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▶ **To cite this version:**

Karen Mouriec, Elisabeth Pellegrini, Isabelle Anglade, Arnaud Menuet, Fátima Adrio, et al.. Synthesis of estrogens in progenitor cells of adult fish brain: Evolutive novelty or exaggeration of a more general mechanism implicating estrogens in neurogenesis?. Brain Research Bulletin, 2008, 75 (2-4), pp.274-80. 10.1016/j.brainresbull.2007.10.030 . hal-00266593

HAL Id: hal-00266593
https://hal.science/hal-00266593

Submitted on 5 Mar 2010

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Synthesis of estrogens in progenitor cells of adult fish brain: Evolutive novelty or exaggeration of a more general mechanism implicating estrogens in neurogenesis?

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Running title

Estrogens and neurogenesis in fish

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Acknowledgements : The research from our laboratory presented in this review was supported by grants form the CNRS, the Ministry of Research and Education and the European Union (EDEN Project). We thank Miranda Maybank for improving the English style.

Introduction

For a long time, it has been a prevailing dogma in neuroscience that the adult brain is unable to generate new neurons. Pioneer studies by Altman [2] or Kaplan and Hinds [31] suggesting that this assumption could be wrong, were received with scepticism or even criticism. Nowadays, it is well accepted that neurogenesis persists in discrete regions of the adult mammalian brain. However, active adult neural progenitors are restricted to two small areas of the telencephalon, the subependymal zone of the lateral ventricle (SEZ) and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus [29,42]. These findings stimulated a considerable number of studies aiming at isolating neural stem cells and understanding their basic biological properties, with the ultimate objective to manipulate them and enhance repair and regeneration.

It is now established that the capacity to produce new neurons as adults is a common feature of all vertebrates and also invertebrates [41]. In non-mammalian tetrapods, neurogenesis is most documented in birds where generation of new neurons appears restricted to a single region, the periventricular zone of the lateral ventricle. This also seems to be the case in lizards [41], whereas in amphibians adult neurogenesis is poorly documented apart from the olfactory bulb and retina [41]. In contrast, there is increasing information for teleost fish, which seem to have an unparalleled capacity to produce new neurons during the adult stages.

Teleost fish are the champions of adult neurogenesis

Teleost fish represent the largest group of actinopterygian fish, a lineage that diverged some 450 millions years ago from the sarcopterygian fish, their tetrapod ancestors. Whereas there is unfortunately little data on adult neurogenesis in the basal representatives of actinopterygians, holosteans (*Amia, Lepisosteus,...*) and chondrosteans (sturgeon), there is quite a lot of evidence suggesting that the adult brain of teleosts exhibits a unique capacity to

generate new neurons. In the late 60's, pioneer studies [34,36,37] demonstrated extensive cell proliferation in the telencephalon, diencephalon, cerebellum and spinal cord of fish. Using tritiated thymidine it was shown that, although the proliferative activity decreased progressively with age and size, the brain of the guppy still exhibited significant proliferative activity in the adult [37]. Since then, the existence of widespread periventricular proliferative zones (PZ) was confirmed in different teleost species [41]. In the stickleback, detailed comparisons of tritiated thymidine incorporation with PCNA and BrdU immunohistochemistry showed the presence of many proliferation zones in the entire brain; although the number of proliferative cells seemed to be more important in the telencephalon, diencephalon and mesencephalon [16]. This study demonstrated that all these PZ were located within or immediately below the ventricular layer, with the exception of the cerebellum in which proliferation occurred in the parenchyma. More recently, several studies further documented the presence of mitogenic zones capable of generating new neurons in many parts of the telencephalon, diencephalon, midbrain and cerebellum of zebrafish [1,27,56,73]. These data confirmed that adult fish exhibit an enormous potential for neurogenesis, compared to tetrapods, and that adult neurogenesis not only occurred in regions homologous to neurogenic regions in mammals, but also in many other regions throughout the entire brain. This is most likely linked to the fact that fish keep growing during their entire lifespan, making it necessary to constantly generate new neurons [8,9]. This, of course, suggests that in addition to the high proliferative activity, other cellular processes such as apoptosis, migration, and differentiation must occur, but these are poorly documented.

