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Brain Aromatase (Cyp19a1b) is a highly sensitive gene to estrogens and xeno-estrogens.

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Bibliographic note :

François BRION completed his PhD thesis in 2001 at the University of Metz investigating the effect of estrogens on the ER-regulated expression of vitellogenin and its use as biological marker of estrogenic compounds in fish. Since this date, he worked at INERIS still focusing on the effects of EDCs on the expression of hormone-regulated genes involved in key reproductive processes in fish. He coordinated a project of the National Research Program on Endocrine Disruptors on the molecular interactions between ER and AhR and their reproductive effects in fish and together with Olivier Kah he is working on the effects of EDCs at the central nervous system in zebrafish (NEED project of the National Research Agency). Since 2009, he also coordinates the NEMO project which aims to set-up a large panel of new *in vitro* and *in vivo* mechanism-based screening tools for studying EDCs effects in the zebrafish (*Danio rerio*). He supervised three thesis in the field of endocrine disruption in fish and is (co)-author of 29 peer-review articles.

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ABSTRACT :

Aromatase is the only enzyme responsible for the irreversible conversion of androgens into estrogens. Teleost fishes have two copies of the *cyp19a1* gene that encode two isoforms of aromatase: *cyp19a1a* encodes ovarian aromatase, while the *cyp19a1b* gene encodes brain aromatase (aromatase B). We have shown that (i) aromatase B is strongly expressed in radial glial cells (RGC), that act as stem cells in mammals and fish and ii) the *cyp19a1b* gene is very sensitive to estrogens, through a mechanism that involves a well conserved ERE. This feature makes this gene an outstanding biomarker of xeno-estrogen exposures and we have developed and validated an *in vivo* assay allowing detection of estrogenic activity with a very high sensitivity. The *in vivo* assay is based on a transgenic zebrafish tg(*cyp19a1b*-GFP) line that expresses GFP in RGCs and we demonstrate the usefulness of the transgenic *cyp19a1b-GFP* as a reliable, sensitive and rapid *in vivo* estrogenic screening assay

Introduction

It is now well established that a number of natural and man-made compounds referred to as Endocrine Disrupting Chemicals (EDCs) interfere with the endocrine system of animals, including wildlife and humans. A growing number of environmental compounds have been proved or suspected to act as endocrine disruptors. EDCs belong to structurally and functionally diverse group of chemicals and include, among others, natural and synthetic steroids, phyto- and myco-estrogens, alkylphenols, phtalates, bisphenolA, polybrominateddiphenyl ethers (PBDE's), several pesticides, dioxins and polycyclic aromatic hydrocarbons (PAHs). In a regulatory perspective, these compounds have been defined as exogenous substances or mixture that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations (WHO, 2002). They have been also defined according to their multiple modes of action on the endocrine system as exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processe (Kavlock et al., 1996).

Because a large proportion of potential EDCs end up in surface waters, aquatic species are particularly vulnerable to their potential adverse effects. In fact, endocrine disruption has been demonstrated to occur in wildlife, particularly in aquatic species (Tyler et al., 1998) and there is strong evidence supporting a causal linkage between exposure and effect (Jobling et al., 1998; Sanchez et al., 2011). Until now, most of the EDCs effects reported on fish been concerned their peripheral actions such as gonadal development, oocyte development, egg production and fertilization, sperm count and liver vitellogenin synthesis, an estrogen-dependent yolk precursor(for review see, e.g. (Hotchkiss et al., 2008). However, the reproductive axis also includes upper levels of regulation inthe central nervous system (CNS) that is likely to be targeted by a wide range of EDCs. For instance, we recently shown that the synthetic estrogen ethinylestradioland the alkyphenolic compound nonylphenol disrupt the ontogeny of GnRH system by inducing in a concentration-dependent manner the number of GnRH3 neurons in zebrafish embryos and larvae (Vosges et al., ; Vosges et al., 2010). These effects were observed as early as 5 days post-fertilisation and occurred at low and environmentally relevant concentrations. Furthermore, the effects ofnonylpenol and ethinylestradiol were shown to be blocked by the pure estrogen receptors (ERs) antagonist ICI 182-780, demonstrating the involvement of functional ERs in mediating their effects (Vosges et al.). The effects of EDCs upon neuroendocrine systems of fish are not limited to the GnRH neurons. For instance, other studies have shown the effects of several EDCs on the dopaminergic and serotoninergic circuits in fish (for a review see (Le Page et al., 2011)).

