



Cell proliferation in the central nervous system of an adult semiterrestrial crab

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Abstract

Neurogenesis occurs in adults of most organisms, both vertebrates and invertebrates. In semiterrestrial crabs of the infraorder Brachyura, the deutocerebrum, where neurogenesis occurs, processes the olfactory sensory information from the antennae. The deutocerebrum is composed of a pair of olfactory lobes associated with cell clusters 9 and 10 (CI 9 and CI 10), containing proliferating cells. Because the location of the neurogenic niche in brachyuran semiterrestrial crabs has not been defined, here we describe a neurogenic niche in the central olfactory system of the crab *Ucides cordatus* and report two types of glial cells in the deutocerebrum, based on different markers. Serotonin (5-hydroxytryptamine) labeling was used to reveal neuroanatomical aspects of the central olfactory system and the neurogenic niche. The results showed a zone of proliferating neural cells within CI 10, which also contains III beta-tubulin (Tuj1)+ immature neurons, associated with a structure that has characteristics of the neurogenic niche. For the first time, using two glial markers, glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS), we identified two types of astrocyte-like cells in different regions of the deutocerebrum. This study adds to the understanding of neurogenesis in a brachyuran semiterrestrial crustacean and encourages comparative studies between crustaceans and vertebrates, including mammals, based on shared aspects of both mechanisms of neurogenesis and regenerative potentials.

Keywords Decapod crustacean · Neurogenesis · Olfactory lobe · Mangrove crab · Glial cell

Abbreviations

AL Accessory lobe
BrdU 5-Bromo-2'-deoxyuridine

CI 9 Cluster 9
CI 10 Cluster 10
DAPI 4',6-Diamidino-2-phenylindole
DGN Dorsal-giant neurons
EM External medulla
GFAP Glial fibrillary acidic protein
GS Glutamine synthetase
HB Hemiellipsoid body
IM Internal medulla
5HT 5-Hydroxytryptamine (serotonin)
L Lamina
NeuN Neuronal nuclei
OGT Olfactory globular tract
OL Olfactory lobe
ORNs Olfactory receptor neurons
PBS Phosphate buffer saline
PH3 Phospho-histone H3
PF Paraformaldehyde
PI Propidium iodide
TM Terminal medulla
Tuj1 III beta-tubulin

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Introduction

Neurogenesis occurs in adults of both vertebrates (Kempermann 2012) and invertebrates (Altman and Das 1967; Harzsch et al. 1999; Sullivan and Beltz 2005; Cayre et al. 2002). We and other research groups have studied questions related to this event in invertebrates such as decapod crustaceans, using different models: crayfish (Sandeman et al. 1998; Schmidt and Harzsch 1999; Sullivan and Beltz 2005; Sullivan et al. 2007; Chaves da Silva et al. 2012), lobster (Harzsch et al. 1999; Schmidt 2001) and crab (Schmidt 1997; Hansen and Schmidt 2001; Hollmann et al. 2011). This study on the brachyuran crab *Ucides cordatus*, a semiterrestrial crab that holds a strategic evolutionary position between aquatic and terrestrial crustaceans (Araújo and Calado 2008) and therefore allowed us to explore neurogenesis from an evolutionary perspective.

Decapod crustaceans, including *U. cordatus*, have a well-organized central nervous system composed of fused ganglia (“brain”): the protocerebrum, deutocerebrum and tritocerebrum (Fig. 1). The deutocerebrum, where central neurogenesis occurs, processes the olfactory sensory information from the antennae. The deutocerebrum is composed of a pair of olfactory lobes (OL) associated with two cell clusters, cluster 9 (CI 9) and cluster 10 (CI 10) (Sandeman et al. 1992). CI 10 contains axons that project unilaterally to the OL and bilaterally through the olfactory globular tract (OGT)

to the hemiellipsoid bodies (HB) in the terminal medulla (TM), a part of the protocerebrum in the eyestalk. CI 9 contains interneurons that innervate only the OL.

Unlike many other decapod crustaceans, which contain two structures in the deutocerebrum related to the olfactory system (the accessory lobe [AL] and the OL), the crab *U. cordatus* contains a single lobe on each side, the OL, a glomerular structure containing synaptic contacts between the olfactory receptor neurons (ORNs), interneurons (CI 9) and projection neurons (CI 10; Sullivan and Beltz 2001). In true crabs, which belong to the infraorder Brachyura, AL is either quite reduced in size and complexity or absent (Sandeman et al. 1992, 2011), as in *U. cordatus* (Hollmann et al. 2015, 2016).

In the crayfish *Procambarus clarkii* and in the spiny lobster *Panulirus argus*, which belong to the infraorders Astacidea and Achelata, respectively, the neural precursors are located in the neurogenic niche on the ventral surface of the AL (Song et al. 2009; Sullivan et al. 2007; Schmidt and Derby 2011). The niche cells can be identified by their immunoreactivity to an antibody against glutamine synthetase (GS), a marker of astrocytes (Anlauf and Derouiche 2013) and glial cells in the brain of crustaceans (Linser et al. 1997; Allodi et al. 2006; Sullivan et al. 2007; Sandeman et al. 2011; Beltz and Benton 2017). In the semiterrestrial crab *U. cordatus*, the locations of neither the proliferation zone nor the neurogenic niche have been reported.

Despite the latest advances in understanding adult neurogenesis in decapod crustaceans, most studies have used aquatic models and little is known of this process in semiterrestrial crabs. In this study, we locate a potential area of neurogenesis in the central olfactory system of the semiterrestrial crab *U. cordatus* and identify at least two different types of glial cells in the deutocerebrum.

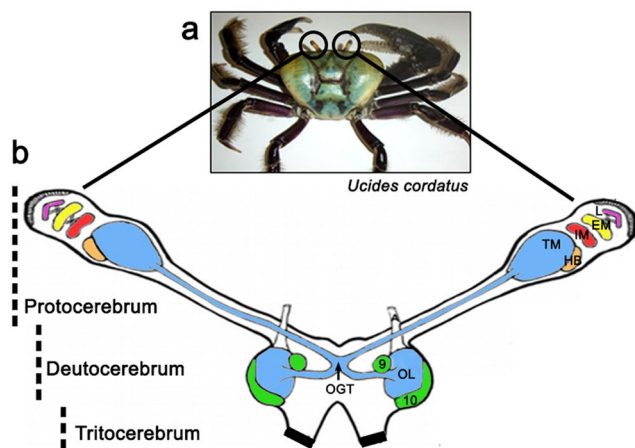


Fig. 1 Neuroanatomical organization of the brain of *Ucides cordatus*. **a** Crab *U. cordatus*. **b** Schematic diagram of the crab brain. The protocerebrum consists of the retina (outermost structure, gray), lamina (L, purple), external medulla (EM, yellow), internal medulla (IM, red), hemiellipsoid body (HB, orange) and terminal medulla (TM, blue). The deutocerebrum has a pair of olfactory lobes (OL, blue) that contain the synaptic contacts of the projection neurons from cluster 10 (CI 10, green), interneurons from cluster 9 (CI 9, green) and projections of olfactory receptor neurons from the antennule. The olfactory globular tract (OGT) connects the OL to the HB in the protocerebrum

Materials and methods

Animals

Fifty-three young adult males of *U. cordatus* (5.9 to 8.3 cm carapace width), in the intermolt phase, were collected from mangroves in Rio de Janeiro, Brazil (22° 54' 10" S, 043° 12' 27" W) and acclimated for 7 days in aquaria. The animals were maintained under controlled conditions (water salinity 20 ppm, 25 °C and 12L:12D) and fed three times a week with leaves of the mangrove *Avicennia schaueriana*.

