Screening Estrogenic Activities of Chemicals or Mixtures In Vivo Using Transgenic (cyp19a1b-GFP) Zebrafish Embryos
François Brion, Yann Le Page, Benjamin Piccini, Olivier Cardoso, Sok-Keng Tong, Bon-Chu Chung, Olivier Kah

To cite this version:
François Brion, Yann Le Page, Benjamin Piccini, Olivier Cardoso, Sok-Keng Tong, et al.. Screening Estrogenic Activities of Chemicals or Mixtures In Vivo Using Transgenic (cyp19a1b-GFP) Zebrafish Embryos. PLoS ONE, Public Library of Science, 2012, 7 (5), pp.e36069. <10.1371/journal.pone.0036069>. <hal-00877371>

HAL Id: hal-00877371
https://hal.archives-ouvertes.fr/hal-00877371
Submitted on 28 Oct 2013

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Screening Estrogenic Activities of Chemicals or Mixtures In Vivo Using Transgenic (cyp19a1b-GFP) Zebrafish Embryos

François Brion¹, Yann Le Page², Benjamin Piccini¹, Olivier Cardoso¹, Sok-Keng Tong³, Bon-chu Chung³, Olivier Kah²*

¹ Unité d’Ecotoxicologie in vitro et in vivo, Direction des Risques Chroniques, Institut National de l’Environnement Industriel et des Risques (INERIS), Verneuil-en-Halatte, France, ² Université de Rennes 1, Institut de Recherche Santé Environnement & Travail (IRSET), INSERM U1085, BIOSIT, Campus de Beaulieu, Rennes France, ³ Taiwan Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan

Abstract

The tg(cyp19a1b-GFP) transgenic zebrafish expresses GFP (green fluorescent protein) under the control of the cyp19a1b gene, encoding brain aromatase. This gene has two major characteristics: (i) it is only expressed in radial glial progenitors in the brain of fish and (ii) it is exclusively sensitive to estrogens. Based on these properties, we demonstrate that natural or synthetic hormones (alone or in binary mixture), including androgens or progestagens, and industrial chemicals induce a concentration-dependent GFP expression in radial glial progenitors. As GFP expression can be quantified by in vivo imaging, this model presents a very powerful tool to screen and characterize compounds potentially acting as estrogen mimics either directly or after metabolization by the zebrafish embryo. This study also shows that radial glial cells that act as stem cells are direct targets for a large panel of endocrine disruptors, calling for more attention regarding the impact of environmental estrogens and/or certain pharmaceuticals on brain development. Altogether these data identify this in vivo bioassay as an interesting alternative to detect estrogen mimics in hazard and risk assessment perspective.

Introduction

Over the last 20 years, numerous examples have documented the adverse reproductive health effects of man-made compounds that, released in the environment, are capable of disrupting the endocrine system in wildlife and human populations [1]. To date, a growing number of structurally and functionally diverse groups of chemicals have been proven or suspected to have endocrine-disrupting chemical (EDCs) activity. Concerns about their effects on human and wildlife reproductive health have stimulated the development and implementation of screening and testing procedures for hazard and risk assessment [2].

EDCs are known to interfere with the endocrine system through multiple signalling pathways. One major mechanism of EDC effects involves their action as estrogen receptors (ERs) agonists. Until now, most studies dedicated to the actions of (xeno-)estrogens have focused on their effects at the level of the gonads and other peripheral tissues [2,3]. However, there is emerging evidence to show that EDCs, notably (xeno-)estrogens, act in the brain, notably on the development and functioning of the neuroendocrine circuits. However, at the present stage, such potential effects of EDCs are not taken into account in risk assessment, mainly because of the lack of readily accessible and validated models.

In this context, the cyp19a1b gene, which encodes a brain form of aromatase (aromatase B) in fish, is of particular relevance for several reasons. First, as documented in different species, this gene exhibits exquisite sensitivity to estrogens [4,5,6]. Second, cyp19a1b expression is strictly limited to radial glial cells (RGC) that act as neuronal progenitors in both developing and adult fish [7]. Furthermore, several studies point to this gene as a sensitive target for estrogen mimics [8,9]. We have developed a transgenic zebrafish tg(cyp19a1b-GFP) line that expresses GFP under the control of the cyp19a1b promoter [10]. As evidenced by careful validation procedures, this line shows perfect co-expression of GFP and endogenous aromatase B in RGC. The reason why cyp19a1b is only expressed in radial glial cells (RGC) is not fully understood. Nevertheless, previous studies showed that the estrogenic regulation of cyp19a1b expression requires a mandatory interaction between estrogen receptors acting through an estrogen response element (ERE) and an unknown glial factor that binds a sequence located upstream from the ERE in the promoter region of the cyp19a1b gene [5]. This results in an intriguing positive auto-regulatory loop through which aromatase, the estrogen-synthesizing enzyme, is up-regulated by E2 (17β-estradiol). This loop explains why aromatase B expression and activity are so high in the brain of sexually mature adult fish with high levels of sex.
steroids [11,12]. In contrast, in embryos, cyp19a1b expression is very low but can be strongly activated by E2 exposure as early as 24 hours post-fertilization, i.e. when both estrogen receptors and cyp19a1b start to be expressed in the brain [13].

This study aims at investigating the potential of a large spectrum of ligands, such as natural or synthetic steroids or ubiquitous environmental contaminants, to alter cyp19a1b-driven GFP expression in RGCs of developing zebrafish. Because the skull is transparent at these early development stages, GFP expression can be easily imaged and quantified in vivo without sacrificing the animals. The main finding of this study is that a number of chemicals can indeed target cyp19a1b-GFP expression through ER-activated mechanisms. These chemicals include established (xeno)-estrogens, but also several aromatizable or non-aromatizable androgens and synthetic progestagens, evidencing the usefulness and the validity of the in vivo tg(cyp19a1b-GFP) zebrafish test for screening compounds, alone or in mixtures.

