# Brain and gonadal aromatase as potential targets of endocrine disrupting chemicals in a model species, the zebrafish (Danio rerio)

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# Brain and gonadal aromatase as potential targets of endocrine disrupting chemicals in a model species, the zebrafish (*Danio rerio*).

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ABSTRACT

Many chemicals in the aquatic environment are able to adversely affect *in vitro* brain and ovarian aromatase expression/activity. However, it remains to be determined if these substances elicit *in vivo* effect in fish. With the view to further understanding possible effects of endocrine disrupting chemicals (EDCs) on aromatase function, we first developed methods to measure brain and ovarian aromatase expression/activity in a model species, the zebrafish, and assessed the effect of estradiol (E2) and androstatrienedione (ATD), a steroidal aromatase inhibitor. We showed that CYP19b gene was predominantly expressed in the brain whereas in the ovary CYP19a mRNA level was predominant. Moreover, aromatase activities (AA) were higher in brain than in ovary. In adult zebrafish, E2 treatment had no effect on aromatase expression/activity in brain, whereas at larval stage, E2 strongly triggered CYP19b expression. In the ovaries, E2 led to a complete inhibition of both CYP19a expression and AA. Exposure to ATD led to a total inhibition of both brain and ovarian AA but had no effect on CYP19 transcripts abundance. Together, these results provide relevant knowledge concerning the characterization of aromatase in the zebrafish, and reinforce the idea that brain and ovarian aromatase are promising markers of EDCs in fish and deserve further *in vivo* studies.

*Keywords* : zebrafish, aromatase, brain, gonad, biomarker.

## INTRODUCTION

To date, most attention on endocrine disrupting chemicals (EDCs) has been focused on compounds that interact with the estrogen receptor (ER). However, the endocrine system may also be disrupted by environmental substances through pathways and mechanisms others than those that are ER-mediated. Function of the hypothalamic-pituitary-gonadal (HPG) axis can be affected by xenobiotics that affect metabolism of sex steroid hormones. In this regard the biosynthesis of steroid hormones provides enzymatic targets for EDCs, particularly the steps catalysed by cytochrome P450-dependent enzymes (Sanderson and Van den Berg, 2003). Among these enzymes, cytochrome P450 aromatase (CYP19) is a crucial steroidogenic enzyme catalysing the final, rate-limiting step in the conversion of androgens into estrogens (Simpson *et al.*, 1994). Recent studies reported alteration of steroidogenesis associated with adverse reproductive effects in wild fish collected from contaminated sites (Noaksson *et al.*, 2001; Orlando *et al.*, 2002; Lavado *et al.*, 2004). However the nature (and the levels) of substances involved in these biological responses remains to be determined. In the laboratory, different substances from diverse chemical family have been shown to disrupt vertebrate aromatase (Monteiro *et al.*, 2000; Sanderson *et al.*, 2002). In fish, however, most of the data were obtained from *in vitro* studies (Monod *et al*., 1993)and little is known about their *in vivo* effects.

The purpose of this study was to assess the effect of the natural steroidal estrogen, estradiol (E2), product of the aromatisation reaction, and androstratienedione (ATD), a steroidal aromatase inhibitor, on aromatase expression and activities in the zebrafish (*Danio rerio*). The zebrafish is a prominent vertebrate model in a variety of biological discipline (Hill *et al.*, 2005) and is extensively used for assessing the effects of estrogenic compounds at various biological levels of organization (Brion *et al.*, 2002, 2004; Fenske and Segner, 2004; Nash *et al.*, 2004). In the zebrafish, two distinct aromatase genes have been isolated (CYP19a and

CYP19b) and their 5'-flanking region characterised (Kazeto *et al*., 2001). While their tissue and ontogenic expression profiles are relatively well described (Trant *et al.*, 2001), data on brain and ovarian AA in control and exposed-zebrafish are missing. With the view to further understanding possible effects of EDCs on aromatase function, detailed information on gene expression and enzyme activities in both the gonads and brain are needed and this was the first aim of this study.

## MATERIAL AND METHODS

### *Fish exposure to E2 and ATD*

Wild type zebrafish (*Danio rerio*) were obtained from our breeding unit. Female zebrafish were exposed for 7 days to 17-β-estradiol (E2, 10 nM), 1,4,6-androstatrien-3,17-dione (ATD, 1 µM) or solvent (DMSO). Exposures were realised under semi-static conditions with a total renewal of the water every day. Each substance was tested in duplicate separated tanks. One replicate was used to measure aromatase activity (N = 10 fish), and the other one to measure CYP19 mRNA levels (N = 7 fish).

Zebrafish larvae of 17 days post fertilisation (dpf) were exposed to either E2 (10 nM) alone or in combination with ICI 182-780 (Tocris, USA) or to solvent alone (DMSO) for 72 hours. Each experimental group was constituted of 20 zebrafish larvae exposed in 100 ml of water. One half of the water was renewed every day.

### *Fish dissection and tissue sampling*

Adult fish were euthanized by an overdose of MS-222, measured and weighted. Brain and gonads were removed, weighted and the gonado somatic index (GSI) was calculated.