All procedures adopted in this study, including the location where the animals were captured, were performed under a permit from the Brazilian National Environmental Committee (Certificate # 14689-1/IBAMA/2008, permission to use the animals # 2440408).

Mallory trichrome stain

Seven crabs were cryo-anesthetized for 20 min and the brains were dissected and fixed in 4% paraformaldehyde (PF) for 6 h at room temperature. The samples were stored in 0.1 M phosphate buffer saline (PBS) (made in crustacean saline: 12 g/L NaCl, 0.4 g/L KCl, 0.25 g/L MgCl₂, 1.5 g/L CaCl₂, 0.2 g/L NaHCl₃), pH 7.4. The samples were embedded in paraffin and 10- μ m sections were cut using a microtome (American Optical), collected on slides with poly-L-lysine and hydrated in decreasing ethanol concentrations. The sections were washed in distilled water and stained with hematoxylin in an aluminum mordant, followed by eosin. Next, they were immersed in Ehrlich's violet crystal stain and finally in Weigert's iodine solution. Then, they were dehydrated in increasing ethanol concentrations and cleared in xylene. The sections were mounted on slides with Entellan® under coverslips and examined under a Zeiss Axioskop 2 Plus light microscope equipped with a color CCD camera (Media Cybernetics, model Evolution™ MP).

BrdU injection

A solution of 10 mg/mL of BrdU (5-bromo-2'-deoxyuridine) was injected into the circulatory system of four crabs once a day, in the late morning, for three consecutive days (Sullivan and Beltz 2005). A volume of solution equivalent to 20 μ L of BrdU per gram of body weight was injected with a syringe into the base of the last pair of pereopods of each crab. Twenty-four hours after the last injection, the brains were dissected and processed as described in the previous item. The samples were stored in 0.1 M PBS and processed using standard immunohistochemical methods.

Immunohistochemistry

Cryostat sections (10 μ m thick) of the brains ($n=4$ for each marker) were placed on slides and washed with 0.1 M PBS, pH 7.4, three times before the heat-shock antigen retrieval in 0.1 M citrate buffer solution, pH 6. The slides were then incubated with 10% goat serum (Sigma® G6767) for 1 h, followed by primary antibodies (monoclonal anti-BrdU Sigma B2531, 1:80; polyclonal anti-GFAP, Sigma G4546, 1:100; monoclonal anti-GS, Abcam® AB64613, 1:100; monoclonal anti-NeuN, Millipore® MAB377, 1:50; monoclonal anti-III beta-tubulin (Tuj1), Sigma T8660, 1:100; polyclonal anti-serotonin (5-hydroxytryptamine, 5HT), Sigma S5545, 1:100; polyclonal anti-phospho histone H3 (PH3), Santa Cruz® SC8656, 1:100; monoclonal anti-tubulin, tyrosinated, Sigma T6557, 1:100) overnight at 4 °C. Then, the slides were incubated with the secondary antibody anti-rabbit Alexa Fluor® 488 (Invitrogen™ MG120, 1:200

dilution) or Alexa Fluor® 546 (Invitrogen A11094, 1:200 dilution) for 2 h at room temperature. The solution used for washing and diluting the antibodies was 0.3% Triton X-100 in PBS. Finally, 4',6-diamidino-2-phenylindole (DAPI) was used to stain the cell nuclei. The slides were mounted with Fluoromount™ for image capture with a Zeiss Axioskop 2 Plus epifluorescence microscope coupled to a Media Cybernetics Evolution camera.

Whole-mount immunohistochemistry was used to identify the proliferation zone and neurogenic niche in the central olfactory system of the brain. Propidium iodide (PI; 10 mg/mL; Sigma) was used as a nuclear stain. Z-stack images were taken at 1- μ m intervals under a Leica TCS SP5 confocal microscope. All images were adjusted, with brightness and contrast applied uniformly in accordance with standards of the field.

Western blotting

In order to confirm the specificity of the antibodies, a pool of four brain samples were dissected and homogenized in a lysis solution (10 μ L protease and phosphatase inhibitor, 100 μ g PMSF and 1% Triton X-100 in PBS). The proteins were separated by 12.5% polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate (SDS-PAGE), using the Laemmli buffer system (Laemmli 1970). The gel was equilibrated for 1 h in blot buffer (25 mM Trizma base, 193 mM glycine, 20% methanol) and transferred to nitrocellulose paper in a wet system for 1.5 h. The nitrocellulose membrane was blocked with PBS-10% albumin for 1 h and then incubated with the following primary antibodies: polyclonal anti-GFAP, Sigma G4546, 1:1000; monoclonal anti-GS, Abcam® AB64613, 1:1000; and monoclonal anti-Tuj1, Sigma T8660, 1:1000. Polyclonal anti-actin, Sigma A2066, 1:5000 dilution, was used as a loading control. Secondary HRP-conjugated antibodies, obtained from Jackson ImmunoResearch, were detected by chemiluminescence (PerkinElmer). The signal was processed with ImageQuant (GE Healthcare Life Sciences).

Semithin sections

Brain samples from two crabs were fixed with 2.5% glutaraldehyde + 0.1 M PBS for 1 h and then post-fixed with 1% osmium tetroxide + 0.8% potassium ferrocyanide + 5 mM calcium chloride in 0.1% cacodylate buffer (pH 7.4) for 1 h. The samples were dehydrated with increasing concentrations of acetone, prepared for gradual infiltration in Epon™ resin overnight and then placed in an oven for 48 h at 60 °C for polymerization. Semithin sections were cut in the ultramicrotome and observed under the Zeiss Axioskop 2 Plus light microscope equipped with a color CCD camera (Media Cybernetics, model Evolution MP).

Results

General view of the central olfactory system

The main olfactory structures in the deutocerebrum of *U. cordatus* are the OL and CI 9/CI 10. The OGT connects the deutocerebrum to the protocerebrum (Fig. 2a). Figure 2(b, c) displays the glomerular neuropil of the left and right OL, respectively, receiving afferents of ORNs. CI 9 and CI 10 lie on the ventral surface of the brain. CI 10 mostly consists of projection neurons, which connect their axons with olfactory receptor neurons (ORN) in the antenna. During each molt, the crabs grow, increasing the number of ORN and also the number of projection neurons, in order to “accommodate” the newly generated ORN (Sandeman et al. 1998).

