Cyp19a1 (Aromatase) Expression in the Xenopus Brain at Different Developmental Stages

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Cytochrome P450 aromatase (P450arom; aromatase) is a microsomal enzyme involved in the production of endogeneous sex steroids by converting testosterone into oestradiol. Aromatase is the product of the cyp19a1 gene and plays a crucial role in the sexual differentiation of the brain and in the regulation of reproductive functions. In the brain of mammals and birds, expression of cyp19a1 has been demonstrated in neuronal populations of the telencephalon and diencephalon. By contrast, a wealth of evidence established that, in teleost fishes, aromatase expression in the brain is restricted to radial glial cells. The present study investigated the precise neuroanatomical distribution of cyp19a1 mRNA during brain development in Xenopus laevis (late embryonic to juvenile stages). For this purpose, we used in situ hybridisation alone or combined with the detection of a proliferative (proliferating cell nuclear antigen), glial (brain lipid binding protein, Vimentin) or neuronal (acetylated tubulin; HuC/D; NeurobTubulin) markers. We provide evidence that cyp19a1 expression in the brain is initiated from the very early larval stage and remains strongly detected until the juvenile and adult stages. At all stages analysed, we found the highest expression of cyp19a1 in the preoptic area and the hypothalamus compared to the rest of the brain. In these two brain regions, cyp19a1-positive cells were never detected in the ventricular layers. Indeed, no co-labelling could be observed with radial glial (brain lipid binding protein, Vimentin) or dividing progenitors (proliferating cell nuclear antigen) markers. By contrast, cyp19a1-positive cells perfectly matched with the distribution of post-mitotic neurones as shown by the use of specific markers (HuC/D, acetylated tubulin and NeurobTubulin). These data suggest that, similar to that found in other tetrapods, aromatase in the brain of amphibians is found in post-mitotic neurones and not in radial glia as reported in teleosts.

Cytochrome P450 aromatase (P450arom; aromatase), the product of the cyp19a1 gene, is a microsomal enzyme that converts C19 androgens, such as testosterone or androstenedione, into C18 oestrogens, oestradiol (E2) or oestrone, respectively (1–3). Increasing evidence suggests that the production of oestrogens in the vertebrate brain is involved in important physiological and behavioural processes (1,4–6). The best documented function of aromatase (also called oestrogen synthetase) is its role in the organisation of sexually dimorphic structures during development of the hypothalamus in the male rodent (1,7–10). During adult life, such regions will also be activated by local oestrogen synthesis to regulate sexual behaviour (11–13).

The distribution of cyp19a1 mRNA has been extensively investigated in the brain of mammals and birds (14–16). However, for technical reasons, the use of antibodies in the mammalian brain has proven problematic, whereas, in contrast, aromatase antibodies have been extensively and successfully used in birds (17,18). In mammals and birds, consistent with its roles in the regulation of reproductive and behavioural functions, cyp19a1/aromatase is mainly expressed in the hypothalamus, the bed nucleus of the stria terminalis and the amygdala (4–6). In songbirds, aromatase is also involved in the seasonal development and activation of vocal centres (19,20). According to current knowledge, cyp19a1/aromatase expression is predominantly in neurones, although expression in astrocytes has been occasionally reported, particularly after chemical and/or mechanical lesions (21,22). In fish, where two cyp19a1 genes (cyp19a1a and cyp19a1b) were identified, a very high levels
of aromatase activity was also described in the brain of many teleost species (23–26). Unexpectedly, in teleost fishes, aromatase is only expressed in radial glial cells and its expression is highly dependent on oestrogens and some aromatisable androgens (23).

In amphibians, aromatase is encoded by a single cyp19a1 gene leading to two transcripts in the gonads and a single one in the brain. These transcripts differ in their 5'-translated region but contain an identical open reading frame (27). Importantly, cyp19a1 transcripts detected in the brain of amphibians encode a biologically active protein (28–30). Reverse transcriptase–polymerase chain reaction (RT-PCR) experiments were performed to study the presence of cyp19a1 mRNA during brain development (27,31,32). Interestingly, the cyp19a1 gene was shown to be strongly expressed from early developmental stages and remains at high levels until metamorphosis. In addition, no sex-specific expression of cyp19a1 gene was observed (27,31,32). The present study provides critical information on the precise sites of expression of cyp19a1 in the brain of _Xenopus laevis_ during development.

