons than un-fought males. In addition, dominants had more new MB neurons than subordinates two weeks after a fight. Dominance status can also enhance neurogenesis in the hippocampus of the adult rat (Kozorovitskiy and Gould, 2004) and chickadee (Pravosudov and Omanska, 2005) and in the brain olfactory centers of the crayfish (Song et al., 2007). For both the rat (Kozorovitskiy and Gould, 2004) and the crayfish (Song et al., 2007), social status did not affect the proliferation of progenitors. Instead, enhanced survival of newborn cells resulted in more new neurons in dominant animals relative to subordinates. Similarly, dominance status in adult male crickets appears to enhance cell survival rather than proliferation. At 14 days, both dominant and subordinate male crickets had more new MB neurons than socially isolated males. However, the number of BrdU+ cells at 24 h (i.e. proliferation) did not differ significantly between fought and un-fought males. Two weeks after an agonistic trial, dominants had more new neurons than subordinates, indicating that dominance status may be a more potent regulator of cell survival.

We propose that some newborn neurons are lost through apoptotic cell death. Control and subordinate male crickets killed at 14 days had significantly fewer new MB neurons than control and subordinate males killed at 7 days. Such a decrease in new neurons was not observed for dominants. Our findings differ from previous reports, where both a TUNEL assay (cf. Cayre et al., 2000) and Feulgen staining (cf. Scotto-Lomassese et al., 2000) failed to reveal dying cells in cricket mushroom bodies. However, both studies were performed in female crickets and no quantitative data were provided. In support of our conclusion, Mashaly and colleagues observed some pyknotic Kenyon cells and degenerating cellular components in electron micrographs of the Kenyon cell perikaryial layer and MB calyx regions of adult *Gryllus bimaculatus* crickets (Mashaly et al., 2008). Our results are similar to findings in mammals, where many new neurons die within a few days of birth (Cameron et al., 1993; Dayer et al., 2003). Similarly, in the neurogenic regions of the crustacean brain, cell birth and death occur in conjunction (Harzsch et al., 1999). Further studies are needed to determine how agonistic behavior enhances cell survival in the cricket.

Agonistic behavior between conspecific male crickets is triggered by antennal contact (Hardy and Shaw, 1983; Tregenza and Wedell, 1997), and olfactory information from antennal chemoreceptors, as well as information from other sense organs, receives higher order processing in the mushroom bodies (Strausfeld et al., 1998). Removal of a cricket's antennae decreases the proliferation of MB NCCs (Scotto-Lomassese et al., 2002; Cayre et al., 2005a) whereas electrical stimulation of the antenna increases their proliferation (Cayre et al., 2005b). Chemosensory antennal input may have played a role in our present results, because both dominants and subordinates showed an increase in MB neurogenesis relative to isolated controls. Other visual or mechanical sensory inputs activated during the fight may have also played a role.

We do not yet know the functional significance of the enhanced neurogenesis in fought male crickets. The mushroom bodies play a role in associative olfactory learning and memory in flies and bees (Zars, 2000; Heisenberg, 2003; Schwartzel and Muller, 2006) and in spatial learning in cockroaches (Mizunami et al., 1998). Suppression of MB proliferation by brain irradiation impairs the olfactory learning abilities of female *A. domesticus* crickets (Scotto-Lomassese et al., 2003). It is possible that increased MB neurogenesis could enhance olfactory learning and thus affect an animal's subsequent behavior. We did observe a decrease in mean fight level for males re-fought after 24 h or 48 h. This decrease in the aggressiveness of the second fight was due to an increase in pairs exhibiting pre-establishment. We do not know if this short-term change was due to rank-specific effects on male behavior or due to a general decrease in the level of aggressiveness of both males. The long-term effects of enhanced neurogenesis, however, remain to be tested.

We examined the effect of age on agonistic behavior and neurogenesis in male crickets. More D_4 male pairs failed to engage in agonistic behavior than D_{10} males. However, for D_4 and D_{10} pairs that did fight, there was no significant difference in mean fight level. As for D_{10} males, second encounters of D_4 males were less aggressive than first encounters due to an increase in pre-establishment. We decided to compare D_4 and D_{10} males because it had been reported that the negative impact of an impoverished environment on the proliferation of MB progenitors could be observed in D_4 but not in D_{10} or D_{20} female crickets (Scotto-Lomassese et al., 2000). We found no significant effect of age on NCC proliferation; however, it is possible that age-related differences may become apparent if older males were used.

For the first time, we report that neurogenesis in adult crickets also occurs in 'non-neurogenic' regions of the brain and in other CNS ganglia, including the TAG. Cayre and colleagues previously reported sparse BrdU labeling of non-MB brain cells in female *A. domesticus* crickets (Cayre et al., 1996). As these cells were immunoreactive for antibodies raised against *A. domesticus* glial cells by John Edwards and colleagues (Meyer et al., 1987; Meyer et al., 1988), Cayre and colleagues concluded that these BrdU+ cells were replicating glial cells (Cayre et al., 1996). The proliferative cells of the MB NCCs, however, did not label with these anti-glial antibodies (Cayre et al., 1996).

We found 20–60 BrdU+ distributed precursor cells in the 'non-neurogenic' regions of the brains and TAGs of male crickets killed at 24 h. At this time, none were immunoreactive for HRP. At 7 days, approximately half of BrdU+ DPCs were HRP+, indicating some DPC progeny can acquire a neuronal phenotype. But what is the nature of the proliferative DPCs that give rise to these new neurons? DPCs may include distinct glioblasts and neuroblasts or they may be multipotent progenitor cells (i.e. neuroglioblasts) that give rise to both neurons and glia (see Condron and Zinn, 1994). Recent work suggests that stem cells in the neurogenic regions of the adult vertebrate brain may be glia (Doetsch, 2003; Garcia et al., 2004; Seri et al., 2004). Proliferative cells in the crayfish brain also label with glial markers (Sullivan et al., 2007). The specific nature of the neural precursor cells in the cricket nervous system requires further study.

Unlike the MB NCCs, agonistic interactions enhance the proliferation of DPCs in the brain and TAG of male crickets. This suggests that NCCs and DPCs are either functionally distinct or may be differentially exposed to factors that enhance proliferation. Although we did not find a significant difference in brain DPC neurogenesis for control and fought males killed at 14 days, neurogenesis was enhanced in TAGs of fought males and this effect was not dependent on social status. The TAG is the last ganglion of the ventral nerve cord and receives a number of sensory inputs from a pair of sensory appendages called cerci. Just as the activation of antennal sensory inputs can stimulate proliferation of mushroom body precursors, cercal sensory information may play a crucial role in modulating DPC proliferation and neurogenesis in the TAG.

Future studies are necessary to determine the precise functional role of these newborn TAG and brain neurons in adult crickets and of the factors generated during agonistic interactions that enhance neurogenesis.

LIST OF ABBREVIATIONS

BrdU	5-bromo,2′-deoxyuridine
BrdU+	5-bromo,2′-deoxyuridine positive
CNS	central nervous system
d	mean nuclear diameter
DPC	distributed precursor cells
GMC	ganglion mother cell
HRP	horseradish peroxidase
MB	mushroom body
N	number of BrdU+ cells in the mushroom body
n	total number of BrdU+ nuclei
NGS	normal goat serum
NCC	neurogenic cluster cells
PB	phosphate buffer
PBS	phosphate buffered saline
PBST	PBS containing TritonX
SGZ	subgranular zone
SVZ	subventricular zone
t	section thickness
TAG	terminal abdominal ganglion

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