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Steroidogenesis in the brain of *Sepia officinalis* and *Octopus vulgaris*

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1. ABSTRACT

The presence of vertebrate-like steroids, steroidogenic enzymes and steroid receptors has been reported exclusively in cephalopods gonads. The role played by these steroids has been also recently characterized. We here provide the first evidence of steroidogenic activity in the brain of cephalopods and the localization of 3ß-hydroxysteroid dehydrogenase (HSD) activity in the lobes of nervous system of both *Sepia* and *Octopus*. Two key steroidogenic enzymatic activity, 3ß-HSD and 17ß-HSD, are present in the nervous system. These activities convert pregnenolone to progesterone and androstenedione to testosterone respectively. Binding experiments seem to assign a functional role to the androgens in the brain of cephalopods. According to the present results, the absence of any progesterone binding moiety supports the hypothesis that progesterone is just a metabolite product along the steroidogenic chain leading to androgens. The presence of these molecules in specific lobes of central nervous system is discussed in terms of the possible role steroids can play in the sexual differentiation of the brain and in the influence of coded behaviours of cephalopods, such as learning processes.

2. INTRODUCTION

Steroids are ancient molecules spread among vertebrates and invertebrates, as well as plants (1). They are used by animals as hormones controlling reproduction, development and/or homeostasis, but they may also fulfil many other functions such as chemical communication, chemical defence or even digestive physiology (1).

In invertebrates, two main classes of steroids, ecdysteroids and vertebrate-type steroids, have been detected and their presence has been reported for almost all invertebrates (2). The complete demonstration for an endogenous synthesis of vertebrate-like steroids, however, has been reported only for molluscs (3) and echinoderms (4). In particular cephalopods and gastropods, seem to possess the required enzymes to produce progesterone, testosterone and estradiol from cholesterol (3).

In the mollusk cephalopod *Octopus vulgaris*, the occurrence of sex steroid hormones progesterone, estradiol and testosterone was firstly reported in the male reproductive system (5); steroidogenic cells and the key steroidogenic enzyme 3ß hydroxysteroid dehydrogenase

(3ß-HSD) were also demonstrated to be present. The same authors detected sex steroids binding molecules by binding assay and Scatchard analyses.

Di Cosmo *et al.* (6, 7) extended their studies on the female reproductive systems of *Octopus* demonstrating by Scatchard analysis the presence of both a progesterone-binding molecule (PR), with high affinity and low capacity for ligand in the nuclear proteic extracts of the ovary, and an estradiol-binding component (ER) with high affinity and low capacity in the cytosol, but not in the nuclear extract of the ovary and the oviduct. PR was immunolocalized in the nuclei of follicle cells of the ovary, of the proximal portion of the oviduct and of the outer region of the oviducal gland, while ER immunoreactivity was present in the nuclei of the follicle cells of the ovary, in the nuclei of the epithelium of the proximal oviduct and in the nuclei and cytoplasm of the inner region of oviducal gland and in the cytoplasm of the outer region of the oviducal gland.

Furthermore in the female of *Octopus* Di Cosmo *et al.* (8) demonstrated both an annual variation of the levels of estradiol and progesterone during the reproductive cycle and the presence of steroidogenesis in the ovary using enzymatic histochemistry for 3ß-HSD.

In *Octopus*, progesterone is able to induce the acrosomal reaction in spermatozoa (9) and steroidogenesis of testosterone, progesterone and estradiol in ovary and testes seems to be mediated by the *Octopus* GnRH isoform (10). Finally, a putative ER, has been cloned from *Octopus* ovary RNA, characterized by a high level of identity with human ER for DBD and a low level of identity for LBD, the latter resulting in absence of binding with estradiol (11). This led them to suggest this molecule cannot mediate estrogen actions.

All these data seem to support the involvement of vertebrate sex steroids in the control of reproduction in *Octopus*.

No evidence, however, is yet available for the existence of steroidogenesis in the nervous system of *Octopus*. The concept that steroids may be synthesized in the brain derived from studies by Baulieu and co-workers (for review, see (12)). To test whether steroids were actually made in the brain or, alternatively, accumulate in the nervous system, several groups determined whether or not the enzymes involved in steroidogenesis are present in nervous tissue, allowing for neurosteroids to be synthesized. Results from several laboratories over the past decade established that the enzymes found in classical steroidogenic tissues are indeed present in the nervous system (13).

Interestingly, estrogens and androgens, locally synthesized in hippocampal neurons by cytochrome P450s, act rapidly to modulate neuronal synaptic plasticity (14). In the pathway of steroidogenesis, cholesterol is converted to pregnenolone (by P450scc), dehydroepiandrosterone (by P450-17α), androstenediol (by 17ß-hydroxysteroid dehydrogenase, 17ß-HSD), testosterone (by 3ß-HSD) and finally to estradiol (by P450arom) and dihydrotestosterone (by 5α-reductase). The mRNAs coding for these enzymes is expressed in hippocampus (14).

