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Characterization of Novel Cytoplasmic PARP in the Brain of *Octopus vulgaris*

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Abstract

Recent investigation has focused on the participation of the poly (ADP-ribose) polymerase (PARP) reaction in the invertebrate central nervous system (CNS) during the process of long-term memory (LTM). In this paper, we characterize, localize, and assign a possible role to a cytoplasmic PARP in the brain of *Octopus vulgaris*. PARP activity was assayed in optic lobes, supraesophageal mass, and optic nerves. The highest levels of enzyme were found in the cytoplasmic fraction. Hyper-activation of the enzyme was detected in *Octopus* brain after visual discrimination training. Finally, cytoplasmic PARP was found to inhibit *Octopus vulgaris* actin polymerization. We propose that the cytoplasmic PARP plays a role *in vivo* to induce the cytoskeletal reorganization that occurs during learning-induced neuronal plasticity.

Substantial evidence indicates that *Octopus vulgaris* possesses the capacity for learning, or “that process which manifests itself by adaptive changes in individual behavior” (1). This species has been shown to exhibit several classes of behavioral plasticity including sensitization, habituation, associative learning, spatial learning, and social learning (2–4).

It is known that *Octopus* brain contains separate visual and chemotactile memory stores (5). There is also evidence that short-term memory (STM) and long-term memory (LTM) are established in distinct sites (6). Pioneering experiments on *Octopus* vertical lobe (VL) demonstrated that this lobe is intimately involved in the establishment of memories. Lesions to the VL were shown to impair learning (7). Moreover, high-frequency stimulation of the vertical to median superior frontal tract induces long-term potentiation (LTP) in the VL. As LTP is a leading mechanism for learning and memory, the vertical-superior frontal system is considered essential for the induction and maintenance of LTM and STM (7, 8).

PARPs are an ancient family of mainly nuclear enzymes that catalyze the covalent transfer of ADP-riboses from nicotinamide dinucleotide (NAD) to acidic residues on themselves and other protein substrates. The effect of poly-(ADP)-ribosylation of acceptors is to modify their interactions with other proteins and DNA. Poly-(ADP)-ribosylation plays major roles in the pathogenesis of cancer, inflammation, and neurodegenerative disorders by regulating genomic stability, transcriptional fidelity, energy metabolism, cell death, DNA repair, and other cellular processes (9–12).

The broad distribution of PARP genes indicates that the ancestor of all extant eukaryotes, including invertebrates, encoded proteins of this type and that the ancestral PARP proteins had different functions and activities (13). Despite their highly conserved catalytic domain, PARPs do not share strong homologies and differ in their localization and interaction domains, a feature favoring their involvement in multiple cellular functions. It was recently reported that poly (ADP-ribose) polymerase 1 (PARP-1) is activated in neurons that mediate several forms of LTM in the marine opisthobranch *Aplysia* (10). PARP-1 is a key molecule in chromatin remodeling, which is essential for gene transcription and is a prerequisite for LTM.

In this investigation, we identified, characterized, and assigned a possible function for a cytoplasmic PARP in the CNS of *Octopus vulgaris*. The presence of PARP in the CNS of *Octopus* was initially assessed by immunohistochemistry. Enzyme immunoreactivity was mainly confined to neuronal cytoplasm of OL, vertical-superior frontal system, and optic nerves using PARP-1 catalytic site antibodies (data not shown). Due to cytoplasmic localization of the immune signal, sections of CNS were analyzed with specific anti vault PARP (anti-VPARP) monoclonal antibodies. The results confirmed the cytoplasmic distribution of the enzyme in the same areas analyzed with anti-PARP-1 antibodies (Fig. 1).

To further confirm the cytoplasmic distribution of PARP, enzyme activity was assayed in nuclei, mitochondria, and cytoplasm from OL, supraesophageal mass (SupraE), subesophageal mass (SubE), and optic nerves (ON), which are known to be nuclei-free (Fig. 2) (14). Mitochondrion-free cytoplasm retained 90% activity. The remaining PARP (10%) was fractionated between nuclei and mitochondria. It is worth noting that in all analyzed tissues, including ON, enzyme activity was detected mostly in the cytoplasm.

In *Octopus* OL, ON, and SupraE, the immunoblotting of cytoplasmic proteins separated by SDS-PAGE (12%) produced a single band at a molecular mass of 193 kDa (Fig. 3). This size corresponds to VPARP (15). Further experiments indicated that *Octopus* VPARP is inhibited by 3-aminobenzamide (3-ABA) (Table 1), as positive immunosignal was observed with anti-VPARP monoclonal antibodies but not with anti-PARP-1 N-terminus (DNA binding domain) antibodies. Moreover, cytoplasmic PARP activity was DNA-independent (Table 2).