To gain more insight into the potential function of aromatase in the brain of amphibians, using _in situ_ hybridisation, we have analysed the distribution of cyp19a1 transcripts in the brain of _X. laevis_ from late embryonic to post-metamorphic (juvenile and adult) stages. We provide evidence that the cyp19a1 gene is expressed very early during brain development and in a region specific manner. In addition, our data indicate that cyp19a1-expressing cells comprise exclusively post-mitotic neuronal cells.

### Materials and methods

#### Animals and tissue processing

For the present study, 47 South African clawed frogs (_X. laevis_) and two _Xenopus tropicalis_ were used. Embryo, larvae, juvenile and adult _Xenopus_ were purchased from the CNRS ressource (CRB-UMS3387; http://xenopus.univ-rennes1.fr/). The different developing stages were obtained by _in vitro_ fertilisation and maintained in water at 20 °C. Embryo and larvae were staged according to Nieuwkoop and Faber (NF; 1967). Late embryo (NF35), premetamorphic larvae (NF42; NF47; NF49), prometamorphic (NF52; NF58), metamorphic (NF62), post-metamorphic (juveniles/NF66) and adult stages were fixed in PAF4% overnight at 4 °C. Before the fixation procedure, juveniles and adult stages were deeply anaesthetised in a 0.4 mg/ml solution of tricaine methanesulfonate (MS222; Sigma, St Louis, MO, USA), rapidly decapitated and then fixed. The brains were dissected out and postfixed in fresh 4% formaldehyde (dilution: 1:100) for 1 h. The brains were embedded in paraffin and sectioned transversally at 8 μm.

#### Immunohistochemistry

To characterise the cyp19a1-expressing cells, immunohistochemistry for the brain lipid binding protein (BLBP), proliferating cell nuclear antigen (PCNA), acetylated tubulin (TUB) and HuC/D (HU) was carried out. Following the _in situ_ hybridisation procedures, immunofluorescence for BLBP and PCNA was performed as described previously (33). For other immunodetections, we used, under similar conditions, mouse monoclonal antibodies, which recognise acetylated Tubulin (dilution 1:100; clone 611B1; Sigma) or HuC/D (dilution 1:100; clone 16A11; Invitrogen, Carlsbad, CA, USA) proteins. At the end of the immunodetection procedures, the slides were mounted in Vectashield containing DAPI. As controls, primary or secondary antibodies were omitted.

#### Results

**Spatio-temporal expression of the cyp19a1 gene in the developing brain of _Xenopus_**

The expression of the cyp19a1 gene was studied by _in situ_ hybridisation in the developing brain of _X. laevis_ from late embryonic through juvenile stages. Below, we first report the cyp19a1 expression pattern at the larva prometamorphic stage (Nieuwkoop and Faber stage 58; NF58) from a series of transverse sections through all subdivisions of the brain (Figs 1 and 2; n = 3). The nomenclature used in the present study is essentially the same as that employed by D’Amico et al. (34). Labelling appeared to be specific because no hybridisation signal was observed with the sense-strand probe (Fig. 2L,M). In addition, the conditions used for _in situ_ hybridisation were highly stringent because no cross-hybridisation signal could be observed with cyp19a1 transcripts of _X. tropicalis_ (Fig. 2N), a close relative of _X. laevis_.