Characterization of neurons and glial cells in the central olfactory system

We used several markers to characterize the neurons and glial cells in the central olfactory system. Immunolabeling for NeuN, a marker used to identify mature neurons, was found in cell bodies of projection neurons in CI 10 (Fig. 3a–c). Among the CI 10 neurons were cytoplasmic profiles and cell processes labeled with tyrosinated tubulin, showing the cluster cytoarchitecture (Fig. 3d–f). These profiles and processes were seen in more detail in semithin sections of CI 10 (Fig. 3g, h). Among these neurons, we found round or elongated smaller cells, showing glial morphology as described by Da Silva et al. (2000) and Allodi et al. (1999), as well as axon fibers projecting from the neuron cell bodies in CI 10, composing the OGT.

Immunohistochemical assays for GS (Fig. 4a–d) and GFAP (Fig. 4e, f) revealed glial cells in the central olfactory system: GS+ cells appeared mostly in the OL and GFAP+ cells mostly in CI 10. We also observed GFAP+ cells within CI 9 (Supplementary Fig. 1). No GFAP+ cells were seen in the glomeruli. Western blotting for GFAP and GS was performed to test the specificity of the antibodies (Fig. 4h).

Serotonin labeling in the central olfactory system

Immunohistochemical reactions showed diffuse 5HT labeling in the deutocerebrum, including the dorsal-giant neuron (DGN) cell bodies, close to CI 9 and their projections (Fig. 5a–d). The 5HT labeling also evidenced the glomeruli in the OL (Fig. 5b, e). Figure 5(e, f) shows 5HT+ cells in CI 10.

CI 10 contains both proliferating neural cells and immature neurons

Immunohistochemistry for BrdU, an S phase proliferation marker and for PH3, a mitosis marker, revealed labeled cells located deep inside CI 10, near the OL (Fig. 6), in the same region shown in Fig. 7. These results confirmed that this region is indeed a proliferation zone.

Nuclear staining revealed a group of elongated nuclei of cells within CI 10, concentrically arranged on the inner surface of a cavity (Fig. 7). The lining of this cavity was labeled with 5HT, as a “ring” in the center of the resident cells of CI 10 (Fig. 3f). Figure 7(d) shows a BrdU+ cell adjacent to the cavity and Fig. 7(a, d) shows GS+ processes among niche cells. These features were also described for a deutocerebral neurogenic niche in other crustaceans (Benton et al. 2011, 2014; Chaves da Silva et al. 2012; Beltz and Benton 2017; Wittfoth and Harzsch 2018).

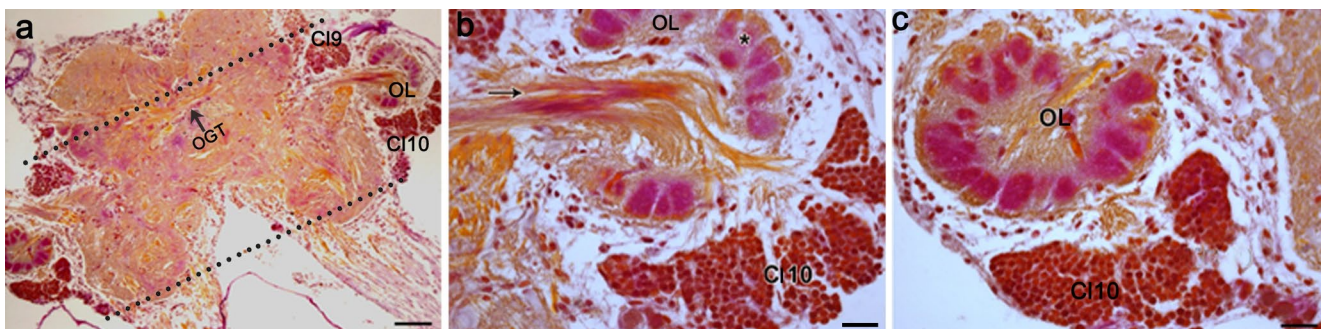


Fig. 2 Central olfactory system in the deutocerebrum: olfactory lobe (OL) and clusters 9 (CI 9) and 10 (CI 10). **a–c** Histological horizontal sections of the brain stained with Mallory. **a** *Ucides cordatus* brain (bilaterally symmetrical) showing the olfactory globular tract (OGT), OL and CI 9 and CI 10. The dotted lines mark the limits of the deutocerebrum. **b, c** Higher magnifications of (a) showing the

left (c) and right (b) OL and CI 10. **b** Axons (arrow) converging into column-shaped glomeruli (asterisk) of the OL. The OL measures approximately 180 μm in diameter. CI 9 has a spherical morphology measuring about 100 μm and CI 10 measures 240 μm \times 90 μm . Red, cell nuclei; orange, axons. Scale bars: **a** 100 μm ; **b, c** 40 μm