Another target of the central nervous system of fish known to be affected by EDCs is brain aromatase B, encoded by the *cyp19a1b* gene. In all vertebrates, cytochrome P450 aromatase (Cyp19a1) converts C19 androgens into C18 estrogens, important hormones involved in the control of many important physiological processes, notably reproduction. In vertebrates, with the notable exception of the pigs, aromatase is produced by a single *Cyp19a1* gene, whose tissue specific expression is driven by alternative promoters (Simpson *et al.*, 1994). Conversely, in many teleosts studied so far, including the model species zebrafish (*Danio rerio*), two structurally distinct *cyp19a* genes are found, *cyp19a1a* and *cyp19a1b*. These two genes generate two structurally and functionally different isoforms referred to as Aromatase A (AroA), predominantly expressed in gonads and Aromatase B (AroB), mainly found in neuronal tissues and responsible for the well-known exceptional aromatase activity of the brain of fish (Pasmanik and Callard, 1985; Gonzalez and Piferrer, 2003).

Expression of the *cyp19a1b* in the brain is regulated by estrogens (Menuet *et al.*, 2005) and accumulating data acknowledge aromatase B as a highly and sensitive target of estrogens and an outstanding biomarker of xenoestrogens exposure. In the developing and adult brain, aromatase B expression is located exclusively in radial glial cells which are stem cells as they are capable of giving birth to newneurons. Given the key role of these cells in neurogenesis, modulation of brain aromatase expression due to EDCs raises the question of their potentialeffect on neurogenesis.

Regulation of *cyp19a1b* expression in the brain of fish.

The structure of the *cyp19a1b*gene pointed out the presence of a well-conserved estrogen–response element (ERE) and a half ERE located within the 500bp of the *cyp19a1b* proximal promoter (Tchoudakova *et al.*, 2001; Tong and Chung, 2003). Transactivation experiments using zebrafish *cyp19a1b* linked to luciferase revealed that, in the presence of estrogen receptors, 17β-estradiol(E2) up-regulates *cyp19a1b*. This up-regulation was however highly dependent on the cell context. Indeed, while no or weak activity of the promoter following E2 treatment was observed in most cell lines studied (e.g., Hela, CHO), a dramatic luciferase activity was reported in cell line providing a glial or a neuro-glial cell context, i.e. U251-MG. Furthermore, deletions or mutations studies indicated that integrity of the ERE is absolutely required for the estrogenic regulation (Menuet *et al.*, 2005). But ERs are not sufficient to drive *cyp19a1b* expression in the brain of fish in the presence of estrogens. In fact, it was shown that a sequence, referred to as GxRE located between -277 and -257 bp from the initiation start site, plays an important role in the cell specific regulation of the *cyp19a1b* gene

and its E2-regulation (Le Page *et al.*, 2008). The cyp19a1b regulation requires glial specific factor(s) that bind to a glial x responsive element (GxRE)acting synergistically with the ERE sequences (Le Page *et al.*, 2008).This mechanism confers to the cyp19a1b gene a high sensitivity to estrogens and a cell-specific expression in radial glial cells (figure 1). In agreement with *in vitro* studies, it isnow well-established that short-term exposure of zebrafish embryos 17beta-estradiol (E2) for two days causes a strong increase in *cyp19a1b* mRNAs, Cyp19a1b protein expression in radial glial cells, and aromatase activity in the head. All these effects can be blocked by an excess of ICI 182 780, indicating the requirement of functional estrogen receptors (ERs) in mediating the effect of E2 on cyp19a1b expression (Menuet *et al.*, 2005). In contrast, the *cyp19a1a* gene that encodes gonadal aromatase is poorly sensitive to estrogens at this stage of development (Hinfray *et al.*, 2006; Cheshenko *et al.*, 2007).

Effect of endocrine disruptors on cyp19a1b expression.

The studies mentioned above clearly demonstrate the estrogenic regulation of the brain aromatase *cyp19a1b* gene in radial glial cell context both *in vitro* and *in vivo* and revealed that aromatase B is very sensitive to estrogens through ER-dependent mechanisms. Many environmental compounds belonging to various chemical families have been shown to bind and activate ERs. Among EDCs, synthetic steroidal estrogens (including the pharmaceutical 17α-ethinylestradiol) are some of the major and most potent estrogenic contaminants in the aquatic environment. Recently, we precisely documented the spatio-temporal expression of AroB within the central nervous system during the early development of control and EE2-exposed zebrafish. The strong and precocious up-regulation of AroB expression was observed in the brain of EE2-treated larvae as early as 5 dpf for EE2 concentration as low as 0.02 nM (figure 2). At 5 dpf, AroB-immunoreactive cells were located in the caudal olfactory bulbs, the telencephalon, the preoptic region, the optic tecta, the rostral hypothalamus and caudal hypothalamus and bordering the fourth ventricle up to the spinal cord. This expression pattern of AroB immunoreactivity in the brain remained unchanged but the intensity of AroB immunoreactivity increased during development demonstrating that up-regulation of AroB was time-dependent and EE2 concentration-dependent. This study confirms and further illustrates the extreme sensitivity of the zebrafish brain aromatase to (xeno)-estrogens by demonstrating that short exposures to low and environmentally relevant concentrations of EE2 are able to strongly induce the expression of AroB in radial glial cells in early life stages.