Tissues (or larvae) used for subsequent determination of mRNA levels were immediately stored at 4°C in a solution of RNA*later*™ (Sigma-Aldrich, France). Samples were kept at 4°C overnight and conserved at −20°C until measurement. For AA measurement, tissues were rinsed in ice cold KCl (0.15 M) and homogenised in a 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM PMSF, 1 mM EDTA and 20% glycerol (v/v) in a ratio of 1:2 (w/v). S9 fractions were isolated by centrifugation of the homogenates (10 000 *g*, 15 min, 4°C). For the brains, S9 were aliquoted and stored at –80°C until used while for the ovaries, supernatants

were subsequently ultra-centrifuged at 100,000 *g* (90 min, 4°C). AA were measured in ovarian microsomes instead of S9 fractions to avoid variation of protein content during the reproductive cycle which may lead to incorrect measurement of AA in this tissue. The microsomal pellets were resuspended in the same buffer as used for the homogenisation. The total amount of proteins was determined by the method of Bradford with BSA (Bovine Serum Albumin, Sigma-Aldrich, France) as standard (Bradford, 1976).

*Determination of CYP19a and CYP19b mRNA levels*

CYP19 mRNA levels were measured by a branched DNA assay (QuantiGene, Genospectra, USA). It directly measures RNA and does not require reverse transcription and cDNA amplification. The bDNA assays were performed according to the manufacturer instructions. Tissues (or 10 pooled whole-body zebrafish larvae) were lysed and incubated in a 96-well plate coated with synthetic oligonucleotide in the presence of a specific probe set designed according to the CYP19a and CYP19b mRNA sequences (gene bank accession number AF183906 and AF183908 respectively). Capture probe allowed capture of the target mRNA to the synthetic oligonucleotide. Blocking Probe linearised the target mRNA and a label probe hybridised to the target mRNA and to a branched DNA (bDNA) coupled with Alkaline-Phosphatase-bound Probes. Addition of a chemiluminescence substrate (Dioxetan) yields a luminescence signal that is proportional to the amount of mRNA present in the sample. Quantification of luminescence was made on a microplate luminometer (Victor$^2$, Perkin Elmer, France). CYP19 expression values were normalised to a housekeeping gene, zf β-actin (gene bank accession number NM 131031). Measurement of target and housekeeping genes were realised in duplicate.

*Aromatase assay*

Brain and ovarian AA were measured in individual fish by the tritiated water assay modified from Thompson and Siiteri (1974). The following additions were made in a final volume of 500 µL : 200 µg of microsomal proteins (ovary) or S9 fraction proteins (brain), 20 µM of NADPH, 1 mM of NADP, 10 mM of G6P, 2 U/ml of G6PDH, 50 mM potassium phosphate buffer, pH 7.4. The mixture was preincubated at 27 °C for 10 min, and the reaction started with the addition of 150 nM of [1β-3H (N)]androst-4-ene-3,17-dione (Perkin Elmer, France). The reaction was stopped after 1 h of incubation by adding 1 ml of chloroform. The aqueous layer was extracted twice with chloroform and once with charcoal (5%, w/v). 150 µL of the aqueous phase was mixed with 750 µL of OptiPhase 'Hi safe' 3 (Perkin Elmer, France) before scintillation counting (Microbeta, Perkin Elmer, France). AA measurements were realised in duplicates. Results were expressed in fmol/mg/min.

*Data analysis and statistics*

Differences between groups were analysed for statistical significance with the Student's t-test. Results are expressed as means ± standard deviation, and differences between groups were considered to be significant if $p < 0.05$.

# RESULTS

## *Baseline aromatase expression and activity in adult female zebrafish*

The transcript abundance for CYP19a and CYP19b genes is shown in the Fig. 1A. In the brain, levels of CYP19b mRNA expression was about 45-fold higher than CYP19a expression, while, in the ovaries, levels of CYP19a mRNA expression was about 3-fold higher than CYP19b expression. Moreover, the level of expression of CYP19b was higher in the brain than the expression of CYP19a in the ovary. At enzymatic level, AA measured in female zebrafish by the tritiated water assay were higher in brain than in ovaries (Fig. 1B). There was about a 4-fold difference between brain and ovarian AA (34.0 ± 25.3 and 8.1 ± 8.2 fmol/mg/min respectively).

## *Effect of exposure to E2 and ATD*

In adult female zebrafish, E2 exposure had no effect on CYP19b expression in the brain but significantly inhibited CYP19a expression in the ovary (Fig. 2A). At enzymatic level, brain AA were not affected, but ovarian AA were totally inhibited (Fig. 2B).

In zebrafish larvae exposed between 17 and 20 dpf to E2 , there was no significant effect on CYP19a expression. However, a 16-fold increase in CYP19b expression was measured (Fig. 3). In addition, co-treatment with the pure antagonist of the ER receptor ICI 182-780 led to a significant inhibition of the E2-induced CYP19b mRNA levels.

In ATD-exposed fish, there were no significant effects neither on the transcript abundance of CYP19b gene in the brain nor on CYP19a expression in the ovary (Fig. 4A). However, ATD totally inhibited both brain and ovarian AA (Fig. 4B).