Since the enzyme localization was restricted to areas of the *Octopus* CNS involved in visual memory, further experiments were conducted to investigate whether cytoplasmic PARP could play a role in learning. Accordingly, we trained a group of octopuses on a simple visual discrimination and subsequently examined VPARP enzyme activity in their brains.

Five yoked octopuses (*Octopus vulgaris* Lamarck, size 100–200 g) were pre-trained to attack a white vertical rectangle (5×2 cm) for a food reward (16). Subsequently, specimens were trained to discriminate between white rectangles and black rectangles, attacks on which led to a weak (4–5 V) electric shock (*T*). In control groups, five yoked animals received the shock but not visual stimuli (non-contingent control *A*) and five yoked animals received visual stimuli without shock (non-contingent control *B*). Octopuses rapidly learned this discrimination and were considered trained when they reached a percentage of success of 90%. Success rate was considered as the ratio of correct attacks to errors and correct responses to errors in 48 trials (Fig. 4).

The mean PARP levels in the brain of the octopuses in each group were calculated and the groups were statistically compared using analysis of variance (ANOVA). VPARP activity was assayed in five yoked but not acclimated animals (group *N*) and in two acclimated animals (*C*) (Fig. 5A). On the basis of results obtained from immunohistochemistry analyses (Fig. 1), homogenates from OL, SupraE, and SubE of each group were assayed for PARP activity. In OL and SupraE, VPARP levels increased significantly ($P < 0.001$) with respect to the control groups *A*, *B*, and *C* (Fig. 5B). Low levels of activity were detected in SubE.

During learning, short-term and long-term changes in brain circuits are associated with neuronal plasticity. Plasticity occurs at many levels in the brain, from structural organization of neuron morphology to the regulation of behavioral state, leading to adaptive responses. Synaptic plasticity is associated with changes in neuron connections. These morphological changes are known to be dependent on *N*-methyl-D-aspartate (NMDA) receptor activity and might participate in the enhancement (long-term potentiation, LTP) or diminution (long-term depression, LTD) of synaptic connections. In neurons, actin filaments are remarkably dynamic. Actin polymerization/depolymerization regulates motility, growth, and shape of synaptic connections. LTP is associated with polymerization of actin, and LTD with actin depolymerization (17).

To investigate the relationship between *Octopus* actin (Oct-actin) and VPARP, we performed a turbidometric assay. Oct-actin polymerization was inhibited in the presence of cytoplasmic fractions enriched in VPARP; in contrast, the addition of 3-ABA, the major inhibitor of PARP, restored the polymerizing ability of Oct-actin (Fig. 6). These results ascribe a novel role to VPARP in cytoskeleton rearrangement.

When considering that our understanding of vertebrate physiology greatly benefited from the subcellular analysis of the cephalopod nervous system, as shown by the discovery of the ionic bases of action potentials and of the Ca^{2+} requirement for neurotransmitter release (18), it will be important to investigate whether VPARP may regulate the state of actin polymerization in other cell systems. Moreover, these findings strongly support the involvement of VPARP in neuronal plasticity mechanisms. This notion is strengthened by the presence of NMDA receptors (19) and VPARP (Fig. 1) in the same areas of *Octopus* brain where both NMDA dependent and independent plasticity occur (20). Field potential recordings from *Octopus* brain slices have shown that a vertebrate-like LTP of glutamatergic synapses may occur in VL (21), but not in OL. Unfortunately, preliminary experiments conducted on brain slices from OL revealed that 3-ABA and its inactive structural analog

(amino benzoic acid) exerted a toxic effect on OL slices. Therefore, we could not assess whether blockage of VPARP directly impairs the initiation of LTP in *Octopus* brain.

Since learning requires a period of consolidation and we detected the VPARP hyper-activation in trained octopuses in both OL and VSF, we suggest that VPARP might work in the neural circuitry responsible for memory consolidation. When attacking its prey, *Octopus* primarily depends upon visual information (5). The information from the eyes proceeds to the optic lobes, the site of visual memory storage, *via* the optic nerves. From OL the input enters in the median superior frontal, passes on to the vertical lobe, and arrives at the subvertical lobe. The main output from the subvertical lobe passes on to the precommissural lobe that controls the motor centers (in particular mantle and funnel *via* magnocellular and palliovisceral lobes, and arms *via* pedal anterior lobes). The motor program triggered by visual input is amplified in the lateral superior frontal and subvertical lobes. This amplifying system serves as a reverberatory channel for short- and long-term memories. However, given the existence of another channel from subvertical lobe to optic lobes, it is conceivable that an efference copy of the motor program, generated in the vertical-superior frontal system, could be discharged to optic lobes (Fig. 7). Since this efference copy is the result of the integration and consolidation in the VSF of inputs coming from optic lobes and other sensory centers, the optic lobes could represent another site for LTM. This hypothesis is supported by the observed VPARP hyper-activation in the optic lobe after training experiments.