In order to investigate the occurrence of steroidogenic enzymes in the nervous system of *Octopus* and *Sepia*, we report in this study the presence and the activity of 3ß-hydroxysteroid dehydrogenase and17ß-hydroxysteroid dehydrogenase as well the presence of sex steroid binding molecules. These results will be discussed in light of the putative role of these enzymes and their products in synaptic plasticity.

3. MATERIALS AND METHODS

3.1. Animals

20 Males of *Octopus vulgaris* and *Sepia officinalis* (body weight between 100 and 500 g) were captured in the harbor of Naples. Sampling started in December-January 2003-2005. Animals were maintained, for a maximum of two weeks, in aquarium tanks with a recirculating seawater system as described in (15, 16). Animals were kept under natural photoperiod (8:16, L:D). Water temperature was set at a value similar to that one of the water in the harbor of Naples (13-15°C). Animals were anesthetized on ice. Soon after the sacrifice nervous systems were removed and treated differently for enzymatic histochemistry, enzyme assay, steroid binding assay.

3.2. Chemicals and reagents

[1,2,6,7-^{3}H] Progesterone (^{3}H-P) (SA = 80.2-111.1 Ci/mmol), [1,2,6,7] ^{3}H-testosterone (^{3}H-T) (SA 80/105 Ci/mmol) were purchased from Amersham Radiochemical Center (Amersham, Bucks, U.K.). [7-^{3}H]-pregnenolone (1mCi/ml) and [4-^{14}C]-androst-4-ene-3,17-dione (0.02mCi/ml) were purchased from Perkin Elmer Life Sciences, trilostane was kindly provided by Dr. G. Margetts, Stegram Pharmaceuticals, UK; quercetin and all other chemicals were from Sigma-Aldrich.

Radioinert reagents used were estradiol-17ß, progesterone, testosterone, diethylstilbestrol (DES), deoxycorticosterone, corticosterone, tamoxifen (Sigma, St. Louis, MO). Radioinert steroids used were progesterone, testosterone, deoxycorticosterone (DOC), corticosterone, 17α-hydroxyprogesterone (Sigma, St Louis, MO); Norit A Charcoal was from Sigma, Dextran T-70 was from Pharmacia (Piscataway, NJ). Maxifluor scintillation cocktail was obtained from Packard (Milan, Italy). Other chemicals were reagent grade or better.

3.3. Buffers

The buffers used were as follows: TEMG buffer (10 mM Tris-Base, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol, pH 7.5); homogenizing buffer (50 mM Tris-Base, 1 mM EDTA, 12 mM monothioglycerol, 10% glycerol, pH 7.5); extraction buffer (1.0 M KCl in homogenizing buffer); washing buffer (10 mM Tris-Base, 12 mM monothioglycerol, 3 mM MgCl2, 0.25 M Sucrose, pH 7.5). Buffers for DNA-Cellulose were as follows: 200 mg/l BSA in TEMG

(Buffer A); 0.5 M NaCl+200 mg/l BSA in TEMG (Buffer B). Buffers were added with the following protease inhibitors: Trypsin-Chymotripsin inhibitor 1 μM (Sigma), Pefabloc (AEBSF) 1mM, Aprotin 0.1 μ M, Leupetin 1 μ M, E64 Protease inhibitor 1 μM (Boehringer Mannheim, [GmBH] Germany). Dextran coated charcoal (DCC) was made up in TEMG (0.5% charcoal and 0.05% Dextran).

3.4. Preparation of cytosol and nuclear extract

All procedures were carried out at 4°C. The brain was dissected, minced and homogenized in 2 vol (wt/vol) of homogenization buffer. Samples were homogenized using a glass potter, filtered through three layers of cheese-cloth and centrifuged at 800 x g at 4°C for 15 min. The supernatant was recentrifuged at 105,000 x g at 4°C for 1 hr in order to obtain the cytosol. The pellet was resuspended in extraction buffer (same dilution as with the homogenization buffer) and placed on ice for 1 hr with regular stirring. The suspension was thereafter centrifuged at 105,000 x g at 4°C for 1 hr. The supernatant constituted the nuclear extract.

3.5. ^{3}H-T, ^{3}H-P binding assays.