We detected cyp19a1 transcripts in a variety of telencephalic and diencephalic areas. Rosstrally, at mid-telencephalic levels, a hybridisation signal for cyp19a1 transcripts was first detected in a few cells located in the ventral portion of the septal regions (arrows in Fig. 1A1,A2–B1,B2). As shown on adjacent sections and using a Vimentin probe, a reliable ventricular marker (33,34,38), the ventricular zone of the septum was devoid of cyp19a1-positive cells (compare Fig. 1A2,B3). Moving caudally, cyp19a1-positive cells were also observed in the bed nucleus of the stria terminalis (arrows in Fig. 1C1,C2). However, at this level, the most obvious cyp19a1-positive cell population was localised ventrally, just anterior to the preoptic recess (arrowheads in Fig. 1C1,C2). Again, no overlapping with Vimentin transcripts could be detected (Fig. 1C3). In the rostral diencephalon, at the level of the anterior commissure, strong cyp19a1 expression was detected in the anteroventral preoptic area (Fig. 1D1,D2). At this level, a uniform presence of cyp19a1 transcripts was detected throughout the dorsal and ventral portions close to the bed nucleus of the stria terminalis and the preoptic recess.
Fig. 1. Expression pattern of cyp19a1 (CYP) (A1–F1 and A2–F2) compared to Vimentin (VIM) (A3–F3) in the Xenopus laevis prometamorphic larva (NF stage 58) brain. Top: dorsal view of the X. laevis brain. Letters correspond to the rostrocaudal location of transverse sections as depicted in the whole brain drawing. Illustrations (A2) to (F2) correspond to higher magnifications of (A1) to (F1) and to adjacent sections of (A3) to (F3). Arrows and arrowheads highlight less conspicuous areas of labelling. For all images, dorsal is to the top. Scale bars = 100 μm.

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preoptic recess. Within the preoptic nucleus, only scattered cyp19a1-expressing cells were observable in more lateral areas (Fig. 1D1,D2). As highlighted with the Vimentin probe on adjacent sections (Fig. 1D3), cyp19a1-positive cells were observed in large numbers outside the ventricular zone of the preoptic recess (Fig. 1D2,D3). Cyp19a1-expressing cells were also found in more caudal aspects of the preoptic area, although the staining was restricted to more dorsal regions (Fig. 1E1,E2). However, very weak labelling was observed in a compact group of small cells in the most ventral aspect of the preoptic recess of the diencephalic ventricle (i.e. in the organum vasculosum laminae terminalis) (arrowheads in Fig. 1E1,E2).

Fig. 2. Expression pattern of cyp19a1 (CYP) (G1–K1 and G2–K2) compared to Vimentin (VIM) (G3–K3) in the Xenopus laevis prometamorphic larva (NF stage 58) brain. Letters correspond to the rostrocaudal location of transverse sections as depicted in the whole brain drawing of Fig. 1. Illustrations [a2] to [k2] correspond to higher magnifications of [g1] to [l1] and to adjacent sections of [g3] to [k3]. (l–n) Control in situ hybridisation experiments using cyp19a1 sense (m) or antisense (l and n) probes on X. laevis (l and m) or Xenopus tropicalis transverse sections. For all images, dorsal is to the top. Scale bars = 100 μm.
cantly expressed more laterally in a group of cells of the amygdala (arrows in Fig. 1E1,E2). In the posterior part of the preoptic area, at the level of optic chiasma, the cyp19a1 gene was expressed moderately in the ventral thalamic region (Fig. 1E1,E2). More caudally, the anterior aspect of the infundibular recess was surrounded by scattered cyp19a1-positive cells (Fig. 2o1,o2). In the mediobasal hypothalamus, cyp19a1 gene expression was strongly detected at all levels (Fig. 2n1,n2−n1). Indeed, cyp19a1-positive cells were observed close to the infundibular in its dorsal, middle, basolateral and basal portions, although positive cells were more abundant in the mammillary (Fig. 2n1−n2) and tuberal (Fig. 2n2−n2) regions. In situ hybridisation on diencephalic adjacent sections using the vimentin marker again suggest that cyp19a1-positive cells are not distributed in the ventricular layer (Fig. 2e3−e3).