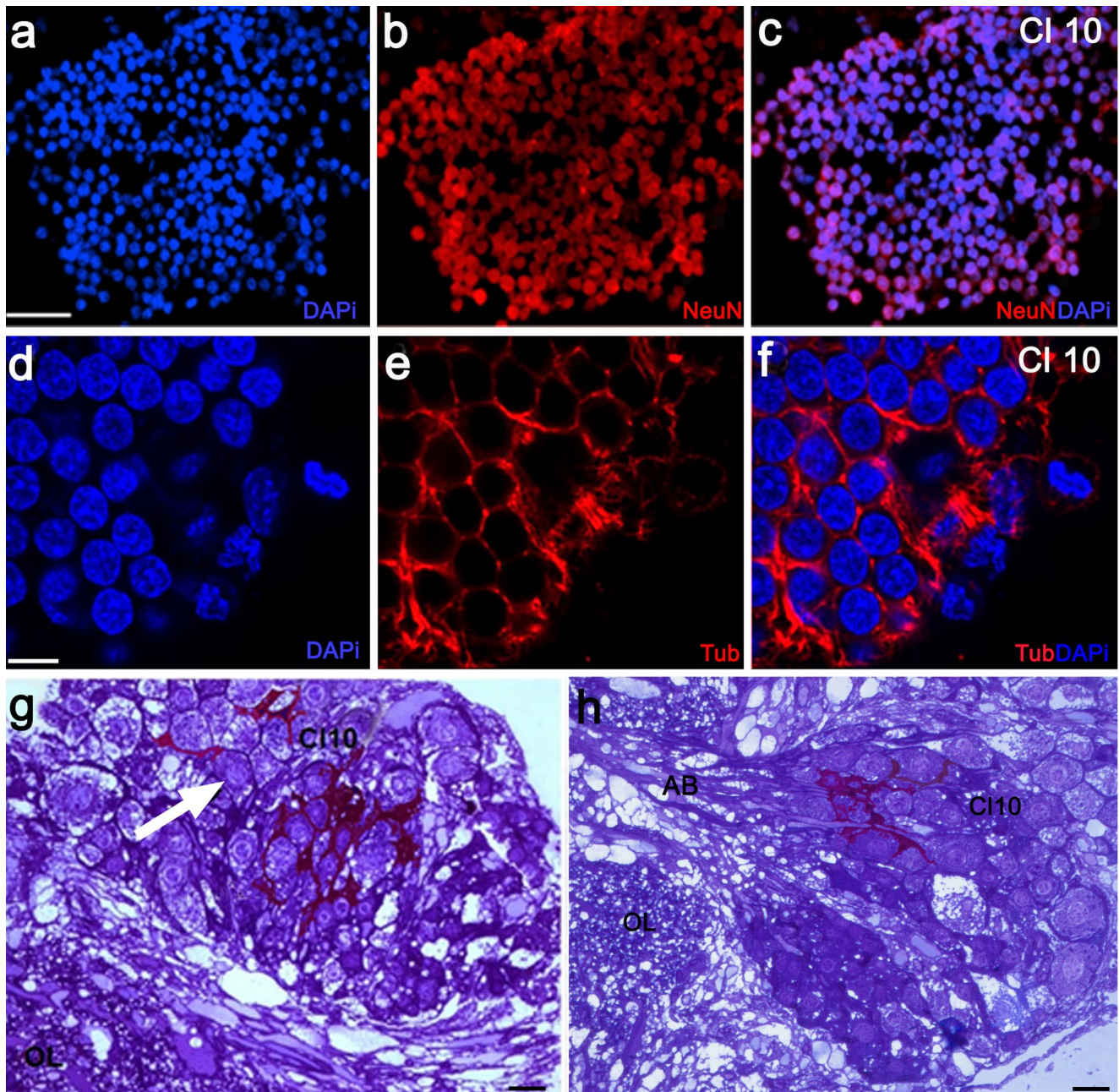


Fig. 3 Cellular components of cluster 10 in the deutocerebrum of the crab *Uca cordatus*. **a–c** NeuN immunofluorescence in red (**b**), DAPI (nuclear staining) in blue (**a**). **c** Merge of images (**a**) and (**b**). **d–f** Tyrosinated tubulin (Tub) immunofluorescence in red (**e**), DAPI in blue (**d**). **f** Merge of images (**d**) and (**e**). **g, h** Semithin horizontal sec-

tions stained with toluidine blue, showing neurons with round nuclei (white arrow) and glial cells (colored in red). **h** Axon bundles (AB) of CI 10 neurons projecting to the hemiellipsoid bodies (not shown). OL olfactory lobe, NeuN neuronal nuclei. Scale bars: **a–c** 80 μ m; **d–h** 10 μ m

Tuj1, used to label immature neurons, revealed cells within CI 10, especially near the outer edge (Fig. 8). Interestingly, we noted DAPI-stained mitotic profiles, as shown in Fig. 8(b). The insert for Fig. 8(b) shows two Tuj1+ close cells at higher magnification, possibly post-mitotic. In Fig. 8(c), Tuj1 specificity is confirmed by Western blotting.

Discussion

This study revealed a zone of proliferating neural cells within CI 10 in adults of the brachyuran semiterrestrial crab *U. cordatus*. Associated with this proliferation zone was a structure with features of a neurogenic niche, lined with a 5HT ring. Additionally, for the first time using two

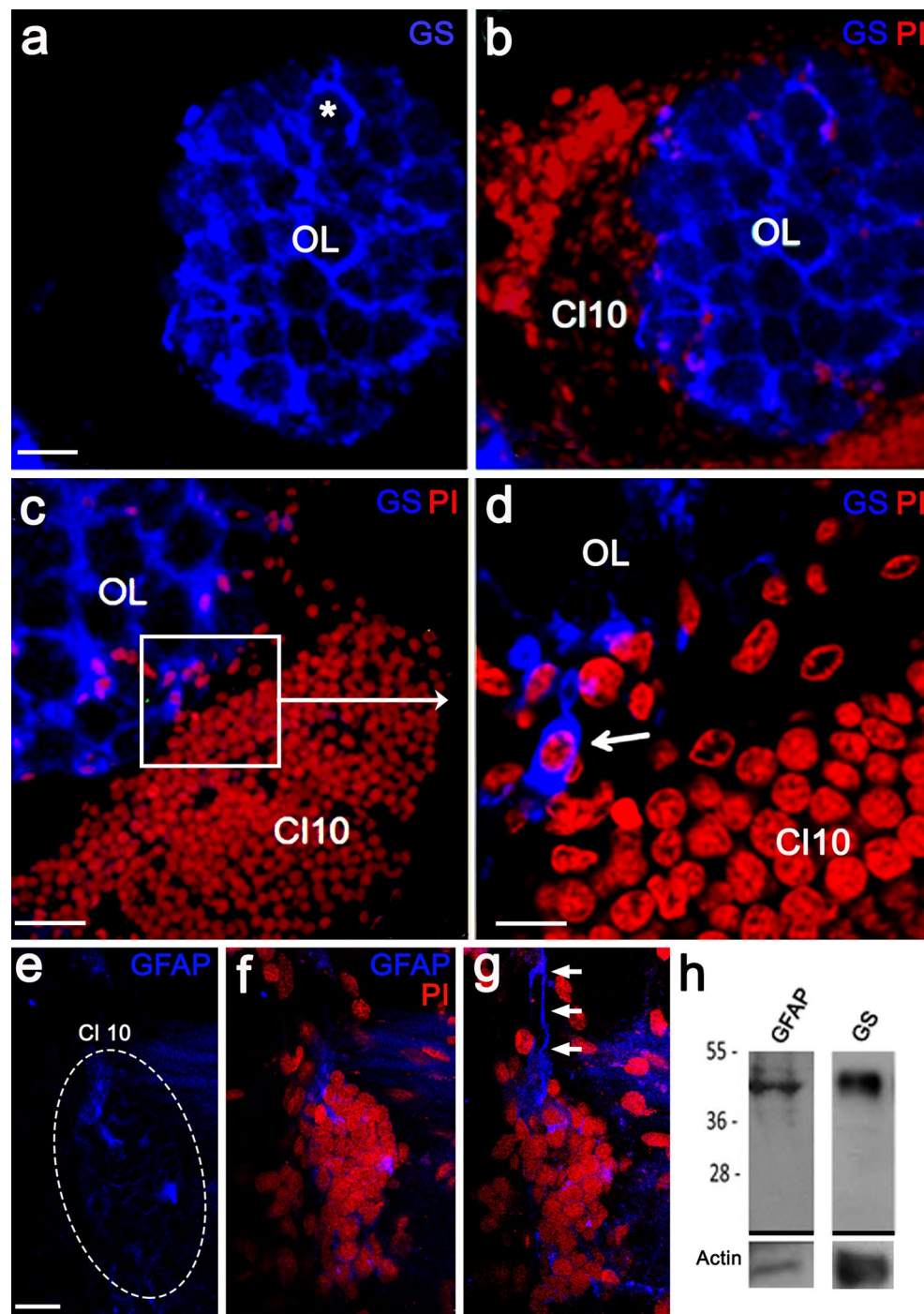


Fig. 4 Deutocerebrum of *Ucides cordatus*, containing two types of glial cells. **a–d** Immunofluorescence for anti-glutamine synthetase (GS, blue) and PI (nuclear staining, red) in the glomerular neuropil (**a** and **b**, asterisk) of the olfactory lobe (OL). **c** GS+ cells in the OL. **d** Higher magnification of the region indicated in the white square in (**c**). Arrow indicates a GS+ cell. **e, f** Immunofluorescence