Abbreviations

3-ABA	3-aminobenzamide
ADP-ribose, ADPR	adenosine diphosphate ribose
CNS	central nervous system
LTD	long-term depression
LTM	long-term memory
LTP	long term potentiation
NMDA	<i>N</i> -methyl-D-aspartate
Oct-actin	Octopus actin
OL	optic lobes
ON	optic nerves
PARP	poly (ADP-ribose) polymerase
PARP-1	poly-(ADP-ribose) polymerase 1
STM	short-term memory
SubE	subesophageal mass
SupraE	supraesophageal mass

VL	vertical lobe
VPARP	vault-associated PARP

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Figure 1.

Vault-associated poly(ADP-ribose)polymerase (VPARG) immunoreactivity in the central nervous system (CNS) of *Octopus vulgaris*. Transverse sections (7 μm) of optic lobes. (A) Optic lobes. VPARG immunoreactivity (antibody dilution 1:100, v/v; Santa Cruz) was localized in the deep retina. In the outer (gr. ou.) and inner granule (gr. in.) cell layers many immunopositive cells are present; there are immunopositive nerve endings in the plexiform layer (plex.). (B) Immunopositive bundles of fibers are present in the optic nerves (arrows). Horizontal sections of supraesophageal masses. (C) Vertical lobe (vl). In all lobules, strong immunopositive amacrine cells can be seen. In the neuropil (n) of the lobules a weak immunopositivity is seen. (D) High magnification of the amacrine cells showing VPARG immunopositive staining. (E) Subvertical lobe (subv.). Small neurons and fibers showed VPARG immunopositive staining. (F) Median frontal lobe. Strong immunopositive neurons can be seen in this lobe. (G, H, I) In control experiments, no specific VPARG immunoreactivity was detected in (G) optic lobes, (H) vertical lobe and subvertical lobe, or (I) median frontal lobe. Scale bars: 50 μm in A, C, E; 25 μm B, F, G, H, I; 15 μm in D. (J) Serial micrograph reconstruction of the horizontal sections (7 μm) of *Octopus vulgaris* CNS. VPARG immunoreactivity was confined mainly to the supraesophageal mass: vertical (VL)-superior frontal (SF) system and inferior frontal (IF) system. Faint staining was detected in the subesophageal mass: pedal lobes (PL) and magnocellular lobes (ML). Scale bars: 100 μm.

Figure 2.

Poly (ADP-ribose) polymerase (PARP) in different subcellular fractions of *Octopus vulgaris* CNS. PARP activity (20 μ g of proteins) in (A) optic lobes (OL), (B) optic nerves (ON), and (C) supraesophageal mass (SupraE) was routinely assayed for 10 min at 25 °C in the presence of 0.51 mmol l⁻¹ [³²P] NAD⁺ (10,000 cpm/nmole). Assay conditions were according to (14). H, homogenate; CF, cytoplasmic fraction; SNF, soluble nuclear fraction; INF, insoluble nuclear fraction; M, mitochondria. The results are mean average of three independent experiments in duplicate with the same animals used for immunohistochemical analysis. Subcellular fractions were prepared according to (14). Integrity of nuclei and mitochondria was checked by optic microscopy. Ninety percent of total activity in optic nerves confirmed the non-nuclear localization of most PARP. In agreement with the immunohistochemistry results, no enzyme activity was detected in subesophageal mass. The peripheral PARP activity was DNA-independent and was inhibited by 70% in the presence of 1 mmol l⁻¹ 3-aminobenzamide (3-ABA) (Table 1).

Figure 3.

Identification of poly (ADP-ribose) polymerase (PARP) by immunoblotting of cytoplasmic fractions from optic lobes (OL) optic nerves (ON), and supraesophageal mass (SM). (A) Cytoplasmic proteins (20 g) were electrophoresed according to (14). Staining was in 0.1% Coomassie G in 10% acetic acid/30% methanol. (B) Western blot analysis was carried out by electrotransferring proteins to a PVDF membrane and incubating the filter with anti-PARP1 antibodies able to recognize the catalytic domain of the enzyme (H-250, Santa Cruz, CA, USA 1:1000). The single immunoband in all cytoplasmic fractions localized at the molecular mass corresponding to that of VPARP (193 kDa). Similar results were obtained with commercial primary monoclonal antibodies against VPARP (P193-4, Thermo Scientific, 1/10). No signal was evidenced with either anti-human PARP-1 N-terminus (DNA binding domain, 1/1000) or the secondary antibodies alone (negative control).

