RESEARCH ARTICLE

Enriched Environment Increases PCNA and PARP1 Levels in *Octopus vulgaris* Central Nervous System: First Evidence of Adult Neurogenesis in Lophotrochozoa

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ABSTRACT

Organisms showing a complex and centralized nervous system, such as teleosts, amphibians, reptiles, birds and mammals, and among invertebrates, crustaceans and insects, can adjust their behavior according to the environmental challenges. Proliferation, differentiation, migration, and axonal and dendritic development of newborn neurons take place in brain areas where structural plasticity, involved in learning, memory, and sensory stimuli integration, occurs. *Octopus vulgaris* has a complex and centralized nervous system, located between the eyes, with a hierarchical organization. It is considered the most "intelligent" invertebrate for its advanced cognitive capabilities, as learning and memory, and its sophisticated behaviors. The experimental data obtained by immunohistochemistry and western blot assay using proliferating cell nuclear antigen and poli (ADP-ribose) polymerase 1 as marker of cell proliferation and synaptogenesis, respectively, revealed cell proliferation in areas of brain involved in learning, memory, and sensory stimuli integration. Furthermore, we showed how enriched environmental conditions affect adult neurogenesis. *J. Exp. Zool. (Mol. Dev. Evol.) 00B:1–13, 2017.* © 2017 Wiley Periodicals, Inc.

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Animals' capabilities to adapt their behavior to infinite environmental situations imply a high degree of structural and functional brain plasticity, usually referred to adding newborn cells to the existent circuits, to reorganize brain connections (Lindsey and Tropepe, 2006). This fascinating process, in adult organisms, recapitulates the whole neural development from neural progenitor to fate determination passing through differentiation, migration, axonal, and dendritic development of newborn neurons, as well as synapses formation and functional integration into the existing neural circuitries (Duan et al., 2008; Sun et al., 2011). All these processes occur in animals with a complex nervous system that exhibit a range of sophisticated behaviors such as mammals (Kempermann et al., '97; Gage et al., '98; Gould et al., '99; Amrein et al., 2011; Lepousez et al.,

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2015), humans included (Eriksson et al., '98; Bergmann et al., 2015), nonmammals vertebrates (Alvarez-Buylla et al., '98; Marchioro et al., 2005; Zupanc et al., 2005; Kaslin et al., 2008; Simmons et al., 2008), and insects and crustaceans (Cayre et al., 2002; Dufour and Gadenne, 2006; Schmidt and Derby, 2011; Fernández-Hernández et al., 2013; Benton et al., 2014; Kim et al., 2014). Mammals and invertebrates show that their adult neurogenesis is not widespread throughout the brain, but it is restricted to specific neural areas, that is, the multimodal associative centers considered the anatomical and functional substrate of the higher cognitive capabilities (Kempermann, 2015).

All stages of adult neurogenesis are affected by a set of extrinsic and intrinsic factors, such as environmental enrichment, exercise, social interactions, stress, hormones, neurotransmitters, growth factors, genetic background, and age (Ming and Song, 2005; Fuchs and, 2014, lügge, 2014). The complex and sophisticated balance of these factors results in combined forces acting on brain plasticity. It allows the integration and the elaboration of stimuli with the consequent organisms' adaptation to a constant changing environment (Glasper et al., 2012; LaDage, 2015).

The effects of the environment on adult neurogenesis are emphasized in numerous studies showing its influence on hippocampus of the laboratory animals kept in specific housing conditions, such as environmental enrichment (Brown et al., 2003; Sale et al., 2009; Curlik et al., 2013; Opendak and Gould, 2015). One definition of enriched environment is "a designed world, aiming at providing a particular, mostly cognitive challenge" (Kempermann et al., 2010), it consists of adding live prey, sibling and new objects to standard housing conditions, in order to provide a set of sensory, intellectual, social, and physical stimulations (Sale et al., 2009; Kumazawa-Manita et al., 2013).

In mammals, experience-dependent plasticity increases in dentate gyrus due to exercise and learning stimuli (Kempermann et al., '97; Garthe et al., 2016); these affect proliferation, maintenance of nervous precursors cells, and promote survival of immature neurons in housed rats (Epp et al., 2009; Kempermann et al., 2010).

Also in insects and crustaceans adult, neurogenesis can be affected by environmental stimulation (Hansen and Schmidt, 2001; Scotto-Lomassese et al., 2002; Ben Rokia-Mille et al., 2008; Ghosal et al., 2009; Ayub et al., 2011). In crickets, adult neurogenesis is regulated by sensory inputs, responsible for cell cycle acceleration, and by hormonal levels, affecting the recruitment of progenitors cells (Cayre et al., 2005a, 2005b). The enriched sensory and social conditions, characterized by the simulation of the wild life, enhance the number of mushroom bodies neuroblasts in housed crickets (Scotto-Lomassese et al, 2000), while the ablation of them implies a damage of learning capabilities (Scotto-Lomassese et al., 2003). Also among the crayfishes, the proliferative potential of the neuronal stem cells can be increased enhancing the quality of the environment of housed animals (Sandeman and Sandeman, 2000; Ayub et al., 2011). Sandeman and Sandeman (2000) showed that animals kept with their sibling in large tanks have a higher number of neurons with respect to animals housed alone in small tanks, furthermore cell body size and their survival result increased.