Methods

Ethics

This study was approved by the ethics committees INERIS (Institut National de l’Environnement Industriel et des Risques) and CREEA (Comité Rennais d’Ethique en matière d’Expérimentation Animale) under permit number EEA B-35-040. All steps have been taken to reduce suffering of animals. Experiments were performed in accordance with European Union regulations concerning the protection of experimental animals (Directive 86/609/EEC).

Chemicals

17β-estradiol (E2), 17α-ethinylestradiol (EE2), estrone (E1), estriol (E3), diethylstilbestrol (DES), hexestrol (HEX), zearalenol (Zea), α-zearalenol (α-Zea), β-zearalenol (β-Zee) and α-zearalanol (α-Zea), genistein (Gen), diadzein (DZ), 4-tert-octylphenol

Figure 1. Upon exposure of embryos to estradiol, the tg(cyp19a1b-GFP) zebrafish expresses GFP only in radial glial cells. (a) Dorsal view of a zebrafish larva treated with 10 nM E2 showing that GFP signal is visible in the brain, notably in the telencephalon (tel), preoptic area (poa), and in the nucleus recessus posterioris (nrp) of the caudal hypothalamus; ob: olfactory bulb. (b) High resolution confocal image showing the RGCs in the telencephalon (tel), preoptic area (poa), nucleus recessus lateralis (nrl) and nucleus recessus posterioris (nrp) of the caudal hypothalamus. (c) High power view of the area shown in (b). Soma (s) are located along the midline except in the case of newborn cells (nb) undergoing migration (see Figure 2). RGCs have long cytoplasmic radial processes (rp) terminating by end-feet (ef) at the brain surface. (a) Bar = 200 μm; (b) Bar = 100 μm (c) Bar = 20 μm.

doi:10.1371/journal.pone.0036069.g001
Animals and Exposures to EDCs

Fertilized cyp19a1b-GFP transgenic zebrafish eggs were exposed to chemicals or to solvent control (DMSO, 0.01% v/v). Each experimental group consisted of 30 embryos exposed in 100 ml of water. Embryos were kept in an incubator at 28°C. cyp19a1b-GFP embryos were observed in dorsal view using an Olympus FLUOVIEW® FV10i confocal laser scanning microscope in multiple field of view mode. The 110 images constituting each of the 9 fields of view were merged plan by plan and the resulting z-stack was reconstructed in a 3D red-green anaglyph image with the ImageJ program (http://rsb.info.nih.gov/ij/).

In Vivo Imaging: Confocal Microscopy

Transgenic cyp19a1b-GFP zebrafish were fixed in paraformaldehyde and embedded in agarose at 8 dpf. The brain was imaged with an Olympus FLUOVIEW® FV10i confocal laser scanning microscope in multiple field of view mode. The 110 images constituting each of the 9 fields of view were merged plan by plan and the resulting z-stack was reconstructed in a 3D red-green anaglyph image with the ImageJ program (http://rsb.info.nih.gov/ij/).

In Vivo Imaging: Wide-field Fluorescence Microscopy

Live tg(cyp19a1b-GFP) embryos were observed in dorsal view and each was photographed using a Zeiss AxioImager.Z1 fluorescence microscope equipped with an AxioCam Mrm camera (Zeiss GmbH, Göttingen, Germany). All photographs were taken using the same parameters: only the head was photographed using a 134 ms exposure time and maximal intensity. Photographs were analyzed using the Axiovision Imaging software and fluorescence quantification was realized using the ImageJ software. For each picture, the integrated density was measured, i.e. the sum of the gray-values of all the pixels within the region of interest. A gray-value of 290 was defined as background value.

Data Analysis

Chemicals were tested in at least two independent experiments. Data are expressed as a mean fold induction above solvent control ± standard error of the mean (SEM). Concentration–response curves were modelled using the Regtox 7.5 Microsoft Excel™ macro (available at http://www.normalesup.org/~vindimian/fr_index.html), which uses the Hill equation model and allows using a Microsoft Excel™ macro [16]. To test the compliance of experimental data with CA and IA models, residues (differences between experimental and theoretical data) were first checked for normality using Shapiro–Wilks test. Then, a Student t-test (df = n–2) was used to test the following H₀ hypothesis: the mean of the residues is equal to 0 (α = 0.05). R²™ (R 2.13.1, software, R Development Core Team) was used for statistical analysis.

RNA Extraction and Quantitative Real-time PCR

After exposure, pools of 10 zebrafish were sonicated (10 sec, three times) in 250 µL Trizol Reagent (Gibco, Carlsbad, CA, USA), and total RNA was extracted according to the manufacturer’s protocol. Reverse transcription was carried out by incubating 2 µg total RNA with 10 mM random examer oligonucleotides, 10 mM DTT, 2.5 mM dNTPs and 100 U MMLX-RT (Promega) in the appropriate buffer for 30 min at 37°C and 15 min at 42°C. Polymerase chain reaction (PCR) was performed in an iCycler thermocycler coupled to the MyIQ detector (Bio-Rad, Hercules, CA, USA) using iQ SYBR-Green Supermix (Bio-Rad) according to the manufacturer’s protocol.