Thus, the question is: Why do fish adult stem cells maintain their mitogenic activity in many regions, whereas in other vertebrates adult neurogenesis is limited to just one or two discrete areas? Recent data in mammals indicate that a combination of intrinsic and extrinsic cues dictates the behaviour of adult neural progenitor [29,51]. In this review, we would like to

examine the possibility that one of the mechanisms participating in the establishment of a highly permissive environment in the adult fish brain could be linked to their unique capacity to produce estrogens, because of a strong expression of the estrogen synthesizing enzyme aromatase (estrogen synthase). Indeed, apart from this exaggerated proliferative activity, another major feature of the adult fish brain is its exceptionally high aromatase activity. Despite the fact that this has been known for some time, the significance of this peculiarity is still not clearly understood [54,55].

Aromatase, the estrogen-synthesizing enzyme is strongly expressed in radial glial cells of the adult fish brain.

The terminal step of estrogen biosynthesis is catalyzed by an enzyme complex termed aromatase (estrogen synthase). This key complex, bound to the endoplasmic reticulum membrane, is formed by the cytochrome P450 aromatase, a heme binding protein produced by the *cyp19* gene, and by an ubiquitous flavoprotein, the NADPH cytochrome P450 reductase [40]. Aromatase is expressed in the brain of all vertebrates [7], but fish exhibit several interesting and unique features compared to mammals and birds. Firstly, it is known that the total amount of enzymatic activity of aromatase (pmol/mg protein) in the brain of fish is one hundred to one thousand times higher than in corresponding regions of mammals and birds [12,54]. Microanatomical and biochemical techniques have shown that an exceptional potential for aromatization takes place in the forebrain of fish, particularly in the telencephalon, preoptic area and the hypothalamus [67]. Secondly, teleosts possess two aromatase genes in their genome. In mammals and birds, aromatase is generated from a single cyp19 gene that is regulated by multiple tissue-specific region promoters and alternative splicing, resulting in transcript variants but identical coding sequences [11]. In most fish, there are two distinct genes, *cyp19a* and *cyp19b*, each of which is regulated differently and encodes a structurally and functionally different aromatase protein [15,66]. Data from a large

sample of fish showed that the two genes have consistently different patterns of expression, CYP19A (Aromatase A) is predominantly expressed in the gonads while CYP19B (Aromatase B: AroB) is mainly expressed in the brain [15,24,48,66], providing a good example of partition of function between duplicated genes[60].

In the brain of birds and mammals, aromatase was for a long time only reported in neurons, notably of the diencephalon and limbic systems [7,72]. However, more recently aromatase was described in radial cells of the developing cortex in the mouse [45] and in astrocytes of the adult human cortex [71]. In contrast, another unique characteristic of teleosts is the fact that AroB expression in adults is strictly confined to radial glial cells. Such cells are characterized by a small nucleus adjacent to the ventricle and long radial processes terminating by **end feet** at the brain surface [59]. Radial cells are strongly involved in embryonic neurogenesis and, in contrast to mammals where they disappear at the end of neurogenesis, they largely persist in the adult brain of non-mammals, notably in fish. Strong expression of AroB in radial glial cells of fish was first shown in the plainfin midshipman [18] and then in trout, zebrafish, pejerrey and bluehead wrasse [44,48,50,63]. In the trout and zebrafish we showed that both AroB mRNA and protein expression, in adult mature fish, are strictly confined to radial glial cells. These AroB-expressing cells are most abundant in the forebrain, notably in the olfactory bulbs, the telencephalon, the preoptic area and the mediobasal hypothalamus, in particular along the lateral and posterior recesses [50]. However, consistent with the distribution of the messengers [48], AroB-positive cells were also observed in the periventricular layers bordering the optic tectum, the torus semicircularis and along the fourth ventricle [48,50].