To date, beside several studies on neurogenesis in the developing cephalopod brain (Marquis, '89; Wollesen et al., 2012), there is a lack of knowledge about adult neurogenesis and factors affecting it in all Lophotrochozoa (Lindsey and Tropepe, 2006); in fact, neurogenesis studies are limited at posthatching stages also in gastropods (Zakharov et al., '98). The unique data available on adult mollusks concern the regenerative capabilities of the protocerebrum in gastropods after a surgical injury (Matsuo et al., 2010; Matsuo and Ito, 2011). Among mollusks, the cephalopods seem to be the most likely candidates for the neurogenic process due to their brain complexity, high cognitive capabilities, and sophisticated behavior.

Cephalopods (nautilus, squid, cuttlefish, and octopus) are a rather small but most intriguing class among mollusks, from which they diverge for their peculiar morphological characteristics, as well as the complex and centralized nervous system, the foot transformed into arms and funnel (Yochelson et al., '73; Salvini-Plawen, '80; Holland, '87; Clarkson, '98; Lee et al., 2003; Shigeno et al. 2008), the mantle into a powerful locomotive organ, and for the direct embryonic development. After hatching, cephalopods, especially octopuses, grow extraordinarily quickly; they have a very short life cycle and mature in about 1 year and rarely live more than 2 years (Packard, '72; Budelmann et al., '97; Nixon & Young 2003, Di Cosmo and Polese, 2014).

Despite their short life, cephalopods are considered "advanced invertebrates" for many reasons, first the size of their brain representing a conspicuous fraction of their body mass (Packard, '72). Starting from the basal molluscan plan of tetraneury (Moroz, 2009; Wanninger, 2009) cephalopods have evolved a complex nervous system (Nixon and Young, 2003), placed in a cartilaginous "cranium" between the eyes. Neurons are packed in fused ganglia located around the esophagus, forming a supraesophageal and subesophageal masses connected to two optic lobes (Young, '71).

The nervous system has a key role in decoding the signals from the sensory organs and selecting an appropriate reaction to the countless environmental stimuli (Godfrey-Smith, 2013), and in cephalopods it provides the unusual capabilities to go over the stereotyped behavioral patterned tuning their responses to different stimuli in real time (Norman et al., 2001; Godfrey-Smith, 2013).

Their exploratory drive is recognized as a sign of their intelligence (Mather, 2008). Arguments in favor of the existence of intelligence are supported by their predation strategies, communication capabilities, and tool use. These abilities are essential in problem solving and exploring environment as performed during foraging activity (Hanlon and Messenger, '96). Moreover, octopuses possess arms capable of a wide range of movements

in the absence of any skeletal support allowing them to face and find solutions to different environmental challenges whenever they happen (Mather, '98; Kuba et al., 2003; Gutnick et al., 2011; Hochner, 2013).

The best way to detect the environment is through sensory organs, and cephalopods have sophisticated ones and also developed eyes and complex visual behavior (Hanlon and Messenger, '96; Yoshida et al., 2015), vestibular system, "lateral line analogue", primitive "hearing" system, (Budelmann and Williamson, '94; Williamson and Chrachri, 2007), chemoreceptors located in epidermis (Budelmann, '96), suckers and mouth (Wells et al., '65; Wells, '78; Boyle, '83; Anraku et al., 2005), and olfactory organ, a small pit of ciliated cells located on either side of the head, below the eyes, close to the mantle edge, recently described in *Octopus vulgaris* by our group (Polese et al., 2015, 2016).

The evolution provides octopus a "tool-kit" for the control of behavior, consisting of stimuli perception, some forms of memory and learning, problem-solving, and a kind of consciousness through a multimodal integration of sensory information with a cross-modal modulation at the nervous level (reviewed in Di Cosmo and Polese, 2014).

Since octopus has a "well-equipped" brain, sophisticated sense organs, and unusual skills, it is conceivable to hypothesize a high degree of brain plasticity (Hochner, 2010; De Lisa et al., 2012a, 2012b; Richter et al., 2015; Shomrat et al., 2015). Given that, we considered *O. vulgaris* a good model to study adult neurogenesis.

In our study, we show, for first time among cephalopods, proliferating cells in the nervous system of adult *O. vulgaris*, and how enriched environmental conditions affect adult neurogenesis. We chose two different markers: proliferating cell nuclear antigen (PCNA), to mark cell proliferation (Derenzini et al., '90, '95; Öfner et al., '92), and a cytoplasmic isoform of poli (ADP-ribose) polymerase 1 (PARP1), previously localized in the frontal–vertical system and in optic lobes (De Lisa et al., 2012b) to mark synaptogenesis. In our experiments, we compared PCNA and PARP1 levels in octopuses housed in enriched environment and octopuses in standard conditions in order to investigate if enhanced housing condition can increase adult neurogenesis. Moreover, since an enriched environment improves animal healthiness (Anderson and Wood, 2001, Anderson, 2003), our study, according to the current law (Directive 2010/63/EU), aims to provide new methods and tools for improvements of welfare of cephalopods used as laboratory animal models (Fiorito et al., 2014; Polese et al., 2014).

METHODS

Animals Collection

Specimens of *O. vulgaris* (male and female, weight ±800 g), collected in Bay of Naples, were transferred to the Department of Biology, as reported in Di Cosmo et al. (2015).