for GFAP (blue) and PI (nuclear staining, red) of cluster 10 (Cl 10). **g** A different plane of section. Arrows indicate a GFAP+ process. **h** Western blotting for GFAP and GS. The reactions were conducted in independent assays. Actin was used as loading control. PI propidium iodide, GFAP glial fibrillary acidic protein. Scale bars: **a–c** 50 μ m; **d** 10 μ m; **e–g** 20 μ m

glial markers, GFAP and GS, we detected two different types of astrocyte-like cells in different regions of the deutocerebrum (Fig. 9).

Adult neurogenesis has been well documented in the brains of many vertebrates, including mammals, fish, amphibians, reptiles and birds (Cayre et al. 2002; Lindsey

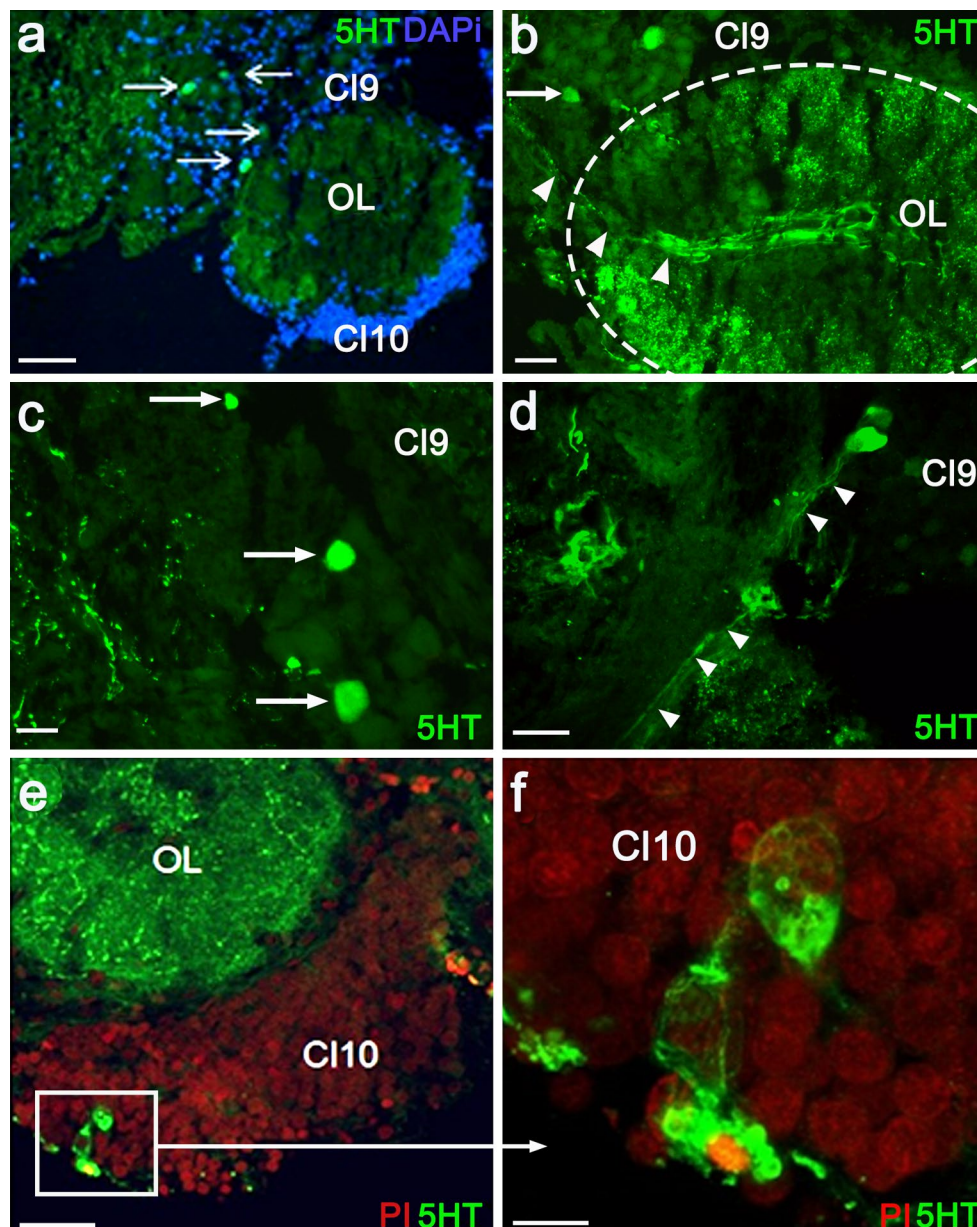


Fig. 5 5HT labeling in the deutocerebrum of *Ucides cordatus* (horizontal sections). **a** Immunofluorescence with anti-serotonin (5HT, green) in the deutocerebrum, showing the OL (**b**) and CI 9 (**c**, **d**); DAPI (nuclear staining, blue). Arrows indicate the serotonergic giant dorsal neurons (DGN). **c** Higher magnification of serotonergic DGNs. **b** 5HT-immunolabeled glomeruli in the OL (dashed circle). An axon bundle (5HT-labeled, arrowhead) can be seen. **d** A DGN

cell body and its axon labeled with 5HT. **e**, **f** 5HT+ cells (green) within cluster 10 (CI 10), with cell nuclei labeled with propidium iodide (PI, red). **f** Higher magnification of the region indicated in the white square in (**e**), showing two 5HT+ cells. OL olfactory lobe, CI 9 cluster 9, 5HT 5-hydroxytryptamine. Scale bars: **a** 80 μm ; **b–d** 20 μm ; **e** 50 μm ; **f** 15 μm

and Tropepe 2006; Barker et al. 2011; Kempermann 2012, 2016; Augusto-Oliveira et al. 2019) but it is rarely explored in the brains of invertebrates. A few decapod crustaceans have been studied; crayfish (Sandeman et al. 1998; Schmidt and Harzsch 1999; Sullivan and Beltz 2005; Sullivan et al. 2007), lobsters (Harzsch et al. 1999; Schmidt 2001) and crabs (Schmidt 1997; Hansen and Schmidt 2001) and it has been reported that the location of adult neurogenesis

varies according to the decapod group (Schmidt and Harzsch 1999). Similar to the true crab *Carcinus maenas* (Schmidt 1997; Hansen and Schmidt 2001), adult *U. cordatus* showed a proliferation zone within CI 10, as revealed by the proliferation markers BrdU and PH3, with no evidence of a migratory stream. Virtually all CI 10 cells were labeled with NeuN, a neuron marker previously used in *U. cordatus* (Wajsenzon et al. 2016) and with Tuj1, a marker for