The following primers were used: EF-1 (fw) 5'-AGCCAGACGT-\linebreak GAGGAGTGAT-3', EF-1 (rev) 5'-CGCATTTGATAGTCAGTGGTAGT-3'; Cyp19a1b (fw) 5'-TCGGCACGGCTGCAACTAC-3', Cyp19a1b (rev) 5'-CATACTATGCTATGAGCAAC-3'; EGFP (fw) 5'-CGACGGCAACTACAGAGACCT-3', EGFP (rev) 5'-TAGTGTACTCCAGCTTGTCG-3'. Expression levels of EF-1 mRNA were used to normalize the expression of other genes. Melting curve and PCR efficiency analyses were performed to confirm correct amplification. Each experiment was performed at least twice in triplicate.

Table 1. Calculated Effective concentrations EC50 for E2, EE2, E1 and Genistein in transgenic cyp19a1b-GFP zebrafish line based on measurement of either cyp19a1b or GFP gene expression by PCR and by image analysis.

| Compound | Method | EC50 (nM) | ± CI 95 |
|----------|--------|----------|--------|
| E2       | cyp19a1b mRNA | 2.8 | 2.61–2.99 |
| E2       | GFP mRNA | 4.1 | 1.55–8.16 |
| E2       | Image analysis | 0.5 | 0.45–0.64 |
| EE2      | cyp19a1b mRNA | 0.04 | 0.031–0.051 |
| EE2      | GFP mRNA | 0.02 | 0.012–0.028 |
| EE2      | Image analysis | 0.01 | 0.01–0.012 |
| E1       | cyp19a1b mRNA | 2.3 | 2.14–1.81 |
| E1       | GFP mRNA | 1.3 | 0.88–2.15 |
| Genistein | cyp19a1b mRNA | 3545 | 3052–3556 |
| Genistein | GFP mRNA | 2466 | 1172–1926 |
| Genistein | Image analysis | 2.4 | 2.04–2.5 |

Results are expressed as mean ± confidence interval at 95% (CI 95%).

DOI:10.1371/journal.pone.0036069.t001

The following primers were used: EGFP (fw) 5'-TCGGCACGGCTGCACTACAGAGACCT-3', EGFP (rev) 5'-TAGTGTACTCCAGCTTGTCG-3'. Concentration–response curves were modelled using the Hill equation model and allows for single compounds (E2, E1 and EE2) and for binary mixtures of estrogens: E1+E2 at fixed ratio of 1:1 and E2+E2 at fixed ratio of 1:1. For each mixture, we performed two independent experiments. The Concentration Addition (CA) [14] and the Independent Action (IA) [15] models were used to model the theoretical concentration-response relationship for binary mixtures

| Image 58x24 to 76x41 |
Figure 2. *In vivo* imaging of 5-dpf old live transgenic cyp19α1b-GFP zebrafish embryos exposed to chemicals inducing GFP expression in radial glial progenitors. Dorsal views (anterior to the top) of the telencephalon (tel), preoptic area (poa), and nucleus recessus posterioris (nrp) of the caudal hypothalamus. For each chemical the concentration used is indicated. CTRL: solvent control, EE2: 17α-ethinylestradiol, E2: 17β-estradiol, E1: estrone, E3: estriol, DES: diethylstilbestrol, HEX: hexestrol, GEN: genistein, α-Zea: α-zearalenol, α-ZEE: α-zearalanol, β-ZEE: β-zearalanol, BPA: bisphenol A, 4-t-PP: 4-t-pentylphenol, 4-t-OP, 4-t octylphenol, NPmix: mixture of nonylphenol, o,p'DDT: 1,1,1-Trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane, MXC: methoxychlor, HPTE 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane, Testo: testosterone, DHT: dihydrotestosterone, 17α-MT: 17α-methyltestosterone, 17β-Trenb: 17β-trenbolone, Noreth.: 17α-Ethynyl-19-nortestosterone, D(-)N: 13β-Ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one, ICI (ICI 182-780).