Brain Dissection

Octopuses were anesthetized by isoflurane insufflation (Polese et al., 2014) and brains were dissected in sterile conditions. Our research is conformed to European Directive 2010/63 EU L276, the Italian DL. 4 /03/ 2014, no. 26 and the ethical principles of Reduction, Refinement and Replacement (protocol no. 0124283-08/11/2012).

PCNA Immunohistochemistry

Brains from wild animals were dissected in sterile conditions and fixed in Bouin for 24 hr at room temperature, dehydrated in ethanol, and then cleared in Bioclear and embedded in paraffin.

Sections (7 μm) were cut at microtome and mounted on albumin-coated slides, then cleared, rehydrated, and, after several rinses, incubated with 1% normal goat serum for 20 min. Sections were incubated with anti-PCNA (P8825, 1:10,000; Sigma–Aldrich, St. Louis, MO, USA) at 4°C overnight in humid chamber, and, after several washes, rinsed in goat anti-mouse secondary antibody biotin conjugated for 1 hr at room temperature. After many rinses, sections were incubated with streptavidin conjugated to horseradish peroxidase for 1 hr; 3% 3,3′-diaminobenzidine tetrahydrochloride with 0.03% hydrogen peroxide in Tris buffer (0.05 M, pH 7.6) was used as chromogen. Slices were dehydrated and mounted in Permount.

Enriched Environment versus Standard Environment

Specimens of *O. vulgaris* (female, $n = 6$, ±800 g), collected in a period of 6 months, were used. They were divided into two experimental groups: *challenged* and *control*. *Challenged* and *control* animals were maintained in aquarium tanks at least for 9 days, each octopus was confined to its own tank to prevent cannibalism and social interactions. They were housed in PVC tanks ($150 \times 50 \times 50$ cm^3), covered with a Plexiglas lid to avoid animals escape, equipped with a den, natural sand, and shells. Water and room temperature were maintained at 16°C, light/dark cycle was set to natural photoperiod. Water was filtered with protein skimmer and biological filters. First 5 days of captivity were considered as acclimatization period, during which several physiological and behavioral parameters were monitored to verify welfare and healthiness of the octopuses (Di Cosmo et al., 2015). During acclimatization phase, animals were fed by experimenter with their natural prey, crabs (*Carcinus mediterraneus*) or mussels (*Mytilus galloprovincialis*) once a day.

According to Kempermann et al., (2010), after the acclimatization period, we altered the standard housing condition adding three objects providing a cognitive challenge. For consecutive 3 days, once a day, they were introduced to three plastic jars closed with a screw lid having a live prey, and they were left in the tank up to the trial of next day. The objects were put into the tank in the opposite position to the animals. During experimental days, octopuses had not feeding opportunities except to open the jars to reach the prey. *Control* animals were not challenged, and they

were fed regularly without any task. At last, *challenged* and *control* octopuses were sacrificed as described in Polese et al. (2014) and their brains were dissected out and stocked at –80°C.

Western Blot Analysis

Total proteins were extracted from homogenate of *O. vulgaris* specific brain areas (vertical–frontal system and optic–olfactory lobes) using RIPA buffer (Sigma–Aldrich) and quantified by BIO-RAD assay, using a BSA standard, according to manufacturer's instructions. After 10% sodium dodecylsulphate–polyacrylamide gel electrophoresis, proteins were transferred on Whatman® nitrocellulose membrane (Sigma–Aldrich) and incubated for 30 min in a blocking solution (nonfat milk 5% in PBS). Membranes were incubated in anti-PCNA antibody solution (P8825, 1:1000; Sigma–Aldrich) in nonfat milk 5%, anti-PARP1 (H-250, 1:1000; Santa Cruz Biotechnology Inc.) in nonfat milk 5% at 4°C overnight. After several rinses with PBS-T (PBS with 0.05% of Tween 20), membranes were incubated with secondary antibodies (1:5000; Sigma–Aldrich) for 1 hr at room temperature. Immunopositive bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate in accordance with the manufacturer's instructions (Pierce Biotechnology Inc.) using a Chemidoc EQ System (Bio-Rad Lab). To normalize quantitative differences between *challenged* and *control* in PCNA and PARP1 levels, membrane was incubated with anti-α-tubulin (T5168, 1:1000; Sigma–Aldrich) and processed at the same conditions of membrane probed with anti-PCNA and anti-PARP1. All data were presented as mean ± SEM and analyzed using Microsoft Excel 2003. Densitometric analysis for western blots was performed using QuantityOne software. Statistical analysis was performed using a two-tailed *t*-test (Microsoft Excel 2003); *P*-value < 0.05 was considered statistically significant.

RESULTS

PCNA Immunohistochemistry

In *O. vulgaris* brain (Fig. 1), PCNA immunoreactivity (-ir) is mainly located in the cell nuclei of the specific lobes of the supraoesophageal mass (Fig. 1) including the optic tract lobes (Fig. 2E) and the optic lobes (Fig. 2F), and suboesophageal mass (Fig. 2A).