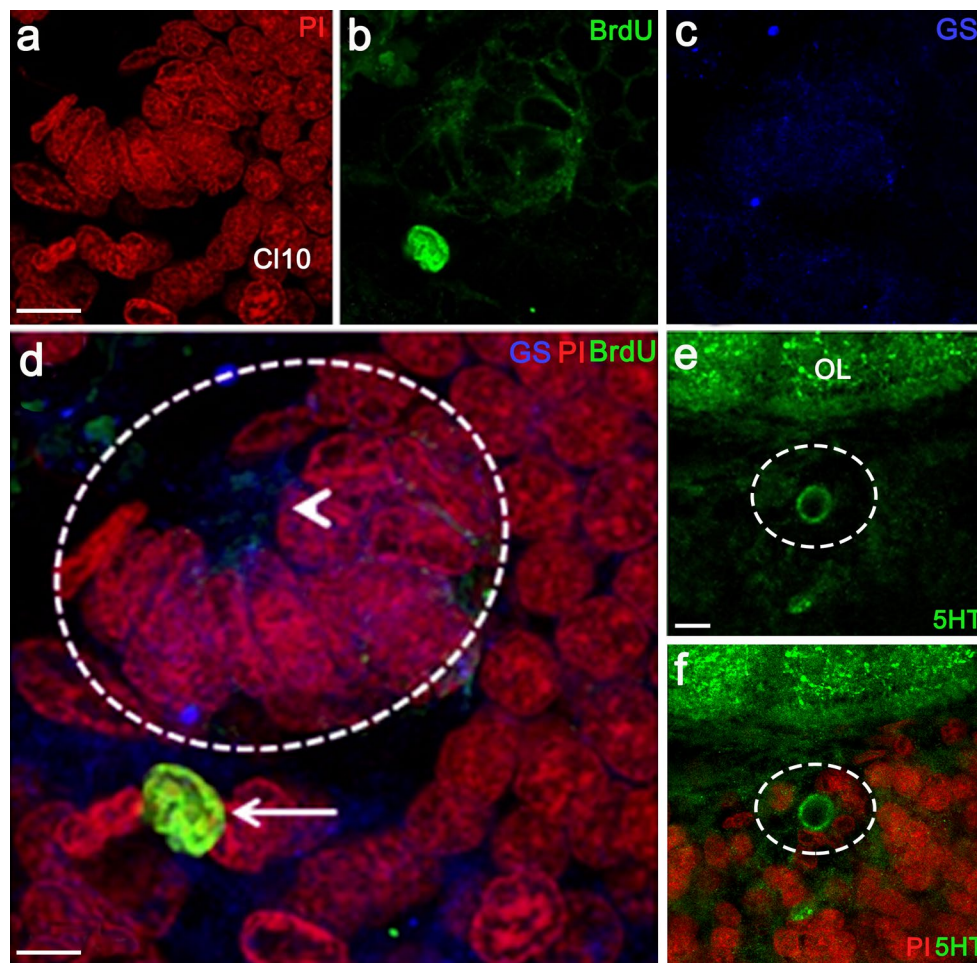


Fig. 6 Immunofluorescence of the proliferation region located deep within cluster 10. **a–c** BrdU+ cells in green and cell nuclei labeled with propidium iodide (PI) in red. **b, c** Higher magnification of the region indicated in the white square in **(a)**. **d–g** PH3+ cells in green,

and cell nuclei labeled with PI in red. **e–g** Higher magnification of **(d)**. **f, g** Merge of PH3 and PI. OL olfactory lobe, Cl 10 cluster 10, BrdU 5-bromo-2'-deoxyuridine, PH3 phospho-histone H3. Scale bars: **a** 80 μ m; **b, c** 50 μ m; **d** 100 μ m; **e–g** 20 μ m

immature neurons also used in invertebrates (Kimble et al. 1990; Medina et al. 2015), suggesting that BrdU+ cells in Cl 10 were mostly differentiating into neurons. Although we have identified Tuj1+ cells, which suggests that neurogenesis has occurred, we did not observe the differentiation of BrdU+ cells into neurons. In addition, a morphologically distinct group of cells in Cl 10 surrounds what appears to be a cavity. This structure closely resembles the “deutocerebral organ” originally described by Bazin (1969) in the brains of several other species of decapod crustaceans and later interpreted as the neurogenic niche by Sullivan et al. (2007) in the crayfish *P. clarkii* and by Schmidt and Derby (2011) in the lobster *Panulirus argus*.

Differently from crayfish (Sullivan et al. 2007; Song et al. 2009) and lobsters (Schmidt and Derby 2011), the neurogenic niche of *U. cordatus*, described here for the first time, did not show a migratory stream. Even though we have seen immature neurons (TUJ1+) distant from the neurogenic

niche within cluster 10 in species of the infraorders Brachyura, Anomura, Astacidea, Palinura and Penaeidae that lack the AL, the neurogenesis in the deutocerebrum seems to be restricted to Cl 10 (Schmidt and Harzsch 1999). Also, although we found proliferative cells, we did not find BrdU+ cells resembling typical large neuroblasts in *U. cordatus*. Therefore, we cannot claim that the niche is self-renewing, as suggested by Song et al. (2009) in crayfish. This may indicate that neurogenesis in the central olfactory system of young adult *U. cordatus* occurs by features distinct from those in crayfish (Song et al. 2009).

In our study, the labeling with 5HT in the lining of the center of the cavity was arranged as the vascular cavity of the neurogenic niche, as shown by Benton et al. (2011) in the crayfish. Previous studies in other species of decapod crustaceans (Sullivan et al. 2007; Sandeman et al. 2011; Schmidt and Derby 2011; Beltz and Benton 2017; Wittfoth and Harzsch 2018; Brenneis and Beltz 2019) described