That aromatase B is expressed in radial glial cells in the brain of adult teleosts is also supported by the fact that such cells were shown to express a variety of markers; in particular Brain Lipid Binding Protein (BLBP), a nervous system-specific member of the large family of

hydrophobic ligand binding proteins, which is exclusively expressed in radial glial cells and astrocytes throughout the developing brain [1,5,6]. BLBP displays very high expression in radial glial cells of zebrafish [1] where it is often co-expressed with AroB (K. Mouriec and N. Diotel, unpublished data). In addition, the exclusive expression of AroB in radial glial cells was confirmed by the fact that none of these cells express markers of post-mitotic neurons such as HuC/D, a RNA-binding protein selectively expressed in neurons, or acetylated tubulin [18,56].

Radial glial cells are progenitor cells in adult fish

The role of radial glial cells in embryonic neurogenesis is well established in birds and mammals. First known for serving as scaffolds for neuronal migration during embryonic neurogenesis [59], radial glial cells are now considered as progenitor cells able to generate glial cells (astrocytes, oligodendrocytes and ependymal cells) and neurons [25,26,52,53]. More recently, data showed that radial glial cells are the source of all brain neurons not only during development, but also in adults [26]. In birds, adult neurogenesis results from asymmetric division of the radial glial cells which give rise to newborn neurons migrating tangentially and radially along radial extensions to reach the telencephalon [4]. In adult mammals, astrocytes from the subventricular zone act as stem cells which divide to generate precursors ultimately generating neurons, some of which migrate to colonize olfactory bulbs [42]. Nevertheless, radial glial cells disappear after the embryogenesis in mammals [52,53]. In contrast, radial glial cells persist in other vertebrates, notably in birds [3] and fish [48,59]. However, it is only recently that the implication of radial cells in adult neurogenesis has been envisioned. Recent data in zebrafish have further confirmed the very high proliferative activity of the brain, already documented in other teleost species [1,27,56,73]. Using BrdU immunohistochemistry and aromatase B as a marker of radial glial cells it was found that, at short survival times (12 and 24 hours), a large majority of cells exhibiting BrdU labelling

corresponded to AroB-positive radial cells [56]. The radial nature of proliferative cells in the telencephalon and diencephalon was also indicated using antibodies to BLBP [1]. In addition, it was shown that, over time, newborn cells clearly move away from the periventricular proliferative zones, as indicated by double BrdU/PCNA staining [1,56,73], using radial processes as scaffolds [56]. Whilst in most of the forebrain newborn cells appear to move radially, actively dividing cells from the ventral subpallium generate rapidly dividing progenitors and neuroblasts that reach the olfactory bulb via a rostral migratory stream [1]. In the zebrafish, many of the newborn cells differentiate into neurons, as shown by combining BrdU and the use of several neuronal markers such as Hu or Acetylated-Tubulin [1,27,56,73] Thus, at least a subset of AroB-positive radial glial cells represents progenitor cells and is capable of actively dividing to generate new neurons. As mentioned above, aromatase is the only synthetic enzyme of estrogens; indicating that, provided aromatizable androgens are available, such cells will produce locally high amounts of estrogens that will act in a paracrine or autocrine fashion in these periventricular proliferative regions (Figure 1). Although preliminary results (K. Mouriec, unpublished data) indicate that E2 modulates cell proliferation in zebrafish, this remains to be thoroughly evaluated. However, increasing evidence suggests that estrogens may promote neurogenesis in other vertebrate models.

Emerging new functions of estrogens in embryonic, adult or reparative neurogenesis

Due to its well-documented synchronizing effects on the reproductive axis, estradiol (E2) is best know as a female sexual steroid. However, it is now considered, in both males and females, as a hormone exhibiting a myriad of neurotrophic and neuroprotective functions that are essential for neuronal development, survival and plasticity throughout life [12,24,62]. This variety of effects is correlated to the diversity of the cellular and molecular mechanisms underlying estrogen actions. Indeed, estrogenic effects may rely on activation of specific intracellular estrogen receptors, ERα and ERβ. These intracellular estrogen receptors

modulate gene expression and produce long-term genomic effects on interaction with plasma membrane sites that produce rapid non-genomic actions, and also on a growing number of receptor-independent mechanisms [69].