Among the lobes of the supraoesophageal mass, the vertical, subvertical, and frontal possess most immunoreactive cells (Fig. 2A–D). In the vertical lobe, the PCNA immunoreactivity is restricted to the nuclei of the amacrine interneurons. These immunoreactive cells are located in the cortical regions of the vertical lobules and also within the neuropil as both scattered and clustered in niches (Fig. 2B and C). In the subvertical and frontal lobes the immunoreactivity is detectable in the nuclei of the small neurons (Fig. 2C and D). Posterior buccal and dorsal basal lobes show scattered immunopositivity (Fig. 2C and D). Concerning the optic tracts, we found positive cells with PCNA-ir

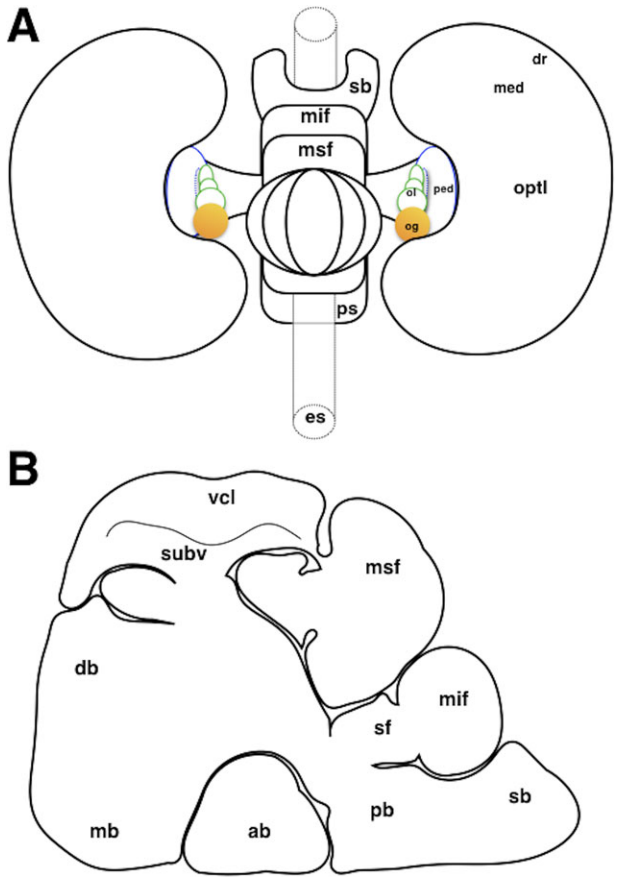
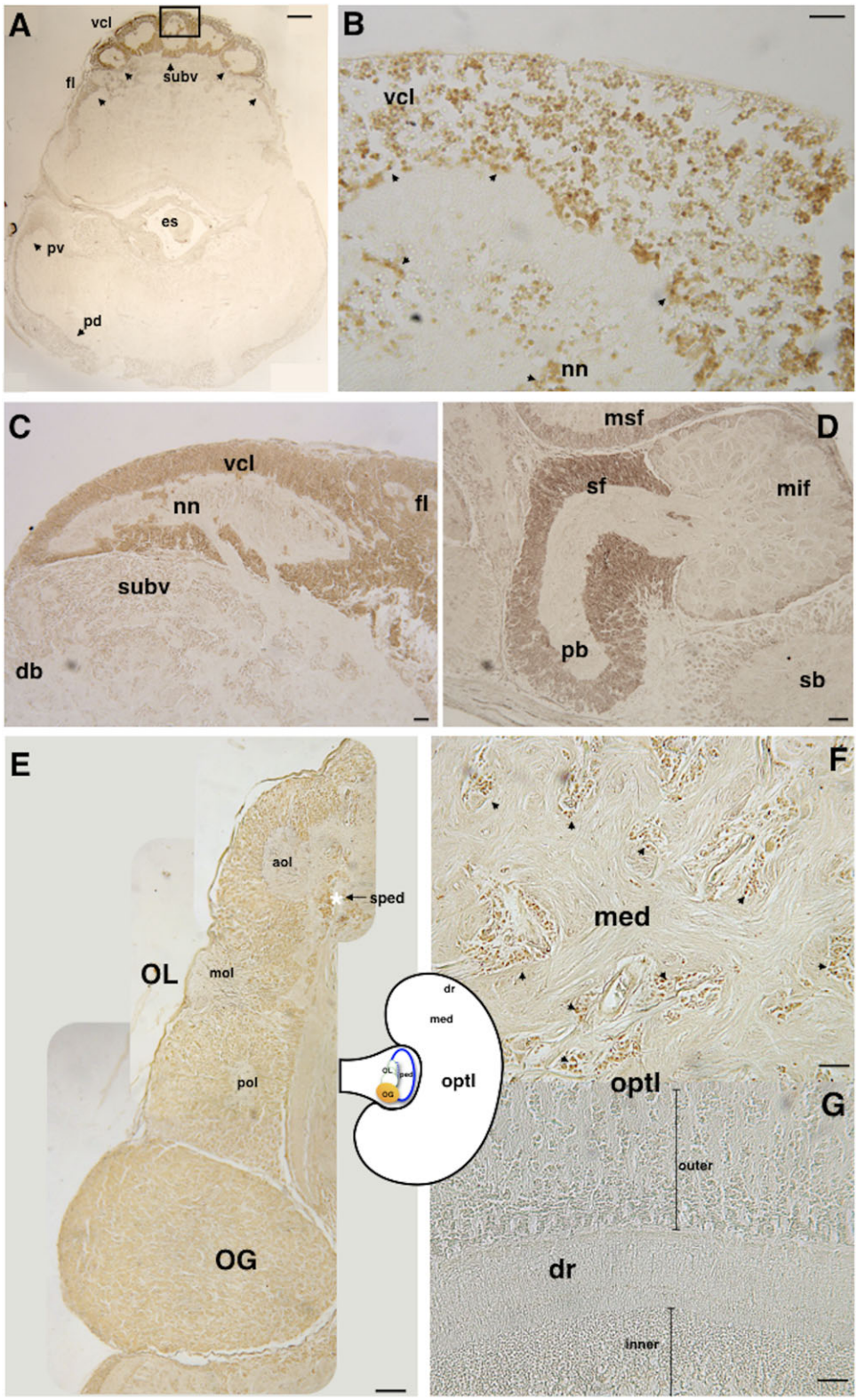