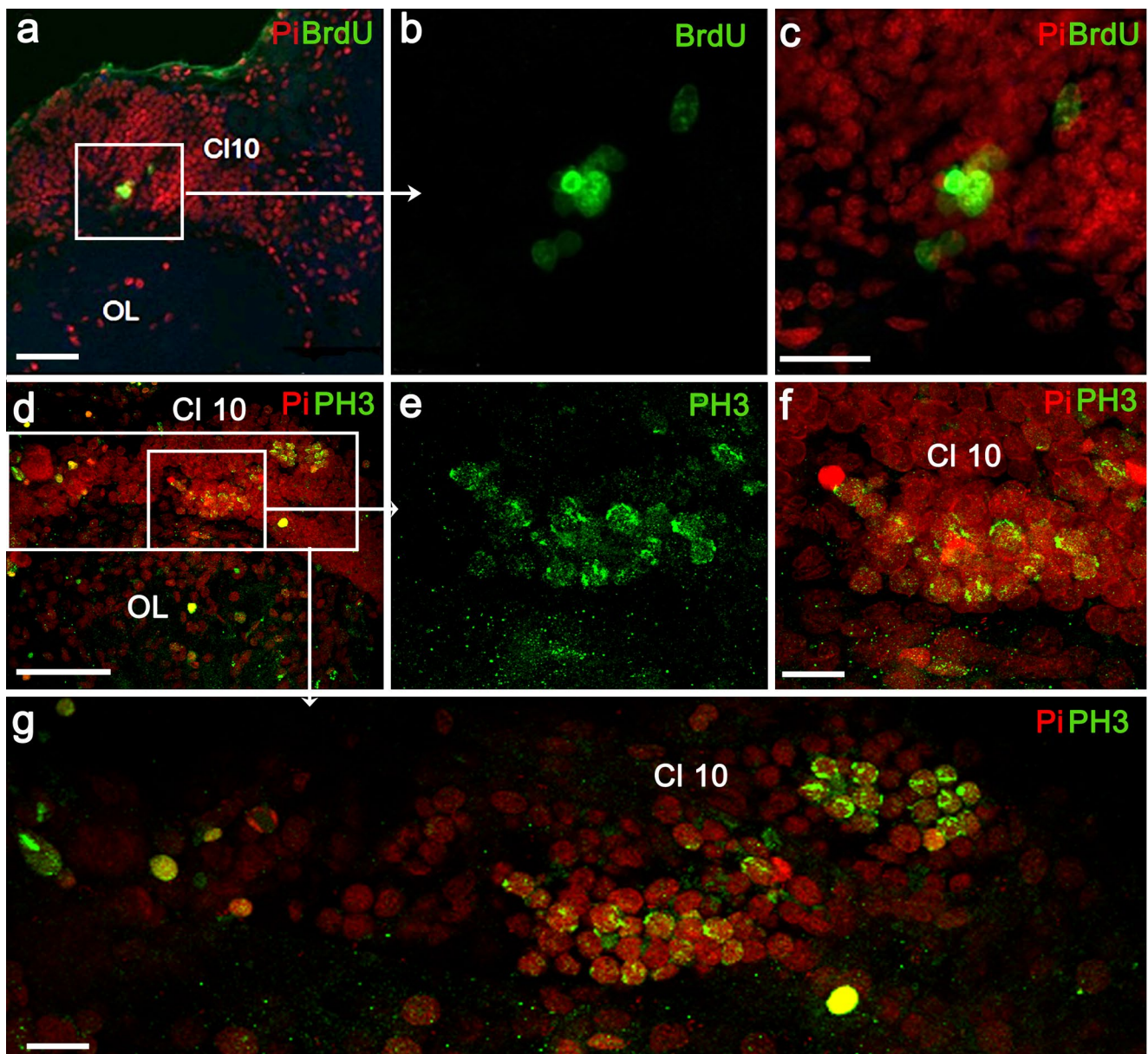


Fig. 7 Neurogenic niche within *Ucidus cordatus* cluster 10. **a–d** Group of elongated cells (PI, red) showing the characteristic morphology of a neurogenic niche of crustaceans. A BrdU+ cell (green) is shown close to the neurogenic niche. **a** PI channel. **b** BrdU channel. **c** GS channel. **d** Merge showing the neurogenic niche

(dashed circle) within cluster 10 (CI 10). Note the central cavity (arrowhead) and a BrdU+ cell (arrow). **e, f** The serotonin (5HT) ring surrounding the cavity of the neurogenic niche (dashed circle). OL olfactory lobe, BrdU 5-bromo-2'-deoxyuridine, PI propidium iodide, 5HT 5-hydroxytryptamine. Scale bars: **a–c** 10 μ m; **d–f** 5 μ m

the proliferation zone and the neurogenic niche with characteristics similar to those found in CI 10 of *U. cordatus*: (1) the niche contains a central cavity lined by a 5HT+ ring; (2) the niche cells surrounding the central cavity are morphologically different from those in the vicinity of the niche, being mostly elongated; and (3) the niche is close to the proliferation zone, where cells are labeled with BrdU. These similarities in both morphology and cell populations to maintain adult neurogenesis in *U. cordatus* and other

crustaceans suggest a common strategy for the generation of new neurons in adult brains.

The 5HT labeling used here identified the glomeruli synaptic zone in the OL and DGN, as also shown in the brain of lobsters and crayfish (Sandeman et al. 1995, 1988; Schmidt and Ache 1997). We found the DGN organized as 2 to 4 pairs of large cells bilaterally to CI 9, which send dense innervations to both lobes, as also reported by Schmidt and Ache (1997). Interestingly, the DGN is the major source of 5HT in the brains

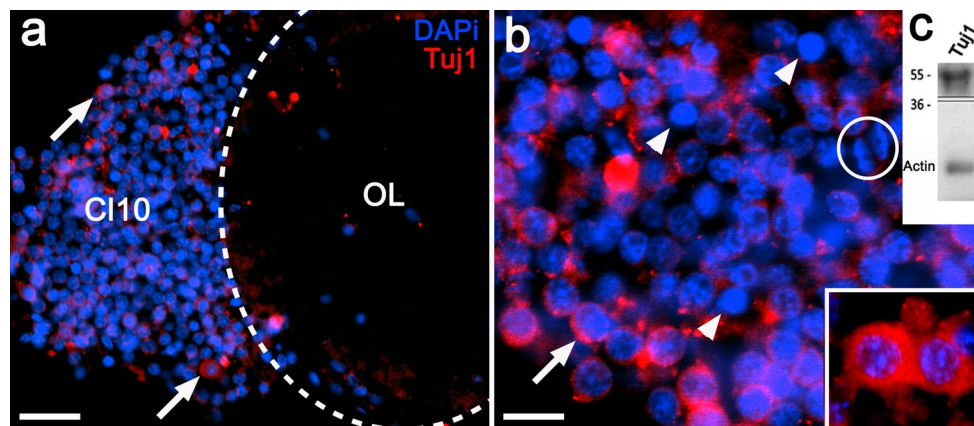


Fig. 8 Tuj1+ immature cells in cluster 10. **a** Tuj1+ cells (red) and DAPI (nuclear staining, blue) in cluster 10 (CI 10). Arrows indicate Tuj1+ cells. **b** High magnification of CI 10 showing a Tuj1+ cell (arrow). Insert shows two Tuj1+ adjacent cells in a higher magnification (possibly post-mitotic). Note the mitotic profile (circle) of CI 10

nuclei cells. Arrowheads show nuclei of cells not labeled with Tuj1. **c** Western blotting for Tuj1. Actin was used as loading control. OL olfactory lobe, Tuj1 III beta-tubulin. Scale bars: **a** 20 μm ; **b** 10 μm ; (insert) 5 μm

of crustaceans and may project fibers to line the central cavity. Remarkably, electrical stimulation of the DGN has shown that 5HT released from this neuron directly alters the rate of adult neurogenesis in the crayfish brain (Sandeman et al. 2009).