Steroid binding measurements were performed using [1,2,6,7] ^{3}H-testosterone and [1,2,6,7] ^{3}H-progesterone as ligands. For Scatchard analysis (17), increasing amounts (0.3-5.0 nM) of ^{3}H-T and (0.3 – 40 nM) of ^{3}H-P were incubated with aliquots of cytosol and nuclear extract in the presence or absence of 200-fold excess of unlabeled testosterone or progesterone respectively to determine non specific and total binding. Incubations were carried out for 16 hours at 4°C. Bound from free steroid were separated by adding 600 μl of TEMG, containing 0.05% (w/v) dextran T-70 and 0.5% (w/v) charcoal. The mixture was vortexed and kept in an ice bath for 5 minutes. It was then centrifuged at 800xg for 10 minutes. The supernatant was transferred to vials containing 4.5 ml Maxifluor scintillation fluid. Radioactivity was measured in a Packard spectrometer (Packard 1600, CA) at 45% efficiency counting. Data of specific binding were plotted according to graphical procedure in (17).

3.6. Enzyme assay

Intact brains and optic lobes were incubated in 2ml sterilized sea water in the presence of ^{3}H-pregnenolone (1μCi) for 3β-HSD activity assay, or ^{14}C-androstenedione (1μCi) for 17β- HSD. After 2 hrs at room temperature under stirring, samples were homogenized in 500μl bidistilled water and extracted in ethyl acetate (250μl × 2). The organic layers were analyzed by HPLC in reverse-phase conditions (5μ Sphereclone ODS-2, 250 × 4.60 mm), UV detection at 280 nm; ϕ 1ml/min; eluant AcOH 0.1%, $AcONH_4$ 2mM and MeOH 63% for pregnenolone metabolites; MeOH 16%, tetrahydrofuran 13% for androstenedione metabolites. The eluates were fractionated every minute from 25' to 60' for quantitation of ^{3}H-progesterone (RT 40') and from 30' to 75' for ^{14}C-testosterone (RT 54') and then counted by a liquid scintillation counter. When necessary incubations were carried out in the presence of trilostane (100μM) for 3β-HSD activity assay and androsterone (100μM) or quercetin (20μM) for 17β- HSD activity assay.

3.7. Protein measurement

Protein content was determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Milan, Italy) with BSA as a standard.

3.8. Enzymatic histochemistry of 3ß-hydroxysteroid dehydrogenase

Slices of brain were washed in 0.1 M cacodylate buffer, pH 7.4, for 10 min at 4°C and then for 5 min at room temperature. Slices were incubated at 37°C for 1–3 hr in a reaction mixture consisting of 0.1 M cacodylate buffer, pH 7.4, containing 0.2 mg/ml of steroid DHA, dissolved in diethyl ether, 0.5 mM ßNAD^{+}, and 0.15 mM nitro-BT tetrazolium salt. Sections were then washed in washing buffer, fixed for 10 min in formaldehyde vapors, and mounted in glycerin. Controls were routinely run in all experiments omitting DHA, ßNAD^{+}, or both.

4. RESULTS

4.1. Enzymatic activity

4.1.1. 3ß-HSD activity

The presence of 3ß-hydroxysteroid dehydrogenase (3β-HSD) activity, both in cuttlefish and octopus, was investigated by assaying the ability of the nervous system to convert pregnenolone to progesterone.

Incubation of brain and optic lobes of cuttlefish with tritiated pregnenolone resulted in the formation of labeled progesterone in comparable amounts in both regions (Figure 1A, open bars). A significantly higher activity was found in octopus, where conversion of pregnenolone to progesterone was 3-fold higher in the optic lobes with respect to the brain (Figure 1B, open bars)

3β-HSD activity was reduced by the application of a specific 3β-HSD inhibitor, trilostane, to the incubation mixture. In cuttlefish, the yield of progesterone decreased of 31% in the brain and 60% in the optic lobes with respect to the control (Figure 1A, dashed bars). In *Octopus* the yield of progesterone decreased of 97% in the optic lobes and 60% in the brain with respect to the control (Figure 1B, dashed bars).

4.1.2. 17ß-HSD activity

The presence of 17ß-hydroxysteroid dehydrogenase (17ß-HSD), the enzyme that controls the interconversion of 17-ketosteroids, such as androstenedione, with the corresponding 17ß-hydroxysteroids, such as testosterone, was detected by incubation of nervous system with ^{14}C-androstenedione and measuring radiolabeled testosterone formed in brain and optic lobes. Comparable amounts of labeled testosterone were produced in the brain and optic lobes of both cuttlefish and *Octopus* (Figure 2A and 2B open bars).