Figure 1. Adult *Octopus vulgaris* CNS diagram showing all lobes involved in cells proliferation. (**A**) Dorsal view, anterior face up, in which are visible two lateral optic lobes connected via optic tracts to the supraoesophageal mass; (**B**) sagittal section of supraoesophageal lobes. vcl:, vertical cortical layer; subv, subvertical; msf, median superior frontal; mif, median inferior frontal; sf, subfrontal; pb, posterior buccal; sb, superior buccal; db, dorsal basal; ol, olfactory lobe; og, optic gland; ped, peduncle lobe; optl, optic lobe; med, medulla; dr, deep retina; ps, posterior suboesophageal mass; es, esophagus. [Color figure can be viewed at wileyonlinelibrary.com]

nuclei distributed in all the lobules of the olfactory lobes, mainly in the anterior and the posterior lobules (Fig. 2E). In the peduncle lobes, positive nuclei were restricted to the spine neurons that are located on both side of the peduncle spine (Fig. 2E). The optic glands do not show any immunoreactivity (Fig. 2E). In the optic lobes, numerous immunopositive neurons are localized in the medulla islets (Fig. 2F), but no PCNA-ir has been observed in the outer and the inner layers cells of the deep retina (Fig. 2G). At least, scattered immunopositive neuron nuclei were detectable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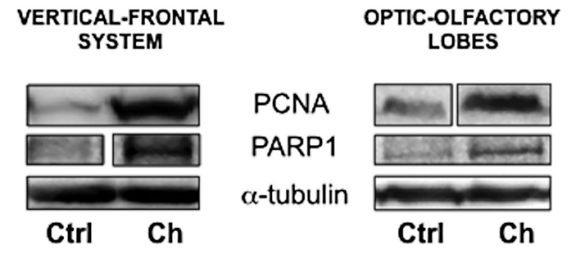

Figure 3. PCNA and PARP1 western blot analysis, and immunoreactive bands comparison of challenged versus control in, respectively, vertical–frontal system on the left and optic–olfactory lobes on the right. α-Tubulin was used to normalize quantitative differences between challenged and control in PCNA and PARP1 levels. ch, challenged; ctrl, control.

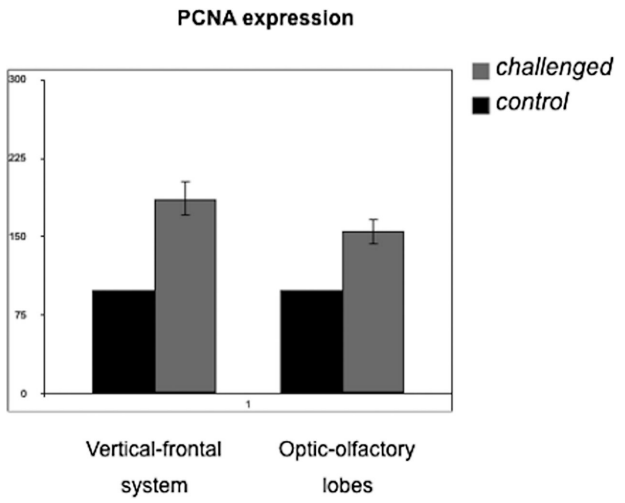

Figure 4. Densitometric analysis of the PCNA immunoreactive bands in the vertical–frontal system and optic–olfactory lobes in challenged and control.

in the palliovisceral and pedal lobes of the suboesophageal mass showing a few PCNA-ir interneuron nuclei (Fig. 2A).

PCNA and PARP1 Western Blot Analysis

Western blot analysis for PCNA and PARP1 were performed on the vertical–frontal system and the optic–olfactory lobes in *challenged* versus *control* animals (Fig. 3). These areas were chosen based on the detection of the main proliferative areas with PCNA-ir in the CNS of wild *O. vulgaris* (see above).

We found immunoreactive bands of 36 kDa corresponding to PCNA protein in total proteins extracts from both vertical–frontal system and optic–olfactory lobes. The densitometric analysis (QuantityOne Software) of the PCNA immunoreactive bands revealed a significant increase in both the vertical–frontal system (*Ch.* 155 $\pm$ 22.65; *Ctrl.* 100) and optic–olfactory lobes (*Ch.* 186.33 $\pm$ 28.92; *Ctrl.* 100) in *challenged* octopuses (both $P < 0.05$ vs. *control*) with band intensity, respectively, 1.5- and 1.8-fold higher with respect to *control* animals (Fig. 4).

Anti-PARP1 shows immunoreactive bands of 193 kDa in total proteins extracts from the same areas of *challenged* and *control* animals. The densitometric analysis of the vertical–frontal system increased not significantly in *challenged* octopuses (*Ch.* 201.33 $\pm$ 98.10; *Ctrl.* 100; $P > 0.05$ vs. *control*), while in the optic–olfactory lobes PARP1 immunoreactive bands intensity significantly increased by 1.6-fold with respect to the *control* (*Ch.* 161.33 $\pm$ 20.50; *Ctrl.* 100; $P < 0.05$) (Fig. 5).