doi:10.1371/journal.pone.0036069.g002
| Substance       | EC₅₀ (nM) | SD   | Max. fold induction | SD | CV (%) | REP       | n |
|-----------------|----------|------|---------------------|----|--------|-----------|---|
| **Synthetic estrogens** |          |      |                     |    |        |           |   |
| EE2             | 0.013    | 0.004| 18                  | 6.6| 31.4   | 36.6      | 6 |
| HEX             | 0.012    | 0.002| 24                  | 2.1| 18.6   | 39.1      | 3 |
| DES             | 0.01     | 0.004| 22.8                | 1.7| 36.6   | 45.8      | 3 |
| **Natural estrogens** |        |      |                     |    |        |           |   |
| E1              | 1.3      | 0.23 | 18.8                | 6.4| 18.1   | 0.36      | 3 |
| E2              | 0.48     | 0.27 | 16.4                | 8.5| 57.4   | 1         | 4 |
| E3              | 83.9     | 22.9 | 22.8                | 1.7| 36.6   | 45.8      | 3 |
| 4tOP            | 595      | 131.5| 11.0                | 4.2| 45.0   | 8.0E-04   | 3 |
| **Alkylphenols** |          |      |                     |    |        |           |   |
| 4tPP            | 2541     | 503  | 10.0                | 3.5| 19.8   | 1.88E-04  | 4 |
| 4NPmix          | 406      | 94.4 | 9.3                 | 0.3| 20.9   | 1.17E-03  | 4 |
| 4-n-NP          | n.e.     |      |                     |    |        |           | 2 |
| **Bisphenol**   |          |      |                     |    |        |           |   |
| Zearalenone     | 16       | 3.46 | 20                  | 1.0| 18.5   | 0.030     | 3 |
| **Phyto & myco-estrogens** |       |      |                     |    |        |           |   |
| α-Zearalanol    | >500     | 6.9  | 0.1                 | 7.4| -      | 2         | 2 |
| β-Zearalenol    | >500     | 5.5  | 1.0                 | 9.7| -      | 2         | 2 |
| Genistein       | 2501     | 6.1  | 8.1                 | 0.3| 0.2    | 1.91E-04  | 3 |
| **Pesticides**  |          |      |                     |    |        |           |   |
| op’DDT          | 257      | 25.4 | 11.4                | 0.6| 9.9    | 1.86E-03  | 3 |
| MXC             | 85       | 19.7 | 9.0                 | 1.5| 23.3   | 5.63E-03  | 3 |
| HPTE            | 477      | 49.2 | 7.4                 | 1.6| 10.3   | 9.99E-04  | 4 |
| Chlordecone     | n.e.     |      |                     |    |        |           | 2 |
| Endosulfan      | n.e.     |      |                     |    |        |           | 2 |
| **Androgens**   |          |      |                     |    |        |           |   |
| Testosterone    | 1031     | 313  | 11.3                | 2.3| 30     | 4.63E-04  | 3 |
| 17α-MT          | 35.4     | 19.0 | 4.5                 | 0.013|      | 0.013    | 2 |
| 11-Ketotestosterone | n.e. | -    | -                   | -  | -      | 2         | 2 |
| DHT             | 2003     | 697  | 20.9                | 3.7| 35     | 2.38E-04  | 3 |
| 17β-trenbolone  | 508      | 13.3 | 4.9                 | 9.38E-04|     | 9.38E-04  | 2 |
| **Progestagens**|          |      |                     |    |        |           |   |
| Norethindrone   | 9.01     | 0.58 | 20.1                | 4.2| 6.4    | 0.053     | 3 |
| D(–)Norgestrel  | 77.1     | 17.63| 19.0                | 3.3| 22.8   | 6.19E-03  | 2 |
| Progesterone    | n.e.     |      |                     |    |        |           | 2 |
| **Other compounds** |      |      |                     |    |        |           |   |
| Spironolactone  | n.e.     |      |                     |    |        |           | 2 |
| Dexamethasone   | n.e.     |      |                     |    |        |           | 2 |
| Rifampicine     | n.e.     |      |                     |    |        |           | 2 |
| Corticosterone  | n.e.     |      |                     |    |        |           | 2 |
| TCDD            | n.e.     |      |                     |    |        |           | 2 |
| BaP             | n.e.     |      |                     |    |        |           | 2 |
| BaA             | n.e.     |      |                     |    |        |           | 2 |
| diBaA           | n.e.     |      |                     |    |        |           | 2 |
| Chrysène        | n.e.     |      |                     |    |        |           | 2 |
| Benzophenone    | n.e.     |      |                     |    |        |           | 2 |
| EtOH            | n.e.     |      |                     |    |        |           | 2 |
| MetOH           | n.e.     |      |                     |    |        |           | 2 |
| KMnO₄           | n.e.     |      |                     |    |        |           | 2 |

Results are expressed as mean ± standard deviation (SD). 
N = number of independent experiments, n.e.: no effect, CV(%) = coefficient of variation inter-assay for EC₅₀. For each experiment, 10–15 transgenic zebrafish embryos were analyzed per condition.
doi:10.1371/journal.pone.0036069.t002

**Table 2.** Effective concentrations (EC₅₀), maximum fold of induction measured above solvent control and relative estrogenic potencies (REP) of various compounds belonging to different chemical families.
Figure 3. Dose-response curves of GFP induction in transgenic cyp19a1b-GFP embryos by various ligands (17α-ethinylestradiol is used as a reference).

(a) Natural estrogens and pharmaceutical compounds: EE2: 17α-ethinylestradiol; E2: 17β-estradiol; E1: estrone; E3: estriol; DES: diethylstilbestrol; HEX: hexestrol; GEN: genistein; α-ZEA: α-zearalenol; α-ZEE: α-zearalanol; β-ZEE: β-zearanol. The hexestrol curve in red is hardly visible because it is very similar to that of DES.

(b) Industrial chemicals: BPA: bisphenol A; 4-t-PP: 4-t-pentylphenol; 4-t-OP, 4-t octylphenol; NPmix: mixture of nonylphenol.

(c) Insecticides: o,p'DDT: 1,1,1-Trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane; MXC: methoxychlor; HPTE 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane.

(d) Androgens: Testo: testosterone; DHT: dihydrotestosterone; 17α-MT: 17α-methyltestosterone; 17β-Trenb: 17β-trenbolone; Noreth.: 17α-Ethynyl-19-nortestosterone (norethindrone); D(-)N: 13β-Ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one (levonorgestrel), ICI (ICI 182-780); R1881 (metribolone): androgen receptor agonist.

doi:10.1371/journal.pone.0036069.g003

Figure 4. GFP expression in zebrafish embryos exposed to various ER, AR and PR ligands alone or in combination with ICI. Results are expressed as fold induction above control (means ± SEM, n = indicates the number of 5-dpf old zebrafish examined).

doi:10.1371/journal.pone.0036069.g004
calculation of EC50. For a given chemical, EC50 was defined as the concentration inducing 50% of its maximal effect. Relative estrogenic potencies (REP) were determined as the ratio of EC50 of E2 to that of the test chemical. Correlation analyses between the EC50 of the tg(cyp19a1b-GFP) and in vitro assays were conducted on log-transformed EC50 data.