The use of androstetone, an inhibitor of type 3 17ß-HSD resulted, in of both sepia and octopus brain and optic lobes, in no appreciable inhibition of testosterone

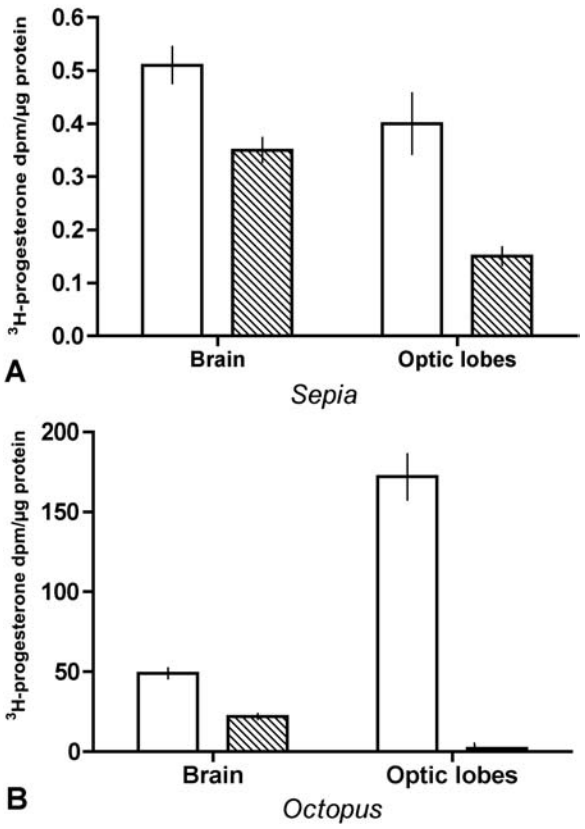

Figure 1. 3ß-HSD activity in CNS of cuttlefish and octopus. ^{3}H-progesterone formed after incubation with ^{3}H-pregnenolone in absence (open bars) and in the presence of trilostane100µM (dashed bars). Data shown are mean values ± S.E.M. (A) 3ß-HSD activity in cuttlefish; n= 5 for brain and 6 for optic lobes; in the presence of trilostane n= 6 for brain and 7 for optic lobes. (B) 3ß-HSD activity in octopus; n= 4 for brain and optic lobes; in the presence of trilostane n= 3 for brain and optic lobes. Statistical significance: $P < 0.05$ in all cases (Anova method).

production with respect to the control (data not shown). Addition of quercetin, a flavonoid inhibitor of type 5 17ß-HSD, caused a significant decrease of testosterone production of 88% and 86% in brain and optic lobes respectively in cuttlefish and 67% in the brain and 51% in optic lobes in *Octopus*. (Figure 2 A e 2B, dashed bars)

4.2. ^{3}H-P and ^{3}H-T binding

^{3}H-progesterone binding activity was tested on both cytosolic and nuclear extracts of the CNS of both *Octopus vulgaris* and *Sepia officinalis*, but no evidence of binding was detected over a concentration of radiolabelled ligand ranging from 0.3 to 40 nM.

^{3}H-testosterone binding activity was detected in the nuclear extract of the CNS of both the animals. In the nuclear extract, saturation was reached at a value between 2.5 and 5 nM of ^{3}H-T when the total protein content was kept at 2 mg/ml. Only one high affinity binding component was observed, with an average Kd value of 2.1 ± 0.3 nM (Figure 3). Scatchard analysis was performed three times and Kd values were submitted to ANOVA analysis. Kd values did not show any significant variation (data not shown).

4.3. Distribution of 3ß-HSD in *Sepia* and *Octopus* brain

The CNS in *Sepia* and *Octopus* is highly centralized and arranged around the esophagus: it consists of supraesophageal and subesophageal masses, laterally connected by the periesophageal magnocellular lobes and a pair of large optic lobes, joined to the supraesophageal mass by optic tracts, on which lie the peduncle and olfactory lobes (18-24). Optic tracts are short in *Sepia* but longer in *Octopus*.

The enzymatic histochemical reaction for 3ß-HSD consisted in the precipitation of tetrazolium salts when the enzymatic reaction of substrate (Pregnenolone), with the specific enzyme, in presence of the co-factor ßNAD$^+$, took place. Specific negative controls were performed omitting the steroid or the cofactor (Figure 4A).

4.3.1. Optic and optic tract lobes

In the optic lobes of *Octopus* and *Sepia*, positive staining was present in almost all the radial and tangential layers of the plexiform zone. (Figure 4B) Positive fibers running through both the outer and inner granule cell layers pointed to the plexiform zone, but no positive cell bodies were seen.

No positive neurons were present in the medulla of the optic lobe of both the cephalopods, while a sparse positivity was present throughout the neuropil.

Although faint in *Sepia*, a strong staining was present in the spine of the peduncle lobe (Figure 4C), and, sometimes, few positive large neurons stained for 3ßHSD. No staining was observed in the small neurons. Conversely, in both *Octopus* and *Sepia*, very little positivity was seen in the basal zone of the peduncle lobe and neurons never stained in this zone.