DISCUSSION

In the present study, we show, for the first time, the occurrence of proliferative activity in adults *O. vulgaris* brain and the influence of the environment on it. Using immunohistochemistry against the PCNA, we demonstrated the presence and the distribution of

Figure 2. PCNA-ir in supra and suboesophageal masses, optic tract lobes and optic lobes in wild animal. (**A**) Transvers section of supra and suboesophageal masses, PCNA-ir is confined mainly to the supraoesophageal mass (arrows), and scattered immunopositive neurons nuclei are detectable in the suboesophageal mass (arrows) scale bar = 500 μm; (**B**) high magnification of vertical lobe lobule showing PCNA-ir restricted to the amacrine neurons nuclei (arrows) scale bar = 50 μm; (**C**) sagittal sections of the supraoesophageal mass showing PCNA-ir in the vertical, subvertical, basal and frontal lobes, scale bar = 100 μm; (**D**) sagittal sections of the supraoesophageal mass showing PCNA-ir in the posterior buccal, subfrontal and frontal lobes, scale bar = 100 μm; (**E**) reconstruction of horizontal section of the optic tract lobes where PCNA-ir is distributed in all the olfactory lobe lobules, mainly in the posterior and anterior lobules, and the neurons of the spine of the peduncle lobe (*), non PCNA-ir in the optic gland, scale bar = 100 μm; (**F** and **G**) horizontal section of the optic lobe, PCNA-ir is localized in the medulla's islets, while no PCNA-ir is detectable in the deep retina, scale bar = 100 μm. vcl, vertical cortical layer; fl, frontal lobes; subv, subvertical; pv, palliovisceral; pd, pedal lobe; nn, neuropile niches; msf, median superior frontal; mif, median inferior frontal; sf, subfrontal; pb, posterior buccal; sb, superior buccal; db, dorsal basal; OL, olfactory lobe; aol, anterior olfactory lobule; mol, median olfactory lobule; pol, posterior olfactory lobule; OG, optic gland; sped, spine of the peduncle lobe; optl, optic lobe; med, medulla; dr, deep retina; es, esophagus. [Color figure can be viewed at wileyonlinelibrary.com]

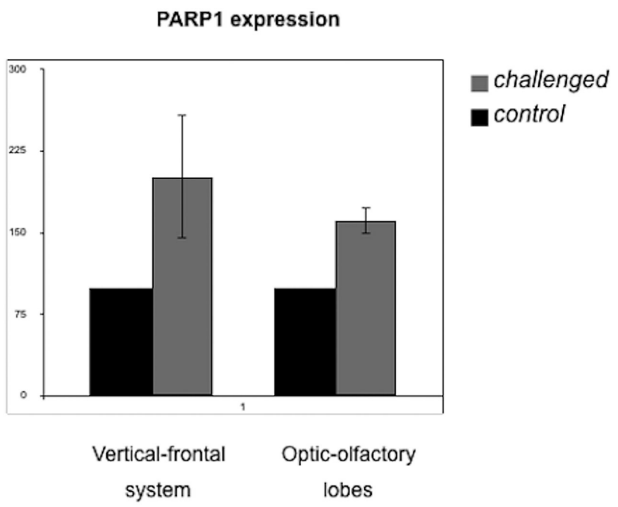

Figure 5. Densitometric analysis of the PARP1 immunoreactive bands in the vertical–frontal system and optic–olfactory lobes in challenged versus control.

proliferative neurons in specific areas of the central nervous system (CNS). To assess the effects of the environmental enrichment on the neurogenic process, we performed western blot assays on the brain areas that have previously shown a PCNA-ir, coming from animals kept in an enriched environment versus control animals kept in standard condition, evaluating not only the cell proliferation by measuring PCNA levels but also synaptogenesis using PARP1 known as an inducer of the cytoskeletal reorganization during the neuronal plasticity process (De Lisa et al., 2012a).

Brain Proliferating Areas

Learning Centers. CNS comprises a central part, encircling the oesophagus, and paired optic lobes laterally connected by a distinct optic tract. The central part is divided into suboesophageal and supraoesophageal masses, linked by the perioesophageal magnocellular lobes. *Octopus vulgaris* shows complex behaviors as a result of advanced cephalization associated with hierarchical functional organization of brain's lobes allowing the development of advanced cognitive capabilities (Young, '91).

The learning and memory abilities of *O. vulgaris* have been deeply investigated in several studies (Maldonado, '63; Wells et al., '65; Young, '65; Sanders, '70; Boal, '96; Boal et al., 2000; Kuba et al., 2006; Hochner, 2010; De Lisa et al., 2012b; Shomrat et al., 2015; Richter et al., 2016). The brain areas involved in such processes are the sites where environmental signals are integrated with other sensory inputs. Since the *O. vulgaris* brain areas associated with learning and memory show a convergence with mammalian and insect brain areas where adult neurogenesis occurs (Hochner, 2010), it was quite predictable to find proliferative activity in those areas also in *O. vulgaris*. In octopus CNS, the anatomical substrate of the higher cognitive capabilities are the lobes forming the vertical–frontal system in the supraoesophageal mass, sites of the most cell proliferation detected (Fig. 2A–D).