In addition to the prometamorphic larva stage (NF58; Figs 1 and 2), expression of the cyp19a1 gene was also investigated at other developmental stages, from late embryonic through juvenile stages (Fig. 3; n = 35). Cyp19α1 expression was never observed in the late embryo (NF35; data not shown) but was detected for the first time in the developing brain at the early larval stage (NF42; Figs 3a and 3a). Indeed, two small populations of cyp19α1-positive cells were found in the primordia of both preoptic area (arrow in Fig. 3a) and hypothalamus (arrow in Fig. 3a). Interestingly, at late larval stages or for premetamorphic larvae (NF47, NF49 and NF52), the strongest expression was observed in two regions: the preoptic area (Fig. 3c,e,o) and the caudal aspect of the hypothalamus (Fig. 3v,f,v). This expression pattern remained similar at later developmental stages, in the prometamorphic (NF58, Fig. 3u), metamorphic (NF62; Fig. 3x,i), juvenile (NF66, Fig. 3w,n) and adult (Fig. 5o and data not shown) stages. Indeed, across development, the overall pattern of expression did not change dramatically. Messengers appeared in certain brain areas (preoptic and hypothalamic areas) at early larval stages and the signal increased progressively with age in these same areas. In addition, in late larval and post-metamorphic stages, we also found cyp19α1 expression, although at lower levels, in the other sites (ventral septum, bed nucleus stria terminalis, amygdala and ventral thalamus) described previously for the prometamorphic stage (NF58, Figs 1 and 2; arrows in Fig. 3; data not shown). It is also interesting to note that, although we studied five animals at each developmental stage, the overall expression pattern of cyp19α1b was very consistent from one animal to the other.

Fig. 3. Distribution of cyp19α1 (CYP) transcripts at different brain developmental stages. In situ hybridisations for cyp19α1 at late embryonic stage 42 (NF42; a, i); at premetamorphic stages NF47 (c, o), NF49 (i, l) and NF52 (o, n); and at prometamorphic NF58 (i, l), metamorphic NF62 (k, l) and post-metamorphic NF66 (juvenile; m, n) stages. For all developmental stages, transverse sections at the levels of the preoptic area (POA) (a, c, e, o, i, k, m) and hypothalamus (n, d, f, r, k, i, l, n) are shown. Arrows highlight less conspicuous areas of labelling. (−−−) For all images, dorsal is to the top. Scale bars = 100 μm.
Cyp19a1 is not expressed in radial glial cells or in mitotic progenitor cells but rather in post-mitotic neurones

In situ hybridisation on adjacent sections using the Vimentin probe (Figs 1 and 2) suggested that cyp19a1-positive cells were not localised in the ventricular layers (i.e. in radial glial or in progenitor cells). To firmly demonstrate this point, in situ fluorescent hybridisation using the cyp19a1 antisense probe was combined with immunofluorescence against cytoplasmic BLBP or the PCNA, which are established markers of radial glia or dividing progenitor cells.
cyp19a1 for Cyp19a1 (CYP) is not expressed in radial glial cells and/or in progenitor cells. Therefore, the neuronal marker HU was used because HuC/D proteins are RNA-binding proteins known to shuttle between the nucleolus and cytoplasm (42) and immunohistochemistry using HU antibodies displays nuclear and/or peri-nuclear staining (43,44). Because HU immunofluorescence signals did not persist when combined with in situ hybridisation conditions, we carefully examined cyp19a1 and HU labelling on transverse adjacent sections of the preoptic area (Fig. 5D,E; n = 3). As shown with the double-staining HU/DAPI (Fig. 5i), HU immunostaining was markedly detected in neuronal cell bodies away from the ventricular layer, similar to the localisation of cyp19a1-expressing cells (Fig. 5o). Using the Neurofibrilin post-mitotic neuronal marker (4S) on transverse adjacent sections (Fig. 5i), we also detected a similar distribution between cyp19a1 and Neurofibrilin transcripts (compared Fig. 5o, r; n = 3). Importantly, a comparison of cyp19a1 labelling with neuronal markers (HU and Neurofibrilin) also suggested that cyp19a1 was not expressed in all neurones but, instead, in sub-populations. Finally, we never observed cyp19a1 expression in oligodendrocytes, as assessed using the proteolipoprotein marker (data not shown). Taken together, our data demonstrate that the cyp19a1 gene is expressed in post-mitotic neurones and not in progenitors or radial glial cells.