Positivity was present in the neuropils of all lobules of the olfactory lobes, but no stained cell bodies were present.

Finally, in *Octopus* as in *Sepia*, staining was virtually absent from both stellate and supporting cells of the optic gland and so was the optic gland nerve. Control sections were negative.

4.3.2. Supraesophageal mass lobes

3ßHSD staining was fairly present in all the neuropil of the lobes of *Octopus* and *Sepia* supraesophageal mass. However, the strongest staining was present in the neuropils of the memory systems, namely the vertical lobe system, and, in *Octopus*, the inferior frontal lobe system. In the vertical lobe system of *Octopus* and *Sepia*, strong positivity was present in the neuropils of superior frontal, vertical and subvertical lobes. Interestingly, in the vertical lobe neuropil, only the fibers at the very center and at the base of the neuropil stained for 3ßHSD. Some positive cells were present in the ventral part of the lobe, lining the subvertical lobe (Figure 4D). The neuropil of superior (both

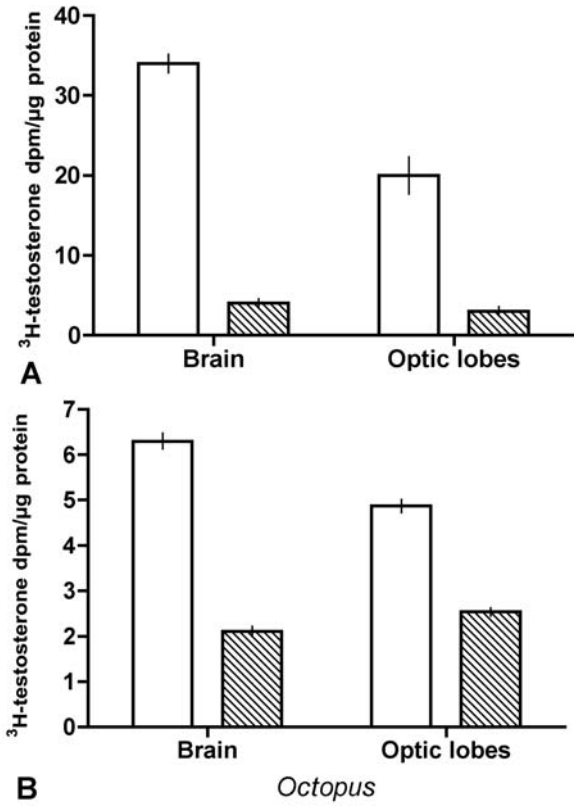

Figure 2. 17ß-HSD activity in CNS of cuttlefish and octopus. ^{14}C-testosterone formed after incubation with ^{14}C-androstenedione in absence (open bars) and in the presence of quercetin 20µM (dashed bars). Data shown are mean values ± S.E.M. (A) 17ß-HSD activity in cuttlefish; n= 6 for brain and optic lobes, in the absence and presence of quercetin. Statistical significance: P = 0.01 for both cases (Anova method). (B) 17ß-HSD activity in octopus; n= 6 for brain and 7 for optic lobes, in the absence and presence of quercetin. Statistical significance: P < 0.01 for both cases (Anova method).

medial and lateral) frontal lobes showed a typical staining in bundles of fibers (Figure 4E). In *Octopus*, very few amacrine cells stained the cortex of the median superior frontal lobe.

Positivity was also present throughout the neuropil of both anterior and posterior subvertical lobe. Many positive fibers ran between vertical and subvertical lobes.

In the inferior frontal lobe system, which was exclusive of octopods, the staining was present in the neuropil of all the lobes: lateral and medial inferior frontal, subfrontal and posterior buccal lobes. Interestingly, neurons in the ventral posterior buccal stained intensely, as did both the inferior frontal to subfrontal and subfrontal to posterior buccal tracts.

In *Sepia*, positivity was present in the neuropil of the inferior frontal lobes.

Positive neurons were present in the dorsal basal lobe of both the cephalopods and intense staining was also present in their neuropil. Stained fibres were also present in the medial and lateral basal lobes.

As in peduncle lobe, in *Octopus* and *Sepia* the spines of the anterior basal lobe showed intense staining for 3ßHSD (Figure 4F), but no positive neurons are present. Very few staining was present in buccal lobe neuropil. Control sections were negative

4.3.2. Subesophageal mass lobes

As in supraesophageal mass lobes, neuropils of subesophageal mass lobes of *Octopus* and *Sepia* showed the highest staining. However, in pedal lobes of both animals, mainly in the posterior and the dorsal lateral pedal lobes, many positive large neurons were present.