Estradiol is present in the brain of vertebrates from developmental stages until adulthood and is known for influencing neuronal differentiation, survival and plasticity [10,22,64,69]. However, a number of recent data suggest that estrogen could also influence the behaviour of progenitors and influence neurogenesis.

In rodents, it is known that brain aromatase activity is maximal during the embryonic period [40]. While aromatase was mostly believed to influence the construction of male specific structures according to the aromatization hypothesis [47], it was recently reported that aromatase is strongly expressed in radial cells of the embryonic neocortex and that such aromatase-expressing radial cells also express ERα [45]. Furthermore, in vitro E2 administration increased proliferation, while in utero blockade of estrogen receptors decreases proliferation of these embryonic cortical progenitor cells [45]. These data, whilst not forgetting the situation in adult fish [56], suggest a new functional role for E2 as a proliferative agent during critical stages of cerebral cortex development [45]. Such findings are in agreement with the fact that the brains of adult ERβ-/- knockout mice show regional neuronal hypocellularity, especially in the cerebral cortex [30]. While in rodents, the source of aromatizable androgens necessary for estrogen production in the cortex is not known, recent studies in songbirds (at posthatch day 1and 5) indicated that all the genes required for *de novo* estrogen synthesis from cholesterol are expressed in the developing brain of both sexes, with a spatial distribution similar to the known pattern of proliferating neuronal precursors along the lateral border of the lateral ventricle [43].

De novo synthesis of estrogens from cholesterol is also documented in neurogenic regions in the adult rodent, notably in the hippocampus, where the E2 concentrations can be higher than in the blood [28].

There is also rapidly accumulating data showing that estradiol modulates adult neurogenesis in the dentate gyrus of normal [17,20,21] or diabetic rats [61,62], a mechanism that would implicate ERα and ERβ [46]. Furthermore, the anatomical and functional implications of brain aromatase expression in the neurogenesis construction of vocal and auditory circuits in teleost fishes and songbirds, are also well documented [19,57].

In addition, estrogens have long been known for acting as a neuroprotective factor under brain repair situations. Until recently [45,71], aromatase expression had been reported in astrocytes (in rat) or in radial glial cells (in birds) only after chemical or mechanical lesions [23,57,58]. More lately, this increase in estrogen production around the lesions is correlated with increased neurogenesis as demonstrated by several recent studies [39,70]. Furthermore, it was shown that estradiol enhances neurogenesis following ischemic stroke through ERα and ERβ signalling [65].

All these data suggest that estrogens should be taken into consideration among other factors potentially regulating embryonic, adult and reparative neurogenesis. If it is clear that estrogens are locally produced in neurogenic regions, notably in the vicinity or even within progenitor cells, many questions remain open regarding the significance of these data. Open questions notably concern the regulation of brain aromatase expression and the origin, central or peripheral, of aromatizable androgenic precursors.

What triggers aromatase expression in the brain of fish?

The promoter of the zebrafish cyp19b gene has been characterized by several groups [13,32,50,68]. Analysis of the promoter sequence revealed the presence of a TATA box,