In addition to potent agonist ligands of the estrogen receptors, studies in zebrafish have demonstrated that weak estrogenic compounds such as genistein and zearalenone, individually or in combination are able to modulate *cyp19a1b* gene transcription at its promoter level. (Le Page *et al.*, 2006). *In vivo,,* the alkylphenolic compound NP is able to induce cyp19a1b mRNA levels (Kazeto *et al.*, 2004) and aromatase B protein expression in a concentration-dependent manner in radial glial cells of developing embryos, this effect being blocked by ICI 182-780 (Vosges *et al.*).

These data provide the rationale for using the *cyp19a1b* gene as an outstanding biomarker for xeno-estrogens. Recently, a transgenic zebrafish that expresses green fluorescent protein (GFP) driven by the zebrafish brain aromatase cyp19a1b promoter was generated (Tong *et al.*, 2009). This cyp19a1b-GFP transgenic line faithfully mimics the expression and the regulation of endogenous AroB expression in radial glial cells. Based on the use of the cyp19a1b-GFP transgenic, we developed and validated a novel *in vivo* assay to detect and to quantify the estrogenic potencies of chemicals. In our assay, zebrafish embryos are exposed from 0 to 5 days post-fertilization to chemical substances and GFP expression is quantified *in vivo* using fluorescence microscopy and image analysis (figure 3). Several estrogenic compounds belonging to various chemical families have been tested and includes natural and synthetic estrogens, phyto and myco-estrogens, alkylphenolic compounds several pesticides, etc. We showed that exposure of tg(*cyp19a1b*-GFP) embryos to various xeno-estrogens induces GFP expression in a concentration-dependent manner and demonstrated the sensitivity of the assay as evidence by the extremely low concentration of EE2 required to induce GFP expression. For instance, concentrations of EE2 as low as 5 pM are sufficient to up-regulate GFP expression in zebrafish embryos. In addition to xeno-estrogens, we have evaluated the effects of several Aryl hydrocarbon Receptor (AhR) ligands. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), or Benzo[a]Pyrene (B[a]P) did not affect GFP expression *in vivo*. However, when tg(cyp19a1b-GFP) embryo were co-exposed TCDD and E2, we have shown the attenuation of estrogen-induced up-regulation of GFP expression, showing that TCDD has anti-estrogenic effect which is in agreement with previously reported data on the expression of zebrafish aromatase *cyp19a1*b gene in response to TCDD (Cheshenko *et al.*, 2007).

Our data demonstrate the usefulness of the transgenic *cyp19a1b-GFP* as a reliable, sensitive and rapid *in vivo* estrogenic screening assay that could, together with fish-specific estrogenic *in vitro* assays (Le Page *et al.*, 2006; Cosnefroy *et al.*, In press), enhance the efficiency and accuracy of EDCs testing strategies for environmental hazard and risk assessment of chemical substances.

Conclusion

Recent studies have now identified a number of brain targets for xeno-estrogen commonly present in environmentally relevant concentrations in surface waters. The most prominent effect of EDCs, in zebrafish is their impact on the *cyp19a1b* gene that encodes aromatase B in the brain. Detailed molecular and whole animal studies in wild-type and transgenic cyp19a1b-GFP zebrafish demonstrated the extreme sensitivity of the *cyp19a1b* gene to xeno-estrogens. A striking feature of fishes is that aromatase expression is restricted to radial glial cells that behave as progenitors in developing and adult zebrafish. The consequences of disruption of cyp19a1b gene in radial glial cells are still unknown but may have important functional implications. Given that our recent studies demonstrated that estrogens modulate proliferative activity of radial glia progenitors, it is likely that (xeno)-estrogen have similar activity and thus the potential outcome requires thorough investigations. In any case, those studies have led to the development of a very sensitive *in vivo* assay that makes use of *cyp19a1b*-GFP transgenic embryos.