Some 3ßHSD positive cell bodies were present also in the anterior palliovisceral lobe, whose neuropil showed a discrete staining, while scarse positivity was present in the neuropil of brachial lobes. In the latter, no positive neurons were present. Control sections were negative

5. DISCUSSION

The concept that the steroids could be synthesized *de novo* in the nervous system arised by the observations made by Baulieu and colleagues (25).

They discovered that steroids such as pregnenolone and dehydroepiandrosterone (DHEA), and their sulfate and lipoidal esters were present in brain and peripheral nerve than in plasma. Furthermore steroids remained in the nervous system long after gonadectomy and adrenalectomy. These findings suggested that steroids might be either synthesized in the central or peripheral nervous systems or might accumulate in those structures. Such steroids were named “neurosteroids” to emphasize their origin in a tissue completely different from classic steroidogenic tissue.

To test whether steroidogenic activity is present in the nervous tissue, it is, however, necessary to demonstrate the presence, the activity and the distribution of steroidogenic enzymes.

The present report provides the first evidence of steroidogenic activity in the brain of cephalopods and contributes to map 3ß HSD activity, as revealed by enzymatic histochemistry reaction, in the lobes of nervous system of both *Sepia* and *Octopus*. Two key steroidogenic activity, 3ß HSD and 17ß-HSD, from the central nervous system of both *Sepia* and *Octopus*, are capable to convert pregnenolone to progesterone and androstenedione to testosterone respectively. Finally binding experiments seem to assign a functional role to the androgens in the brain of cephalopods. According to the present results, the absence of any progesterone binding moiety supports the hypothesis that progesterone is just a metabolite product along the steroidogenic chain leading to androgens. Further

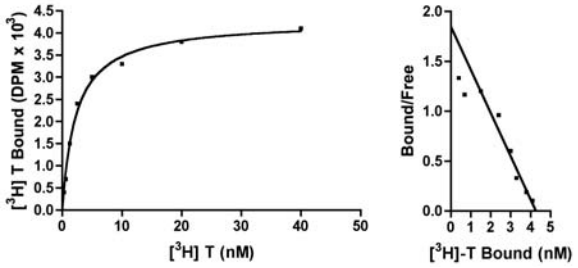

Figure 3. Saturation and Scatchard plot of ^{3}H-T binding in the nuclear extracts of the brain of *Octopus vulgaris*. Only the specific binding is shown in both saturation and Scatchard plot. These data are representative of three separate experiments which gave similar results. The brain of *Sepia* gave similar results.

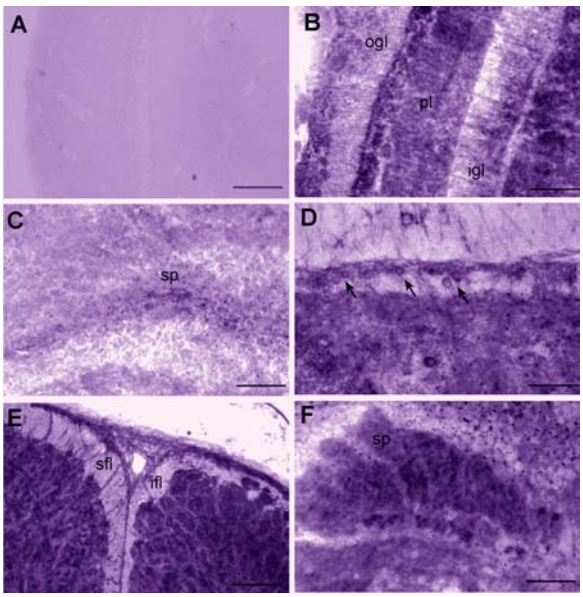

Figure 4. 3ß-HSD enzymatic histochemistry in CNS of cuttlefish and octopus. The localization of 3ß-HSD enzymatic activity was achieved using precipitates of nitrazolium blue salts. A Control section (optic lobe of *Sepia*). The omission of either the steroid or the cofactor did not induce any staining. B Staining of the deep retina in the optic lobe of Sepia. The labelling is limited to the plexiform layer (pl), while no staining is present in both outer (ogl) and inner (igl) cell granule layers. Note the sparse positivity in the medulla. C The spine (sp) of the peduncle lobe of *Octopus* (as well as the one of *Sepia*) shows positive staining in the parallel fine fibres. No neuron staining is present. D Vertical lobe system of *Octopus*. Strong positivity is present in the neuropil of subvertical lobe , while positive fibres are present in the neuropil of vertical lobe. Note positive small cells of the vertical lobe (arrows) lining the roof of the subvertical lobe. E Positive enzymatic activity in the neuropil of both median superior (sfl) and median inferior (ifl) frontal lobes of *Octopus*. F Staining of the posterior anterior basal lobe spine (sp). Staining is confined to the fibers. Scale bars: A, B, E= 50 µm; C,D,F = 35 µm.

investigations, not performed in this paper, will address the presence of pathway of convertion of androgens in estrogens.