Figure 4.

Visual discrimination training of *Octopus vulgaris*. Record of training experiments in which the octopuses ($n = 10$) discriminate between a black and a white rectangle. Percentage (%) of success reported as correct attacks (A) or as correct responses (B). Data are the mean number of attacks or responses per sessions for the trained octopuses.

Figure 5.

Poly (ADP-ribose) polymerase (PARP) activity in *Octopus vulgaris* brain. The enzymatic activity was routinely assayed according to (14). (A) PARP activity levels in the brain of untrained and trained octopuses. Octopuses were divided in five groups: N, not acclimated animals ($n = 10$); T, animals trained to visually discriminate ($n = 10$); A, control animals that receive the same number and pattern of shocks as the animals in group T but no visual stimuli ($n = 10$); B, control animals that receive the same number of presentations of the black rectangle as those in group T but without the shock ($n = 10$); C, acclimated animals ($n = 2$). (B) The graph shows PARP activity levels in homogenates from optic lobes, supraesophageal mass, and subesophageal mass of controls (N, A, B, C) and trained animals (T). All experiments were performed in triplicate. Data are presented as means $\pm$ SEM. Statistical analyses were performed by ANOVA and Bonferroni *post hoc* test ($P < 0,0001$).

Figure 6.

Turbidometric assay of *Octopus vulgaris* actin (Oct-actin). Oct-actin was cloned from *Octopus vulgaris* CNS, expressed in *E. coli*, and purified using a HisTrap FF 1-ml column (GE Healthcare) (data not shown). Polymerization of Oct-actin was monitored by turbidity at 340 nm in a Varian Cary 50 UV/Vis spectrophotometer. Purified Oct-actin (final concentration, 0.15 mg ml^{-1}) was added to the standard polymerization buffer containing 50 mmol l^{-1} KCl, 2 mmol l^{-1} MgCl_2 and 1 mmol l^{-1} ATP in the presence of 0.2 mmol l^{-1} ATP and 0.5 mmol l^{-1} DTT. Turbidity was monitored for 1 min. The same assay was performed in the presence of active poly (ADP-ribose) polymerase (PARP) from optic nerve cytoplasmic fractions (ON/CF) and 3-aminobenzamide (3-ABA), a PARP inhibitor, at a final concentration of 5 mmol l^{-1} . Active PARP (2.7 mg/ml ; $1 \mu\text{l}$) from optic nerve fractions was tested in $500 \mu\text{l}$ of final volume reaction. Volumes increased 2- to 4-fold were also tested. A decrease of the Oct-actin curve slope was observed that was dependent on enzyme concentration. The presence of 3-ABA restored the effect of non-nuclear PARP on Oct-actin polymerization.

Figure 7.

Illustration of the elaboration of motor control and memory consolidation in *Octopus vulgaris*. The sensory inputs enter the median superior frontal vertical system. The motor program is amplified in the median superior frontal (MSF) and subvertical lobes. From the subvertical lobe an efferent copy of the motor program generated in the vertical-superior frontal (VSF) system is discharged to the optic lobes, where it is stored.

Table 1Enzymatic activity for *Octopus* PARP in the presence of 3-aminobenzamide (3-ABA)

3-ABA concentration (mmol l ⁻¹)	Activity (mU/g tissue)
Optic Lobes	
Control	5.63 ± 0.180
1	4.57 ± 0.110
2	4.26 ± 0.150
5	2.26 ± 0.100
10	2.28 ± 0.070
Optic Nerves	
Control	13.41 ± 0.230
1	8.90 ± 0.306
2	7.58 ± 0.301
5	5.45 ± 0.156
10	5.50 ± 0.253
Supraesophageal Mass	
Control	5.83 ± 0.030
1	5.31 ± 0.051
2	2.50 ± 0.100
5	2.25 ± 0.068
10	2.30 ± 0.047

Table 2

The table shows the enzymatic activity for *Octopus* poly (ADP-ribose) polymerase (PARP) in the presence of fragmented calf thymus DNA

Cytoplasmic fraction	mU/g tissue	% Activity/g tissue
Brain – DNA	3.8	100
Brain + DNA	3.6	94.7
Optic Lobes – DNA	5.01	100
Optic Lobes + DNA	5.01	100