Vertical–frontal system. The octopus visual learning area comprises the vertical, the subvertical, and the superior frontal lobes, which are strictly interconnected. The major tactile memory area instead takes place in the inferior frontal lobe (Wells, '78). Both these areas show a strong PCNA immunoreactivity (Fig. 2A–D).

Octopus shows well-developed chemotactile memory systems, evolved in relation to its benthic life style (Hanlon and Messenger, '96) and visual memory systems (Wells and Young, '70; Gutnick et al., 2011; Di Cosmo and Polese, 2014; Polese et al., 2015). It uses the sensory organs to explore the environment and detect visual, tactile, and chemosensory information that are processed in the vertical–frontal system, in optic and olfactory lobes, in order to produce an adequate response (Hanlon and Messenger, '96; Di Cosmo and Polese, 2014; Richter et al., 2015). The cell active proliferation detected in these brain areas suggests that, in *O. vulgaris*, visual, tactile, and olfactory capabilities are supported by adult neurogenesis.

In insects and mammals, adding newborn neurons to the existent circuits is necessary to maintain the neural plasticity underling the higher cognitive capability (Lindsey and Tropepe, 2006), as well as in octopus. In insect brain, cell proliferation is detectable in the mushroom bodies (Cayre et al., 2002; Malaterre et al., 2002; Scotto-Lomassese et al., 2003; Cayre et al., 2007) involved in sensory discrimination, learning, memory, control of complex behavioral repertoires, and spatial orientation (Mizunami et al. '93; Strausfeld et al., '98). In mammals, the dentate gyrus of hippocampus is considered one of the canonical sites of adult neurogenesis (Kempermann and Gage, 2000; van Praag et al., 2002; Choi et al., 2016) involved in memory, spatial navigation, and learning (Leuner and Gould, 2010; Gradari et al., 2016). We found that the neurogenic sites of octopuses, insects, and mammals share anatomical and functional similarities.

In particular, the vertical lobe, a structure characterized by many layers of cells folded in a system of gyri (Young, '71; Shigeno and Ragsdale, 2015), aims to accommodate the large number of cells. The vertical lobe receives fibers from the median superior frontal cells, innervating the amacrine neurons orthogonally and forming a matrix-like structure, relevant for learning and memory processes (Wells, '65, '66; Hochner, 2010; Shigeno and Ragsdale, 2015; Shomrat et al., 2015).

The dense associative network and the laminar organization, in which cell bodies are tightly packed, are evident also in insect's mushroom bodies (Menzel and Giurfa, 2006; Hochner, 2010) and mammalian's hippocampus (Neves et al., 2012; Bartsch and Wulff, 2015).

In light of these considerations, adult neurogenesis seems to be transversely shared across very distant taxa, suggesting that this process is conserved as mechanism needed for learning and memory abilities, and also the overall maintenance of the CNS.

Multisensory Integration Centers. Visual and chemical information detected by the sensory organs are integrated in the optic and olfactory lobes. They seem to be involved, respectively, in coding the visual input and memory storage, and in integration of olfactory stimuli (Young '71; Hanlon and Messenger, '96; De Lisa et al, 2012a; Polese et al., 2015, 2016).

Optic and olfactory lobes. The optic lobes are considered as special development of the supraoesophageal mass, anatomically connected to it via the optic tracts (Young, '71). Characterized by a kidney shape, they show an external cortex called deep retina, consisting of inner and outer layers and a central area, the medulla, in which are distinguishable some spotted islands of cells (Young, '62). Recently, it hypothesized their functional association to the vertical–frontal system as an extra memory storage site (De Lisa et al., 2012a).

The olfactory lobe consists of three lobules, anterior, middle, and posterior, interconnected to each other (Young, '71). Its role is recently clarified by Polese et al. (2015, 2016). It receives fibers from the olfactory organ, through the olfactory nerve (Young, '71), and may act as a switch between growth and reproduction (Di Cosmo and Polese 2014; Polese et al., 2015, 2016).

Our data regarding PCNA show that the immunoreactivity is mainly detected in the medulla of the optic lobes and in all the lobules of olfactory lobe (Fig. 2E). It suggests that adult neurogenesis in octopus is linked to sensory stimulation.

Comparably, in insects, cell proliferation takes place in mushroom bodies, as a center of multimodal integration, where newborn cells migrate into the depth of the cortex placing among the older interneurons (Cayre et al., 2000, 2007). In crustaceans, adult neurogenesis is detectable in the central olfactory pathway where an overall increase in olfactory sensory neurons is evident (Schmidt and Harzsch, '99; Schmidt and Derby, 2011). Among mammals, the cell proliferation affects the olfactory bulb, first processing stage of olfactory information, in which immature neuroblasts differentiate into two types of interneurons that integrate themselves in existent circuits (Petreanu and Alvarez-Buylla, 2002; Nissant et al., 2009; Breton-Provencher and Saghatelyan, 2012).

The active cell proliferation in optic and olfactory lobes of *O. vulgaris* brain is perfectly comparable and evolutionary convergent, showing that, in octopus, as well as in other animals with complex and centralized nervous system, the adult neurogenesis plays a crucial role in the integration of sensory stimuli.