several putative *cis* regulatory elements including an Estrogen-Responsive Element (ERE) and an half ERE and pioneer studies indicated that AroB is up-regulated by E2, its own product [33,35]. Of particular interest is the finding that if embryos are treated for 2, 3 or 5 days with E2 (10nM) a very strong up-regulation of AroB messenger, protein and activity is observed exclusively in the radial glial cells of zebrafish larvae [50]. This effect is entirely blocked by an excess of the pure anti-estrogen ICI 182 780, indicating the requirement of one of the three zebrafish estrogen receptors [49]. The fact that ERs are necessary for the up-regulation of AroB, but not sufficient alone, is further evidenced by the fact that ER are expressed in many cells in the brain parenchyma that do not express AroB [49].This is further indicated by in vitro studies showing that E2 up-regulation of an AroB-reporter gene in the presence of E2 was only observed in certain cell contexts, again indicating the need of specific factors. Interestingly, strong up-regulation of the zebrafish AroB-luciferase reporter in the presence of zebrafish ER was observed only in P19 cells, differentiated into neurons and glial cells by retinoic acid, or in U251-MG human astrocytes indicating that a "neuroglial" cell context is necessary [14,38,55]. These data correlate well with the fact that deletion/mutation of the ERE on the AroB promoter results in the total absence of E2 induction. Interestingly, deletion/mutation of a sequence upstream of the ERE, named GxrE, also prevents the E2 stimulation of the AroB-luciferase even when the ERE is intact. As shown by gel shift experiments, this sequence is able to bind nuclear extracts from P19 cells differentiated into neurons and glial cells by retinoic acid, or in U251-MG human astrocytes, but not from other cell lines [50]. These data suggest that ER could act in cooperation with some unknown neuro-glial factor to promote the up-regulation of the cyp19b gene in neuro-glial or glial cells (Figure 2). So, our hypothesis is that a positive autoregulatory loop explains the high expression of Aro-B in radial glial cells of adult fish. Indeed, high circulating steroid levels will further promote AroB expression in radial cells. Whether such a mechanism is also

implicated in expression of aromatase in other vertebrates is unknown and requires urgent investigation.

Conclusions

These data stress the originality of the teleost fish brain in which progenitor radial glial cells, and not neurons, strongly express the estrogen synthesis enzyme, aromatase. Until recently, it was thought that aromatase expression in radial cells was a unique feature of teleosts, but very recent data suggest that aromatase is expressed in radial glial cells during embryogenesis or under brain repair situations in other vertebrates. In addition, an increasing number of studies point to the role of estrogens in cell proliferation and neurogenesis. Overall, this suggests that the brain of adult fish possibly presents an exaggeration of a more general mechanism involving estrogens in embryonic, adult and reparative neurogenesis.

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Legends to figure:

Figure 1: Schematic representation of the current hypothesis regarding the role of radial glial cells in adult neurogenesis in fish. Radial glial cells divide by asymmetric division and give birth to new cells (1). Such newborn cells can occasionally further divide (2) or migrate along the long radial processes (3) to give birth to neurons (4). It is also possible that newborn cells undergo apoptosis (5). A large number of in vivo and in vitro data have demonstrated that estradiol has the capacity of modulating all these cellular activities.

Figure 2: Schematic representation of the current hypothesis for the strong aromatase B expression in the radial cells of fish. A mandatory cooperation between estrogen receptor and a « glial »-specific factor (Gx), binding onto a GxRE sequence upstream the ERE, results in a high sensitivity of the *cyp19b* gene to E2. This hypothesis also explains why testosterone (T) also up-regulates aromatase B expression.

Figure(s)