5.1. The enzymes

The hydroxysteroid dehydrogenases, which include the 3β−HSDs and the 17β−HSDs, belong to the same phylogenetic protein family, namely the short-chain alcohol dehydrogenase reductase superfamily. There are several isoforms for the 3β−HSDs and several isoenzymes of the 17β−HSDs, each a product of a distinct gene. The number of isoforms and isoenzymes varies in different species, in tissue distribution, catalytic activity (whether they function predominantly as dehydrogenases or reductases), substrate and cofactor specificity, and subcellular localization (26).

In invertebrates, 3β-HSD activity has been identified in *Taenia crassiceps* and *Taenia solium* cysticerci (27), in the gonads of *Octopus vulgaris* (8); a 17beta/3beta-hydroxysteroid dehydrogenase (HSD) activity has been identified in gonads and digestive tubes of the gastropod *Marisa cornuarietis*, the amphipod *Hyalella azteca*, and the echinoderm *Paracentrotus lividus* (28). Moreover, a 3β-HSD activity is hypothesized to be involved in ecdysteroid biosynthesis in the shore crab *Carcinus maenas* (29), in the gonads of the cuttlefish *Sepia officinalis* (30), in the gonads of the mussel *Mytilus edulis* (31).

In this report, we have identified an enzymatic activity, which possesses, for substrate specificity and inhibitor selectivity, the features of a 3β−HSD. Such an activity has been already reported to be present in male and female cephalopod gonads (5, 8) by using biochemical and enzymatic histochemistry methodologies. Particularly, 3β−HSD activity has been localized in the Leydig-like cells in testes and follicle and granular cells in the ovary. It is worth of note that there is a substantial difference masses in the level of activity between optic lobe and supra/subesophageal in Octopus, while the levels are comparable in *Sepia*. Nonetheless, trilostane, a specific inhibitor of 3β−HSD activity, exerts its inhibitory role at different levels, depending on both the animal and the region of the brain. Again, this effect reaches its maximum in *Octopus* optic lobes. Since the different role of such lobes in the nervous physiology of octopus, if these differences are linked to a specific relationship enzyme - nervous activity, it remains to be solved

Like 3β−HSDs, 17β−HSDs play essential roles in steroidogenesis. These enzymes catalyze the final step in the biosynthesis of active gonadal steroid hormones, estradiol and testosterone.

To date 11 different 17β−HSDs have been identified. Unlike the 3β−HSDs there is very little homology among the different 17β−HSD isoenzymes. The 17β−HSDs differ in tissue distribution, catalytic preferences, substrate specificity, subcelluar localization, and mechanism of regulation. Among the many different forms of 17β−HSDs, three forms participate in the final

step of biosynthesis of active steroid hormones in gonads, type 1, 3 and 7. In peripheral tissues and in the ovary, the same enzymatic reaction is catalyzed by another enzyme, namely type 5 17β-HSD, which represents an additional source of androgens (32).

In invertebrates, 17β-HSD activity has been identified in gonads of sea pectin and Gray's mussel (33), in *Mytilus edulis* (31), in corals (34, 35), in the oyster (36), in *Ciona intestinalis* (37), in the snail *Helix aspersa* (38), in the ovaries and hepatopancreas of crustacean (39); a 17beta/3beta-hydroxysteroid dehydrogenase (HSD) activity has been identified in gonads and digestive tubes of the gastropod *Marisa cornuarietis*, the amphipod *Hyalella azteca*, and the echinoderm *Paracentrotus lividus* (28).

Quite interestingly, in cephalopods brain, inhibitors selectivity revealed that a type 5-like 17ß HSD activity is present. This specific isoform is the main testosterone-forming 17HSD enzyme (40): it synthesizes 17ß-diol (5-diol) from dehydroepiandrosterone and testosterone from andostendione (12). Moreover, if confirmed, this would be one of the first report on the presence of such isoform in the nervous system. As happens for 3ßHSD inhibitor, also quercitin, the specific inhibitor of type 5 17ß HSD activity, exerts its action more effectively on Sepia brain lobes, while in octopus, only 50-60 % of the activity has inhibited by this substance. Again, no data are available to justify these differences.

5.2. The binding

The presence of sex steroid binding moieties in cephalopods has been widely already reported. Progesterone, androgens and estradiol binding moieties are present in *Octopus* male gonads and reproductive tracts, while only progesterone and estradiol binding moieties have been reported to be present in *Octopus* female gonads and reproductive tracts.