Motor Centers. The higher and middle motor centers of the octopus brain are the basal lobes, in the supraoesophageal mass, and the lower are the pedal lobes in the suboesophageal mass that directly innervate the effectors. The motor program is elaborated in the peduncle lobe, an area considered as cerebellum analogue, located in the hilum of the optic lobe (Messenger, '67; Young, '71; Hobbs and Young '73). We found a faint and scattered PCNA-ir in the motor centers, while it results more intense in the spine of the peduncle lobe (Fig. 2A and E).

In mammals, the exercise affects the adult neurogenesis, increasing cell proliferation and its maintenance over time in hippocampus (Kempermann et al., 2010; Clark et al., 2012; Inoue et al., 2015). In light of our data, also in octopus, cell proliferation results are detectable in lobes controlling motor-coordination program in the higher, middle, and inferior motor centers (Fig. 2A).

Influence of Enriched Environment

Considering the role of the brain's areas in which adult neurogenesis occurs, we evaluated if the enhancement of the environmental condition increases the cell proliferation and synaptogenesis in *O. vulgaris*.

The "feeding challenge" represented the only feeding opportunity for those octopuses that were forced to learn how to unscrew the lids, open the jars, and reach the preys to eat.

To face this challenge, octopuses with several trials became able to solve the problem, and after the first success they do it in shorter time. This acquired ability to solve a problem is supported by newborn neurons and neuronal circuits reorganization, as it was already demonstrated in insects and mammals (Scotto-Lomassese et al., 2003; Cayre et al., 2007; Burghardt et al., 2012; Lemaire et al., 2012).

In insects and mammals, both the environment and the experience-based learning affect the adult neurogenesis in term of circuitry reorganization. In insects mushroom bodies, neuroblasts proliferation results an increase in specimens reared in enriched environment compared to specimens housed in sensory deprivation, also demonstrated in analogue experiments on crustaceans (Hansen and Schmidt, 2001, 2004; Sullivan et al., 2007; Ayub et al., 2011), and the ablation of neuroblasts implies a damage of learning capabilities (Scotto-Lomassese et al., 2000, 2002, 2003; Cayre et al., 2007). In mammals, both enriched environment and cognitive stimulation influence proliferation and survival of newborn interneurons (Rochefort et al., 2002; Shors et al., 2012; Clemenson et al., 2015), and their capacity to form new synapses (Kondo et al., 2012; Kumazawa-Manita et al., 2013; Lepousez et al., 2015), in olfactory bulb as well as in hippocampus, as shown in numerous behavioral experiments (Kempermann and Gage, '99; Magavi et al., 2005; Snyder et al., 2009; Lepousez et al., 2014). The function of new neurons seems to alter the existent circuitry to enhance the information processing (Kempermann, 2002; Glasper et al., 2012).

In nonmammals vertebrates, environmental cues modulate the neurogenic process, specifically the cell proliferation (Kaslin et al., 2008). In birds, reptiles, and amphibians, the proliferation rate and survival of newborn neurons seem strictly linked to hormonal fluctuations affected by seasonal variations inducing breeding (Small and Moore, 2009; Delgado-Gonzalez et al., 2011; Margotta, 2012), due to the enhanced plasticity needed to sustain the behavioral tasks underlying mating and reproduction (Tramontin and Brenowitz, 2000).

In fish, sensory stimulation plays the main role in regulation of adult neurogenesis (Lindsey et al., 2014). An enriched environment, in which motor, social, and cognitive stimuli are detected by sensory organs, affects the neurogenic process not only in term of cell proliferation but also in the learning capability (Makino et al., 2015).

In *O. vulgaris* adult, neurogenesis induced by feeding challenge has been evaluated quantifying the PCNA and PARP1 levels in the brain lobes that have been demonstrated to be the sites of neuronal proliferation and synaptogenesis (Figs. 3–5).

In the multisensory integration centers, in optic and olfactory lobes, both proteins levels significantly increased in *challenged* versus *control* groups (Fig. 3), confirming that visual and chemotactile stimulations induce adult neurogenesis in the multisensory integration centers.

Also in the vertical–frontal system, PCNA levels significantly increased in *challenged* versus *control* ($^{*}P < 0.05$) (Fig. 4), suggesting that to elaborate the appropriate behavioral response to the set of sensory and cognitive stimuli provided, newborn cells occurred. The PARP1 protein level of *challenged* versus *control* octopuses increases but not significantly (Fig. 5). This result could be related to a slower circuitry reorganization in the vertical–frontal system with respect to the multisensory integration centers (Toni and Schinder, 2015), in which optic lobes have been hypothesized to represent an additional site for memory storage (De Lisa et al., 2012b; De Maio et al., 2013), besides to be the site of visual inputs integration. The octopus olfactory lobe, that also shows adult neurogenesis and synaptogenesis, is the first processing stage of olfactory information (Di Cosmo and Polese 2014; Polese et al., 2015, 2016) that together with the optic lobes are comparable to the sensory integration sites of insects, crustaceans, and mammals. In light of our results, we extend the classic morphofunctional similarities between the octopus vertical–frontal system and insect mushroom bodies (Hochner, Hochner, 2010) in the presence of cell proliferation in this area.

Since cephalopods have been recently included in animal welfare legislation (EU directive 2010/63 EU L276), and that in *O. vulgaris* the environmental stimuli induce adult neurogenesis on the learning and multisensory integration centers; this finding strongly suggests the use of environmental enrichment to enhance their healthiness and welfare when used as laboratory animals.

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