Results

In the cyp19a1b-GFP zebrafish line, GFP expression, perfectly matching cyp19a1b expression [10], can be strongly stimulated by estradiol. As visible in Figure 1A, GFP is strictly limited to RGC of the developing brain. Figure 1B shows the distribution and organization of the RGC with a high level of resolution in the brain of an 8 days-old zebrafish larva treated with 10 nM E2. GFP-expressing RGC exhibit soma located along the brain ventricles and long cytoplasmic radial processes terminating by end-feet at the brain surface. GFP-expressing RGC can make asymmetrical divisions, generating daughter cells that undergo migration along the radial processes (Figure 1c) and rapidly lose GFP expression to gain a neuronal phenotype [7,17].

To investigate if this model is relevant for assessing the potency of EDC to disrupt cyp19a1b in RGC, embryos were exposed for 5 days to increasing concentrations of 45 different compounds belonging to various chemical classes. The calculated EC50 based on measurements of cyp19a1b mRNAs, GFP mRNAs or in vivo imaging showed that the 3 methods yielded similar results (Table 1), indicating that GFP expression reflects the response of the endogenous gene. Figure 2 shows examples of the GFP signal generated by different active compounds. In 5 days-old controls, GFP expression is weakly detectable in the preoptic area, while embryos exposed to active compounds exhibit a stronger fluorescence signal with a much wider distribution from the anterior telencephalon to the caudal hypothalamus.

Selected compounds with well-known estrogenic activity included pharmaceuticals estrogens, natural estrogens, phyto and myco-estrogens, and environmental chemicals. Most of them were able to induce GFP expression in a concentration-dependent manner, but clear differences in terms of both EC50 and maximal induction were observed (Table 2, Figures 2 and 3). As expected, the synthetic estrogens E2, HEX and DES were the most active compounds (Figure 3a) characterized by extremely low EC50s (10 pM) and maximal inductions around 20 times the basal expression in controls. Based on their REP (relative estrogenic potency), they are much more potent than E2 by a factor 37–46. The natural estrogens, E2 and E1 yielded very similar responses (Figure 3a), while E3 was active at much higher concentrations and yielded very similar responses (Figure 3a), while E3 was active at much higher concentrations with an REP 175-fold lower than E2. The myco-estrogen zearalenone (Zea) and its metabolites α-Zea, α-Zee and β-Zee exhibited different response patterns (Figure 3a). Zea induced a full concentration-dependent response curve similar to those of the E2 and E1, while the three Zea metabolites did not elicited complete concentration-response curves. Among isoflavones, genistein was the only active compound, though at relatively high concentrations, and daidzein was inactive.

Among the various industrial chemicals tested, all alkylphenolic compounds were active, yielding similar concentration-dependent responses with the exception of the linear alkylphenol 4-n-NP that was inactive (Figure 3b). NPmix and 4-tert-OP exhibited similar
Figure 7. Effects of binary mixtures of estrogens on cyp19a1b-GFP expression. The combined effects of mixture of E1+E2 (ratio 1:10) and E2+EE2 (ratio 1:1) induced GFP expression in a concentration-dependent manner. Mixture means (green) is the mean of two independent assays, mixture assays 1 (pale blue) and 2 (red). CA: dose response curve generated by the CA model (black). IA: dose response curve generated by the IA model (blue).

doi:10.1371/journal.pone.0036069.g007

Discussion

This study confirms the high sensitivity of the cyp19a1b gene to estrogens and xeno-estrogens in the RGC context [4,6,18]. The tg(cyp19a1b-GFP) embryo assay is sensitive, fast, and cost effective for estrogen mimic screening. Twenty-one out of the 45 compounds tested induced GFP expression in a concentration-dependent manner through ER binding. For several of them, this study is the first to report estrogenic activity in vivo. In addition, this study demonstrates that a wide range of EDC targets RGC in fish brain, raising concern about the consequences of their actions on brain development and functioning.

The synthetic estrogens (EE2, DEX, HEX) were 37 to 49 times more potent than E2 with EC50s similar to those previously determined for estrogenic potencies while 4-tert-PP was 6-fold less active than NPmix. In comparison, the NP mixture was 8-fold more active than BPA. Several organochlorine pesticides were also tested (Figure 3b). The DDT-related compounds, o,p-DDT and MXC, induced strong GFP expression with similar response patterns (Figure 3c). Endosulfan and chlordane were inactive. The strong effect of MXC is of interest since it is known that its estrogenic potency is due to biotransformation into estrogenic metabolites. Among them, bis-desmethyl-MXC (HPTE) was capable of inducing GFP expression in RGCs. Interestingly, the EC50 of HPTE was higher than the EC50 for MXC, which could reflect the additive effect of several estrogenic metabolites derived from MXC and/or a higher excretion rate of HPTE compared to MXC. Again, this demonstrates the xenobiotic biotransformation capacities of embryos.

This is further illustrated by the fact that several natural and synthetic androgens also induced GFP expression. This was the case of the aromatizable androgens, T and 17α-MT (Figure 3d) an effect due to aromatization since it is blocked by co-exposure with the ER antagonist ICI 182,780 (Figure 4). In agreement, confirming previous studies [18], the non-aromatizable androgen 11-KT was totally inactive, while DHT, a non-aromatizable androgen, strongly up-regulates GFP expression (Figure 3d), an effect blocked by ICI 182-780 (Figure 4). Based on their REP, the non-aromatizable synthetic androgens, 17β-trenbolone and R1881, were 4200 and 1000-fold less active than E2, but induced strong GFP expression by a factor of 20 and 13, respectively (Figure 3d). These effects could not be blocked by the androgen receptor antagonist flutamide (Figure 5), but were in contrast blocked by co-exposure with ICI (Figure 4) In addition, two synthetic progestins, norethindrone and levonorgestrel, commonly used in oral contraception and post-menopausal disorders, induced GFP expression in a concentration-dependent manner (Figure 3d), while progesterone was inactive. Based on their respective EC50s, norethindrone was 8.5-fold more active compared to levonorgestrel and both exhibited lower estrogenic potencies compared to E2 (Table 1). Inhibition of progestins-induced fluorescence in embryos co-exposed with ICI revealed the involvement of ERs in mediating this effect (Figure 4). To further evaluate the specificity of the assay in detecting estrogenic activity, several other compounds were selected. The GR agonist dexamethasone, the MR antagonist spironolactone, the PXR agonist rifampicine, several aromatase inhibitors such as anastrozole, androstanediol and 4-hydroxyandrostenedione, the UV-filter benzophenone, ethanol, methanol and potassium permanganate were all unable to induce GFP expression.