Discussion
The present study provides a detailed description of the anatomical distribution of cyp19a1 transcripts during the development of the central nervous system in X. laevis, starting from the late embryonic stage and ending after completion of metamorphosis. We provide evidence that cyp19a1 gene expression is already significant during brain development in X. laevis and presents a regionalised pattern. Cyp19a1 messengers were first detected at the early larval stage in the primordia of the preoptic and hypothalamic areas and their expression strongly increased, before metamorphosis, at late larval stages. No obvious changes were observed at the prometamorphic, metamorphic and post-metamorphic stages, except that these cyp19a1-expressing areas became larger as the brain size increased during development. We also found minor sites of cyp19a1 expression in the septum, bed nucleus of the stria terminalis, amygdala...
and ventral thalamus. Previous RT-PCR analyses in *X. laevis* have monitored cyp19a1 gene expression in the brain from larval stage 48 to juvenile (27,32). Interestingly, PCR-based studies reported a high expression of cyp19a1 in the brain of *X. laevis* during this time without sex-specific expression. Moreover, our in situ hybridisation data in the brain correlate with previous in vitro detection of cyp19a1 expression and/or aromatase enzyme activity in the developing and/or adult brain of other amphibian species, including *X. tropicalis, Rana esculenta* and *Pleurodeles waltii* (24,29,31,46,47).

Importantly, the present study demonstrates that cyp19a1 expression appears in specific brain regions at a very early larval stage (NF42), clearly before the sensitive window for sex differentiation of *X. laevis* that occurs between stage 44 and 54 (48), and this expression remains elevated until post-metamorphic stages (juvenile and adult). High levels of aromatase activity have been measured in the developing brain of all vertebrate species studied, including mammals (1). Our data confirm that the brain is a major cyp19a1-expressing organ in amphibians.

We detected aromatase mRNA in a variety of diencephalic and telencephalic areas, including the medial and lateral septum, bed nucleus of the stria terminalis, amygdala (medial and lateral), preoptic area, ventral thalamus and hypothalamus. Clearly, the major sites of cyp19a1 expression are within brain regions involved in reproductive functions and behaviour. This expression pattern is well conserved compared to that known in other vertebrate species, notably in birds and mammals, in which cyp19a1 mRNA and protein have similarly been detected in the medial preoptic area, the ventromedial nucleus of the hypothalamus, bed nucleus stria terminals and the amygdala (i.e. areas involved in sexual behaviour and neuroendocrine control of reproduction) (14,49,50). Among non-mammalian vertebrates, teleost fishes exhibit exceptionally high levels of aromatase activity in the brain, higher than that in the ovary (51,52), and the functions of such high levels of enzymatic activity still remain open to speculation. Similar to other vertebrates, studies in several different fish species have reported elevated aromatase expression and activity in the telencephalon and diencephalon, notably the preoptic area and caudal hypothalamus (23,53). Expression of cyp19a1 and/or aromatase activity have also been documented in lizard and snake brains, and its distribution was also correlated with regions that control sex behaviours (54–57). Our results support the idea that the general pattern of cyp19a1 expression in the adult and developing brain is well conserved among vertebrates, reinforcing the assumption that its functions during development of the brain in amphibians, as well as at the adult stage, are similar to those identified in other vertebrates (i.e. in brain sexual differentiation and sexual behaviours). Nevertheless, the broad expression of cyp19a1 does not exclude important developmental roles other than brain sexual differentiation. In perfect agreement with our study, a large increase in cyp19a1 gene expression was identified on whole *X. tropicalis* specimens at early developmental stages (NF41) followed by a major aromatase activity at stage NF46 (Langois et al., 2010) and, interestingly, aromatase inhibition at these early developmental stages appears to affect the transcription of genes associated with the thyroid and reproductive axes (47).