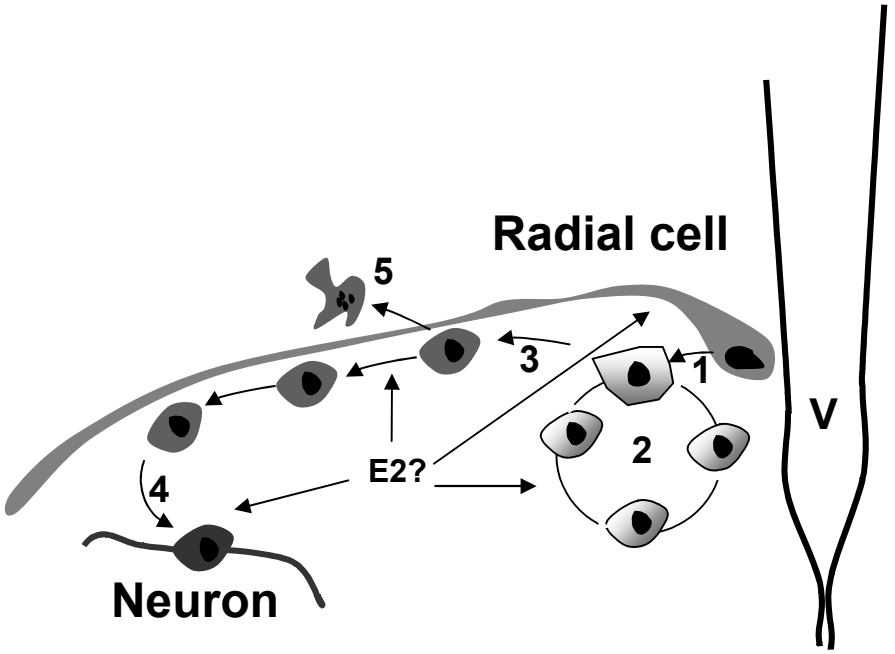


Figure 1

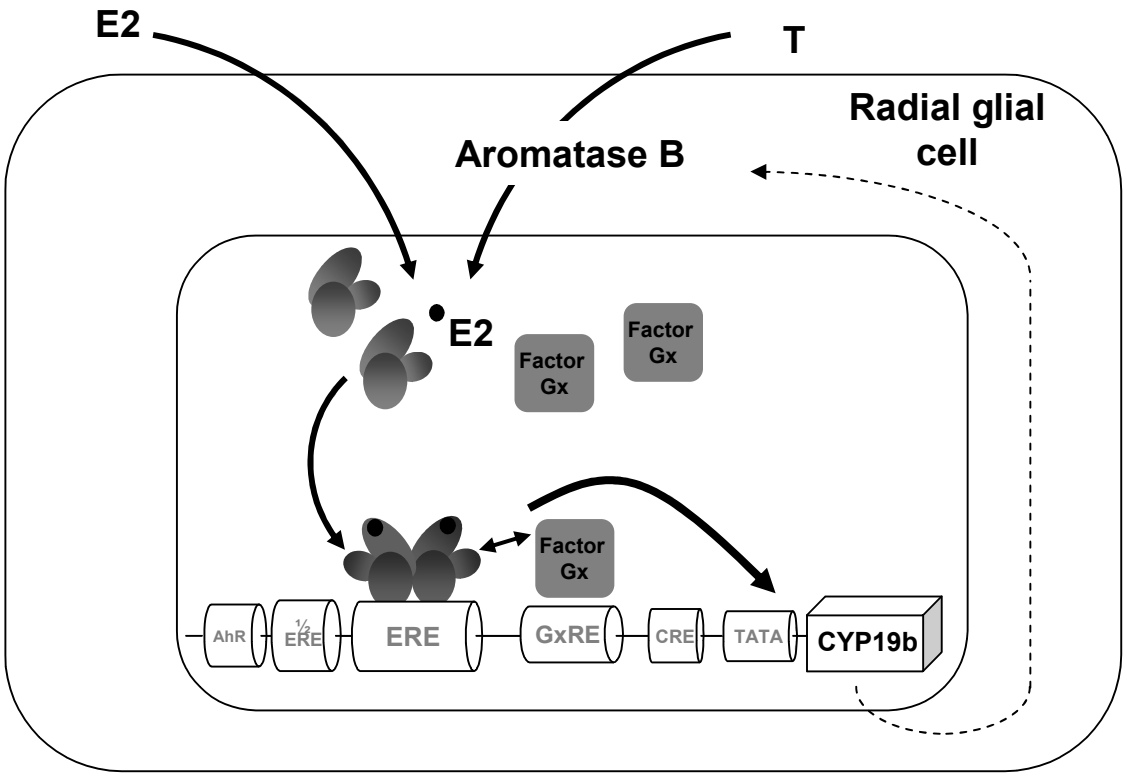

E2
T
Radial glial cell
Aromatase B
E2
Factor Gx
Factor Gx
Factor Gx
AhR
½ ERE
ERE
GxRE
CRE
TATA
CYP19b

Figure 2