Because of the ubiquitous character of dioxin-like compounds as environmental contaminants, agonists of the aryl hydrocarbon receptor (AhR) were evaluated (TCDD, BaP, BaA, diBaA, chrysene). None of the AhR agonist ligands were able to induce GFP expression in RGCs in agreement with previous data [19]. However, co-exposure of embryos to TCDD and EE2, significantly down-regulated the EE2-induced fluorescence confirming the anti-estrogenic effect of TCDD on ER-mediated mechanisms (Figure 6).

Combination effects of binary mixtures of estrogens were assessed using the fixed-ratio method. Experimental designs of mixtures were optimized so that the mixture concentrations covered a large range of effect predicted by the CA model. The combined effects of mixture of E1+E2 (ratio 1:10) and E2+EE2 (ratio 1:1), induced GFP expression in a concentration-dependent manner which were predicted by CA model [14] but not by IA model [15] (Figure 7).
reported in the most sensitive fish and human cell-based in vitro assays [8,20,21]. In transgenic zebrafish stably expressing ERE-Luciferase [21], EC_{50} for EE2 and E2 were 10 and 20 times higher, respectively, than those reported using the tg(cyp19a1b-GFP), further illustrating the sensitivity of the cyp19a1b gene to estrogenic and the sensitivity of this line. Zearalenone and zearalenone metabolites have been well described as ER agonists in both fish and human in vitro systems [22]. In this study, zearalenone exhibited a strong concentration-dependant induction of GFP while zearalenone metabolites induced partial concentration-response, indicating that zearalenone metabolites generally behave as partial agonists of fish ERs [20,23]. In agreement, zearalenone exhibited a comparably strong in vivo effect on reproduction, notably vitellogenin induction zebrafish, despite its low in vitro estrogenic potency [24]. The phyto-estrogen genistein clearly stimulated GFP expression in RGCs in agreement with previous data [25]. Interestingly, in tg(5xERE:GFP) fish genistein induced fluorescence in heart and liver, but not in brain [26].

In this assay, industrial chemicals with known estrogenic activity, such as alkylphenolic compounds (4NPmix, 4+OP, 4-t-PP), BPA, o,p’DDT, MXC, and its estrogenic metabolite HPTE, were active, in contrast with the fact that NP had no effect in ERE-luc zebrafish [21], tg-GFP [27] and 5xERE:GFP [26]. Differences were also noticed regarding the effect of BPA. In 5xERE:GFP larvae, BPA activates ER transcriptional activation only in heart and liver [26], whereas BPA induces GFP expression in RGCs of developing tg(cyp19a1b-GFP) further confirming recent data (15) of BPA on cyp19a1b expression in wild type zebrafish. Importantly, in mammals BPA adversely affects brain development and brain sexual differentiation [28,29].

In addition to the extreme sensitivity of the cyp19a1b gene, the biotransformation capacity of the tg(cyp19a1b-GFP) embryo is a clear advantage over in vitro assays. This is exemplified by MXC whose metabolites OH-MXC and HPTC directly interact with ER and potentially show long lasting addictive effects [30]. Testosterone and 17α-MT, the non-aromatizable DHT, but not 11-KT, were able to induce cyp19a1b expression in RGCs in an ER-dependent manner. While aromatase converts androgens into estrogens that subsequently bind to ERs to activate the cyp19a1b promoter [4,6,18], DHT effect involves conversion into 5α-androstane-3β,17β-diol, a metabolite of DHT with known estrogenic activity. Conversion of DHT into diols requires 5α-reductase and 3β-hydroxysteroid dehydrogenase, both of which are expressed in the brain of developing fish [31] and rodents [32].

17β-trenbolone acetate is a potent androgen extensively used in the United States as a growth promoter in beef. It is a recognized reproductive toxicant in fish [33]. R1881 is the 17-methylated derivative of 17β-trenbolone and is also a potent non-aromatizable androgen agonist of fish and human AR [34]. To our knowledge, this is the first report on the capacity of 17β-trenbolone and metabolites to activate an ER-dependent gene in a vertebrate. The metabolic pattern of 17β-trenbolone acetate revealed the presence of two major metabolites, 17α-trenbolone and trenadione that have low affinity for androgen receptor as compared to 17β-trenbolone acetate [35], however their affinity towards ERs is unknown [36]. Progesterone and 19-Nor-testosterone derivatives, used in contraception, behaved differently in tg(cyp19a1b-GFP) embryos. Progesterone had no activity as expected from its lack of estrogenicity [27,37]. But, we show for the first time that norethindrone and levonorgestrel, both of which are present in surface waters [38], were very active. In mammals, none of these compounds binds ERs, but they elicit estrogenic effects when they are metabolized into 3β,5α-tetrahydro norethindrone or norgestrel derivatives, which are likely responsible for the observed in vitro estrogenic effects of the parent compounds [39,40].