In amphibians, two studies have reported distributions for immunoreactive aromatase; data were reported on the distribution of the aromatase protein in the preoptic and mesencephalic tegmentum of adult male *Peneaus esculentus* (58) and in the choroid plexus, olfactory bulbs and paleocortex of *X. laevis* at larva (NF50) stage (59). As noted in the Introduction, there have been a number of technical complications regarding the use of aromatase antisera in mammals, although there has been greater reliability in some non-mammalian vertebrates. These data, based on heterologous antibodies, do not fully match our present results based on a specific *X. laevis* riboprobe. In the anterior preoptic area of *P. esculentus*, few aromatase positive cells were detected in a similar way to the detection of *Xenopus cyp19a1* transcripts. There are several possible explanations for the discrepancies observed among in situ hybridisation and immunohistochemical studies, including antisera specificity, different frog species or developmental stages. Future studies that combine both procedures will provide significant advantages.

Although, in mammals and birds, aromatase expression under normal conditions has been reported mainly in neurones (60–62), studies in various fish species have consistently demonstrated that transcripts and proteins, corresponding to expression of the cyp19a1b gene (aromatase B), were exclusively detected in radial glial cells (26,41,43,53,63–66). Such cells play a major role in brain development because they are the origin, either directly or indirectly, of all brain cell types, including neurones and astrocytes (67,68), and this is also the case in adult fishes (41). Radial glial cells largely persist in the brain of adult teleost fishes in which they continue to generate new neurones throughout life (41,69–72). Until now, the functional significance of the strong expression of aromatase in the brain of fish, and specifically in radial glial cells, has not been understood. In the *X. laevis* brain, using radial glial, proliferation and neuronal markers, we demonstrate that cyp19a1 was probably exclusively expressed in neurones at all of the developmental stages studied, including post-metamorphic stages (juvenile and adult). Indeed, we demonstrated that radial glial cells and neural progenitors do not express cyp19a1. As noted above, under normal conditions, aromatase expression in the brain was largely reported in neurones, notably in the telencephalon and diencephalon of mammals (60,61,73) and birds (74,75), and in a very limited number of studies, in astrocytes (76,77). In summary, under normal conditions, cyp19a1 expression in amphibian *X. laevis* is very similar to other vertebrates in terms of brain aromatase-positive areas. Regarding the cell types expressing the aromatase enzyme encoding gene, our data suggest that, similar to mammals and birds, cyp19a1 expression is restricted to neurones. This is in sharp contrast with the situation in fish where aromatase is only found in radial glial cells of the developing and adult fish brain. Interestingly, in birds and mammals, reactive astrocytes express aromatase following brain injury and ischaemia (21,22,78–80). Because amphibians are well known for the high capability of their brains to regenerate after injury (81,82), it would be interesting to investigate whether ectopic cyp19a1 expression could be detected in radial glial cells in amphibians during the regenerative process, in a similar way to that occurring in other vertebrates.

In conclusion, the present study fills a gap in the literature regarding in situ cyp19a1/aromatase expression in vertebrates by
reporting information on the detailed sites of the expression of cyp19a1 during brain development in the amphibian *X. laevis*. We show that, similar to other tetrapods, cyp19a1 expression is mainly found in neurones, in contrast to the situation in teleosts. Although the present study represents a significant advance in our knowledge regarding cyp19a1 expression in the developing brain of the *Xenopus*, there are still many issues that remain to be addressed. In particular, it would be of interest to document the potential sexually dimorphic expression of cyp19a1 in the brain regions expressing this gene in adults. It would also be informative to compare *in situ* cyp19a1 expression with that of oestrogens and androgen receptors during brain development and adults. In teleosts, it is well documented that cyp19a1b expression is strongly regulated by oestrogens and some androgens, making this gene a target for xenogenic oestrogens (23,63,83). Whether this is also the case in amphibians requires further investigation given the sensitivity of amphibians to endocrine disruptors (84).

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