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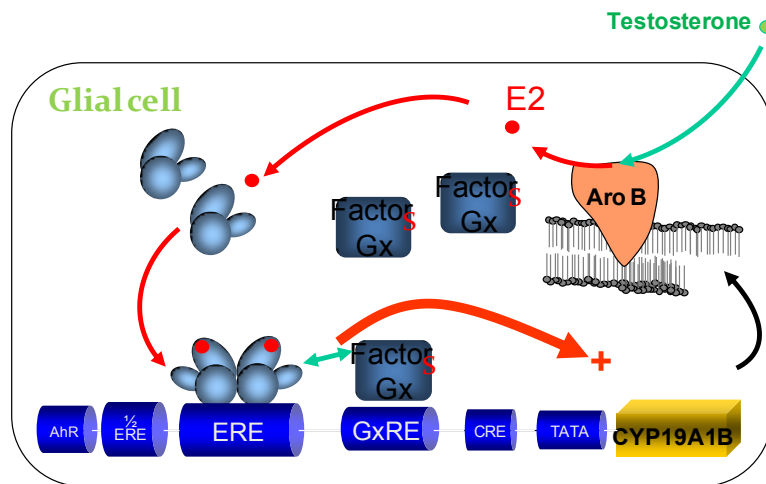
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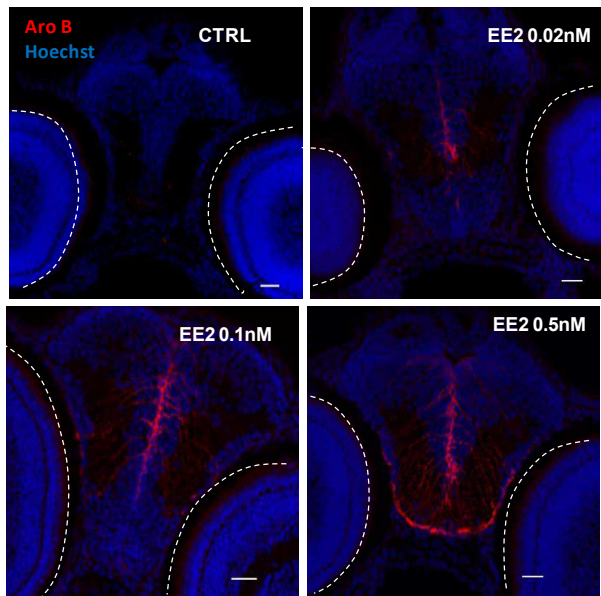
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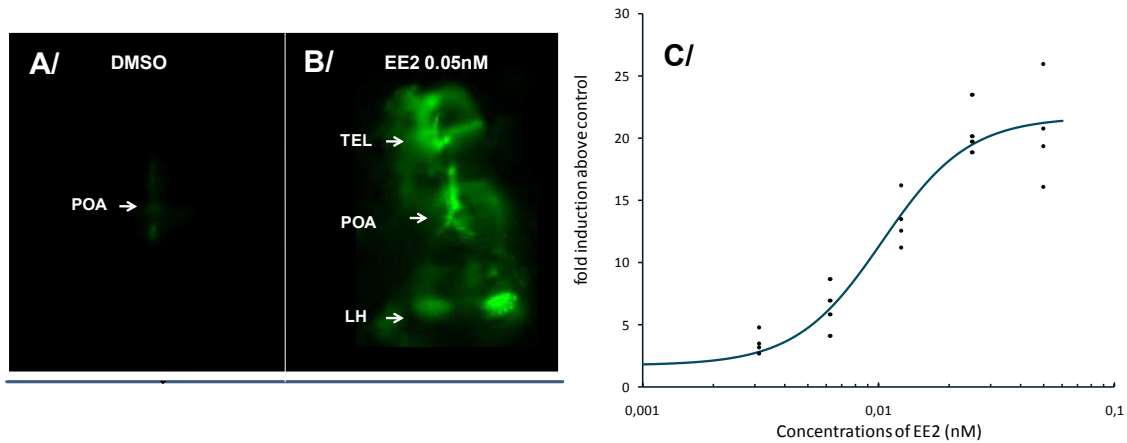
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Figure 1. Schematic representation of regulation of the cyp19a1b gene by estradiol in radial glial cells. AhR: binding site for the aryl hydrocarbon receptor; CRE: item responses to the cyclic AMP ERE: estrogen response element; GxRE: response element factor glial-specific (Gx) TATA: TATA box; E2: estradiol, T: testosterone; X: xenoestrogens. Adapted from Mouriec et al. (2008).

Figure 2. Photomicrographs of expression of AroB-ir cells (red) on transversal sections of 5-dpf old zebrafish larvae control and exposed to nM and 0.5nM of EE2 at the level of the preoptic area (200× magnification). Blue: Hoechst staining. Scale bars = 20µm.

Figure 3. In vivo imaging of live transgenic cyp19a1b-GFP zebrafish embryos A/ In control fish, GFP expression is weakly detectable in the preoptic area. B/ In EE2-exposed embryos a stronger fluorescence signal with a much wider distribution was observed and quantified by image analysis C/ Quantification of GFP induction using image analysis in transgenic cyp19a1b-GFP zebrafish embryos exposed to increasing concentrations of EE2. The effective concentration EC_{50} is 0.013nM and the lowest observed effect concentration is equal to 5pM. Each point is a mean of n=10 individus, N=4 independent experiments