We also addressed the question of the combination effects of mixture of estrogenic hormones. We show that mixture of E2 and EE2 (E2+EE2; 1:1) as well as mixture of E1 and E2 (E1+E2; 1:10) acted in an additive manner on cyp19a1b-driven GFP expression that was predicted by the CA model, in agreement with previous data on vitellogenin synthesis [41] or on zebrafish cyp19a1b-luciferase activity in vitro [8]. It highlights the interest of the tg(cyp19a1b-GFP) in combination with CA models to assess combined effect of estrogenic compounds.

In conclusion, the tg(cyp19a1b-GFP) line clearly emerges as a simple, fast and reliable in vivo assay for monitoring the capacity of any chemical or its metabolites to activate ER-signalling in vivo at very early critical developmental stages. It is based on the use of an endogenous promoter and thus shows a true physiological brain-specific response. Its sensitivity is outstanding and comparable to the most performing in vitro assays [42]. In complement of the in vitro assay using the same cyp19a1b promoter [8], this in vivo assay will permit taking into account the biodisponibility and pharmacodynamics of chemicals. This will enhance the efficiency and accuracy of EDCs testing strategies while meeting the 3R policy (replacement, reduction, refinement) that is enforced by the OECD (Organisation for Economic Co-operation and Development) and the main environmental agencies worldwide [43].

Finally, although the potential consequences of such exposures are unknown, the present data showing direct effects of EDCs on gene expression in radial glial progenitors raise several serious issues in the context of risk assessment. One of them is to evaluate to what extent the present findings may apply to other vertebrates. Some studies indicate that estrogens indeed affect early brain development in rodents [44,45,46,47,48], but there is a lack on data the expression on steroidogenic enzymes, notably aromatase, and estrogen receptors, notably ERβ in the developing brain. Similarly, the roles of steroids in early aromatase expression [49] are unknown. Additionally, the potential production and effects of beta-diol, sometimes referred to as the “second estrogen”, have just started to receive some attention [50], albeit the present work recalls that this alternative pathway should not be forgotten in the context of developing animals.

Acknowledgments

The assistance of the staff of the BIOSIT zebrafish facility was greatly appreciated. Thanks are due to Cyril Turies and Fabrice Senger for their assistance in image analysis and in vivo imaging, respectively, and to Florence Zeman and Alexandre Pétry (Unité de Modélisation en Toxicologie et Ecotoxicologie, INERIS) for their help in mixture design and analysis. We appreciate the help of Sara Powers with the English style.

Author Contributions

Conceived and designed the experiments: FB OK. Performed the experiments: YL BP OC SKT. Analyzed the data: FB OK. Contributed reagents/materials/analysis tools: BCC. Wrote the paper: FB OK.
human and wildlife health: where we are today and where we need to go. Toxicol Sci 105: 235–259.

3. Tyler CR, Jolbing S, Sumpter JP (1998) Endocrine disruption in wildlife: a critical review of the evidence. Crit Rev Toxicol 28: 319–361.

4. Diefen N, Le Page Y, Mourier K, Tong SK, Pellegrini E, et al. (2010) Aromatase in the brain of teleost fish: expression, regulation and putative functions. Front Neuroendocrinol 31: 172–192.

5. Le Page Y, Menuet A, Kah O, Pakdel F (2008) Characterization of a cis-acting element involved in cell-specific expression of the zebrafish brain aromatase gene. Mol Reprod Dev 75: 1549–1557.

6. Menuet A, Pellegrini E, Brion F, Gueguem MM, Anglade I, et al. (2005) Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. J Comp Neurol 483: 309–320.

7. Pellegrini E, Mourier K, Anglade I, Menuet A, Le Page Y, et al. (2007) Identification of aromatase-positive radial glial cells as progenitor cells in the ventricular layer of the forebrain in zebrafish. J Comp Neurol 501: 150–167.

8. Le Page Y, Schole M, Kah O, Pakdel F (2006) Assessment of xenoestrogens using three distinct estrogen receptors and the zebrafish brain aromatase gene in a highly responsive glial cell system. Environ Health Perspect 114: 752–758.

9. Vosges M, Le Page Y, Chung BC, Combrincaur Y, Porcher JM, et al. (2010) 17alpha-ethinylestradiol disrupts the ontogeny of the forebrain GnRH system and the expression of brain aromatase during early development of zebrafish. Aquat Toxicol 99: 479–491.

10. Tong SK, Mourier K, Kuo MW, Pellegrini E, Gueguem MM, et al. (2009) A cyp19a1b-gfp (aromatase B) transgenic zebrafish line that expresses GFP in radial glial cells. Genesis 47: 67–73.

11. Pasmanik M, Callard GV (1985) Aromatase and 5 alpha-reductase in the teleost fish. J Appl Biol 26: 585–595.

12. Schwartz P, Thorpe KL, Bucheli TD, Wettstein FE, Burkhardt-Holm P, et al. (2010) Ontogeny of rapid estrogen-mediated extrasellar signal-regulated kinase signaling in the rat cerebellar cortex: potent nongenomic agonist and endocrine disrupting activity of the xenoestrogen bisphenol A. Endocrinology 146: 5388–5396.

13. Rubio BS, Lenkowski JR, Schaeberle CM, Vandenberg LN, Ronnheim PM, et al. (2006) Evidence of altered brain sexual differentiation in mice exposed perinatally to low, environmentally relevant levels of bisphenol A. Endocrinology 147: 3681–3691.

14. Berenbaum MC (1985) The expected effect of a combination of agents: the Bliss additivity model. Trends Pharmacol Sci 6: 347–351.