A very recent paper reports the cloning and the molecular characterization of a cDNA present in both the gonads and brain of *Octopus* coding for an orthologue gene of the estrogen receptor (11). However, this protein is constitutively active as transcription factor and, mainly, does not bind estradiol. Authors claim that if estrogens have a role in cephalopods it does not happen through this receptor. More recently, however, a distinctive role of progesterone in *Octopus* vitellogenesis has been demonstrated (41).

In the brain of both the cephalopods here investigated, no evidence of progesterone binding has been found. Progesterone action in the brain is mainly related to its neuroprotective effects (42). In addition, progesterone stimulates the myelination of axons during development and the regeneration of myelin after lesions, also referred to as remyelination or myelin repair (43). Although little is known on the neuroprotection in cephalopod brain, the absence of progesterone binding well fits with the absence in such brains of a real myelin.

Indeed, the presence of androgens binding is worth of note. It has been elsewhere reported the presence of androgen receptors in specific areas of vertebrates brain. Circulating levels of androgens in embryos or juveniles determine the sexual differentiation of the brain and spinal cord (44).

Androgen effects on the brain begin at the time of sexual differentiation and continue throughout the life of the organism. In many species androgens act indirectly through aromatization to estrogens. Both androgen receptors and aromatase are found in the brain of those investigated species, although their distribution varies within the brain (45-47). The autoradiographic method has been successfully used to describe androgen-concentrating neurons in mammals (48-50) birds (51, 52), reptiles (53), amphibians (54-56), and fishes (57). Androgens have profound influences on behaviour. In general, androgens stimulate male-associated behaviours in subavian vertebrates (58).

It is interesting that androgen binding was only evident in juvenile octopuses, while completely disappeared in adults, indicating, a role for androgens possibly in a correct, sex-dependent, development and mainteinance of cephalopod brain.

5.3 .The distribution: a key to plasticity

The distribution of 3ß HSD in the nervous system of *Octopus* and *Sepia* strongly suggests that this enzyme is localized in those lobes of supraesophgeal mass that are involved in learning and memory and in the control of the movement. The positive staining was restricted to neurons and fibres. Glial cells are generally accepted to be the major site of steroidogenesis in mammals (59) but the concept of neurosteroidogenesis in brain neurons has been demonstrated (60, 61).

Cephalopods are renowned for their learning abilities (62), and *Sepia* and *Octopus* have been the subjects of an extensive series of experiments investigating their memory and their neural correlates (for summaries see (63, 64)). For *Octopus*, it has been shown that there are two separate memory stores, a visual store in the optic lobes and a chemotactile store in the subfrontal system; in *Sepia*, there is a visual store but not the chemotactile store (65).

The visual learning system in *Sepia* and *Octopus* comprises anterior and posterior superior frontal lobes (called lateral and median superior frontal lobes in *Octopus*), subvertical and vertical lobes, and optic lobes, which are almost certainly the site of the visual memory store (18, 21, 23).

Octopus readily recognizes differences in the chemical nature and texture of objects by touch (64). The inferior frontal lobe system contains the major tactile memory. This part of the brain has four lobes anatomically and functionally resembling those of the visual learning system. The inferior frontal lobe system probably reinforces the tendency to draw in whatever object an arm touches, unless impulses signaling trauma arrive (21).

The results of 3ßHSD enzymatic histochemistry seem to indicate that this key steroidogenic enzyme is

present in the neuropils of both the visual and tactile (in *Octopus*) learning systems. Interesting is the presence of positivity in some neurons of both vertical and posterior buccal lobes.

In vertebrates, the role of neurosteroids in the processes of learning and memory has been extensively studied (for review, see. (66).

The hippocampus, which is essentially involved in learning and memory processes (67), is known to be a target for the neuromodulatory actions of androgens such as testosterone (T) (68-74) and, androgen specific receptor (AR) immunoreactivity and AR binding are present in rat hippocampus, particularly in CA1 pyramidal cells (75, 76). Androgens can also enhance neural excitability in the hippocampus of male rats (77, 78), increase dendritic spine density in the CA1 and CA3 regions of the dorsal hippocampus (79) and modulate some behaviors thought to be mediated by the hippocampus. Finally, many studies indicate that, performance in the inhibitory avoidance, water maze, and Y maze tasks are influenced by androgen milieu (79, 80).

The fact that an androgen-synthesizing pathway is present in the area involved in the generation of learning in cephalopods let to speculate that such ancient molecules, together with their receptors, kept the ability to influence basic coded neural activities throughout the animal evolution. The treatment of animals with androgens, before specific training paradigms, will give us more insights on the relationship steroids-behaviour in cephalopods.

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