Glial cells are a component of the neurogenic niche and migratory route in crayfish (Sullivan and Beltz 2005; Sullivan et al. 2007; Sandeman et al. 2011; Beltz and Benton 2017). The majority of studies reporting glial cells related to crustacean neurogenesis in the central olfactory system have found glial cells labeled with GS (Sullivan et al. 2007; Sandeman et al. 2009; Schmidt and Derby 2011; Beltz and Benton 2017) and to our knowledge, no other glial marker has been used for this purpose. Additionally, little information is available on the classification of glial cells in general in decapod crustaceans (Harzsch et al. 1999; Allodi et al. 1999; Da Silva et al. 2001, 2003, 2004; Harzsch 2003).

The characteristics that define glial cell types in invertebrates are not yet well established (Hartline 2011; Ortega and Olivares-Bañuelos 2020). In crustaceans, these cells have mostly been classified based on the morphology, including cell size, functionality, or location (Da Silva and Allodi 2000; Radojic and Pentreath 1979; Hartline 2011; Zhang et al. 2016) and, as in other arthropods, on the glial cell nucleus periphery-clumped chromatin (Pentreath 1987; Allodi et al. 1999), rather than through the use of glial cell markers (Linsler et al. 1997; Allodi et al. 1999, 2006; da Silva et al. 2004). Here, we showed two different subtypes of astrocyte-like cells in the deutocerebrum of *U. cordatus*: one, GFAP+, located within CI 10 and another, GS+, distributed throughout the OL. We also showed GS+ cells in the border between CL 10 and the OL. Because in the semithin sections of the OL we see cells surrounding neuronal cell bodies and because we only see GFAP+ in CI 10 (but not in the niche), we can assume that glial cells within CI 10 are

GFAP+. GFAP labeling was also described previously in the visual system of the same crab (da Silva et al. 2004).

It has been reported that the OL and AL, in the deutocerebrum and the lateral protocerebrum are areas that underwent significant remodeling during the evolution of Malacostraca (Sandeman et al. 2014). The AL first appeared in early Reptantia and then lost its supposed role as a secondary olfactory center, in both *Brachyura* and *Anomura* (Krieger et al. 2015). The development and subsequent loss of the AL may have been linked to lifestyle and habitat (Sandeman and Scholtz 1995; Sandeman et al. 2014). Crayfishes subject to water currents (such as in streams and rivers) may have variable sizes of aesthetascs on the antennules (Mead 2009). We may suppose that this variation in the aesthetascs is reflected in the size variation of the OL and of the associated clusters, which may be related to the neural zones of proliferation. Further, terrestrialization had significant consequences for the physical process of odor capture (Waldrop et al. 2016). For example, terrestrial brachyurans differ from aquatic species in their antennule morphology, with fewer aesthetascs and reductions in the brain area dedicated to aesthetasc-mediated olfaction (Krieger et al. 2015). At this point, we may infer that highly adaptive species that live in variable places, such as the aquatic/terrestrial habitat of *U. cordatus*, rely on adult neurogenesis and therefore, the presence and the specific features of the neurogenic niche described here are important for the capacity to adapt to and exploit this environment.

To conclude, our results show that neurogenesis in the semiterrestrial crab *U. cordatus* shares features with both aquatic crayfish and semiterrestrial crabs. These morphological and cellular features must be explored to comprehend how they are related to crustacean habitat and how they may adapt to changing environments. These findings provide a comparative

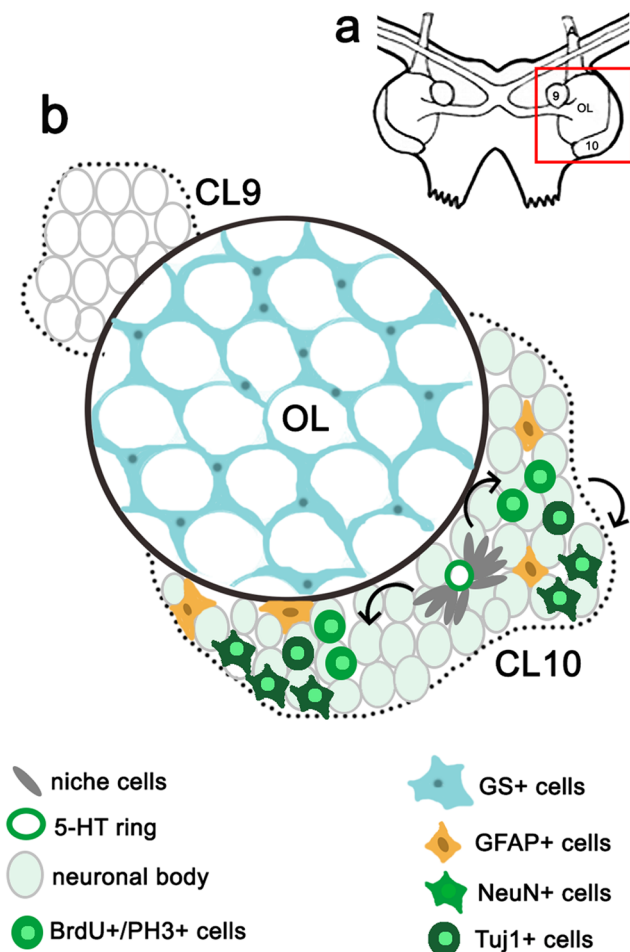


Fig. 9 Neurogenesis in the olfactory lobe of the crab *Ucidès cordatus*. **a** Scheme of the crab brain. The red square shows in **(b)** a higher magnification of the olfactory lobe (OL). The neurogenic niche is composed of niche cells (in gray) and the central cavity, outlined by a serotonin ring (5HT, green). Proliferating cells (BrdU+/PH3+) originating from the neurogenic niche reside in CL10 and differentiate into immature neurons (Tuj1+). The neurogenic niche is located within cluster 10 (CL10), which contains glial cells (GFAP+) and neurons (NeuN+). GS+ cells are located exclusively in the OL glomeruli. BrdU 5-bromo-2'-deoxyuridine, GFAP glial fibrillary acidic protein, GS glutamine synthetase, PH3 phospho-histone H3, NeuN neuronal nuclei, 5HT 5-hydroxytryptamine, Tuj1 III beta-tubulin

view of an understudied group of crustaceans and help to understand neurogenesis from an evolutionary perspective.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures adopted in this study, including the location where the animals were captured, were performed after approval by the National Environmental Committee (Certificate # 14689-1/IBAMA/2008, permission to use the animals # 2440408) and by the Ethics Commission on Research Animals of the Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro (protocol DHEICB 005). This article does not contain any studies with human participants performed by any of the authors.

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