15. Bliss CI (1939) The toxicity of poisons applied jointly. Ann Appl Biol, 26: 585–595.

16. Rothenaigner I, Krecsmarik M, Hayes JA, Bahn B, Lepier A, et al. (2011) Clonal regulation of brain aromatase (cyp19a1b) by estrogen receptors during zebrafish development. Dev Dyn 238: 2641–2651.

17. Rothenaigner I, Krecsmarik M, Hayes JA, Bahn B, Lepier A, et al. (2011) Clonal regulation of brain aromatase (cyp19a1b) by estrogen receptors during zebrafish development. Dev Dyn 238: 2641–2651.

18. McCarthy MM (2009) The two faces of estradiol: effects on the developing nervous system. Brain Res Br J Dev Neurosci 30: 53–61.

19. Zsarnovszky A, Le HH, Wang HS, Bolchez SM (2003) Ontogeny of rapid estrogen-mediated extrasellar signal-regulated kinase signaling in the rat cerebellar cortex: potent nongenomic agonist and endocrine disrupting activity of the xenoestrogen bisphenol A. Endocrinology 146: 5388–5396.

20. Rubín BS, Lenkowski JR, Schaeberle CM, Vandenberg LN, Rönnheim PM, et al. (2006) Evidence of altered brain sexual differentiation in mice exposed perinatally to low, environmentally relevant levels of bisphenol A. Endocrinology 147: 3681–3691.

21. Miyashita M, Shimada T, Nakagami S, Kurikara N, Miyagawa H, et al. (2004) Enantioselective recognition of mono-demethylated methoxychlor metabolites by the estrogen receptor. Chemosphere 54: 1237–1267.

22. Umotzen K, De Regu JL, Anglade I, Vaillant C, Pellegrini E, et al. (2011) Activity and expression of steroidogenic enzymes in the brain of adult zebrafish. Eur J Neurosci 34: 45–56.

23. Sugiyama N, Andersson S, Lathe R, Fan X, Alonso-Magdalena P, et al. (2009) Spatiotemporal dynamics of the expression of estrogen receptors in the postnatal zebrafish brain. Mol Psychiatry 14: 223–232, 117.

24. Anklade GT, Jensen KM, Mackvyn EA, Kahl MD, Korte JJ, et al. (2009) Effects of the androgenic growth promoter 17-beta-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environ Toxicol Chem 22: 1322–1338.

25. Wilson VS, Cardon MC, Gray LE, Jr., Hartig PC (2007) Competitive binding comparison of endocrine-disrupting compounds to recombinant androgen receptor from fathead minnow, rainbow trout, and human. Environ Toxicol Chem 26: 1799–1802.

26. Bauer ER, Daxenberger A, Petri T, Saurerwein H, Meyer HH (2000) Characterisation of the affinity of different androgens and synthetic hormones to the human androgen receptor, human sex hormone binding globulin and to the bovine progestin receptor. APMM 108: 832–846.

27. Chen H, Hu J, Yang J, Wang Y, Xu H, et al. (2010) Generation of a fluorescent transgenic zebrafish for detection of environmental estrogens. Aquat Toxicol 96: 53–61.

28. OECD (2006) Draft Report of Pre-validation and Inter-laboratory Validation Methods for Environmental Endocrine Disruptor Screening Estrogen Mimics in Zebrafish Embryos.

29. McCarthy MM (2009) The two faces of estradiol: effects on the developing nervous system. Brain Res Br J Dev Neurosci 30: 53–61.

30. Brinton RD (2009) Estrogen-induced plasticity from cells to circuits: predictions from corticolimbic interactions. Trends Neurosci 32: 1222–1228.

31. Bradstreet KD, Morgan AR, Bhattacharya SK, Sumpter JP (2006) Endocrine disrupters: mechanisms of action and detection methods. Anal Bioanal Chem 378: 582–587.

32. Larrea F, Garcia-Becerra R, Borja-Cacho E, Cooney AJ, Jackson KJ, Lemes AE, et al. (2002) The intrinsic transcriptional estrogenic activity of a non-phenolic derivative of levonorgestrel is mediated via the estrogen receptor-alpha. J Steroid Biochem Mol Biol 82: 333–341.

33. Lephart ED (1996) A review of brain aromatase cytochrome P450. Brain Res Brain Res Rev 22: 1–26.

34. Borrelli J, Duranti E, Viader F, Duc I, Delansorne R, et al. (1995) Lack of estrogen potential of progesterone- or 19-nor-progesterone-derived progestins as opposed to testosterone or 19-nor-testosterone derivatives on endometrial Ishikawa cells. J Steroid Biochem Mol Biol 55: 77–84.

35. McCarthy MM (2009) The two faces of estradiol: effects on the developing brain. Neuroscientist 15: 599–610.

36. Bluetooth RD (2009) Estradiol-induced plasticity from cells to circuits: predictions for cognitive function. Trends Pharmacol Sci 30: 212–222.

37. Wang L, Andersson S, Warner M, Gustafson JA (2001) Morphological variations in the brains of estrogen receptor beta knockout mice reveal a role for ERbeta in migration of cortical neurons in the developing brain. Proc Natl Acad Sci U S A 100: 703–708.

38. Wang L, Andersson S, Warner M, Gustafson JA (2001) Morphological abnormalities in the brains of estrogen receptor beta knockout mice. Proc Natl Acad Sci U S A 98: 2792–2796.

39. Leavitt PD (1996) A review of brain aromatase cytochrome P450. Brain Res Brain Res Rev 22: 1–26.

40. Sugiyama N, Barros RP, Warner M, Gustafson JA (2010) ERbeta: recent understanding of estrogen signaling. Trends Endocrinol Metab 21: 345–352.