# DISCUSSION

## *CYP19 genes expression and AA in adult female zebrafish*

In contrast to mammals, many teleosts fish possess two forms of the aromatase gene in their genome, as it was demonstrated for zebrafish (Kishida and Callard, 2001). CYP19a and CYP19b transcripts, as measured by the bDNA assays, were both detected in ovarian and brain tissues of adult female zebrafish which is in agreement with previously reported CYP19 genes expression measured by RT-PCR (Trant *et al.*, 2001; Fenske and Segner, 2004). In the ovary, CYP19a mRNA level was higher compared to CYP19b, whereas CYP19b expression was predominant in the brain. Cerebral CYP19b was shown to be expressed at particularly high levels in adult female (about 600 fold CYP19a expression in the ovary) which is well in accordance with the difference observed by Trant *et al.* (2001). At the cellular level, ovarian CYP19a expression was localised in granulosa cells surrounding follicules (Goto-Kazeto *et al*., 2004); in the brain, P450aromataseB (AroB) was found to be express exclusively in radial glial cells at high levels in males and females (Menuet *et al*., 2005). Teleosts fish are well known for their exceptionally high cerebral levels of aromatase ( Pasmanik and Callard, 1988; Callard *et al.*, 2001). However, the functional outcomes of elevated aromatase expression in the brain of teleosts fish are still unresolved. Since teleosts fish shows a continuous neurogenesis throughout life, one hypothesis is that the high levels of neuroestrogens synthesis in adult may be related to the remarkable neuroplasticity and regenerative potential of the adult fish central nervous system (Callard *et al.*, 2001; Forlano *et al.*, 2001).

Further characterisation was achieved by measuring brain and ovarian AA in female zebrafish. In agreement with the CYP19 transcripts abundance, we showed that brain AA were significantly higher than ovarian AA which is in accordance with the general pattern of

AA described in other teleosts fish (Pasmanik and Callard, 1988; Gonzalez and Piferrer, 2002). These differences can be accounted for differences in the levels of mRNA expression in the two tissues (Trant *et al*., 2001; our study) and/or be due to the higher catalytic activity (Vmax) of the brain aromatase compared to the ovarian aromatase (data not shown).

*Effect of exposure of zebrafish E2 and ATD on CYP19 expression and AA*

At adult stage, E2 had no significant effect on CYP19b expression and AA in the brain of female zebrafish. In contrast, short-term exposure of zebrafish larvae to E2 strongly up-regulated CYP19b expression. This result confirms previous data obtained by RT-PCR on RNA from total zebrafish embryos and larvae exposed to E2 (Kishida and Callard, 2001). *In toto* hybridisation and immunohistochemistry experiments further revealed that E2 causes strong expression of AroB messengers and proteins in radial glial cells of zebrafish embryos and larvae (Menuet *et al.*, 2005). Additionally, the E2 dependant induction of the CYP19b gene was blocked by co-treatment with an excess of the pure ER antagonist ICI 182-780 indicating that functional ERs were involved. In teleosts fish brain, CYP19b gene is known to be under the control of a positive autoregulatory feedback loop driven by E2, the product of aromatization (Callard *et al.*, 2001). On a molecular basis, it was recently demonstrated that the E2-dependent regulation involves a direct transcriptional action of ERs requiring the synergistic effect of ERE and ½ ERE in the promoter region of the CYP19b gene (Menuet *et al.*, 2005). The absence of effect of E2 on aromatase in adult zebrafish brain is not surprising since the endogenous estrogenic stimulation on aromatase expression/activities is already high due to the positive autoregulatory feedback loop driven by E2 (Callard *et al.*, 2001). Our results clearly demonstrated that the CYP19b expression is very sensitive to (xeno)-estrogen in zebrafish larvae in comparison with adult fish and reinforce the idea that AroB is a

promising marker of estrogenic compounds in zebrafish early life stages (Menuet *et al.*, 2005). In contrast to the dramatic alteration of the CYP19b gene expression, E2 had no effect on CYP19a expression in zebrafish larvae. In zebrafish fry exposed to high concentration of EE2, conflicting results were obtained since RT-PCR analysis revealed no effect or down-regulation of the CYP19a gene (Trant *et al.*, 2001; Kazeto *et al.*, 2004). At adult stage, both CYP19a expression and AA were totally inhibited in the ovary of E2-exposed fish and our results suggest that the inhibitory effect of E2 on AA in ovary of mature female is mediated through a transcriptional inhibition of the CYP19a gene. However, the exact mechanism of action of E2 on CYP19a expression remains to be determined. It may be attributed to a negative feedback action of E2 on gonadotropins release, which are known to stimulate aromatase gene expression and activities in ovarian follicles (Gen *et al.*, 2001; Kagawa *et al.*, 2003). However, a direct effect of E2 on the ovary cannot be excluded. Indeed, it has been demonstrated that the inhibitory effect of E2 on steroidogenic enzymes mRNA levels in undifferentiated testis of rainbow trout did not imply follicle stimulating hormone (Baron *et al.*, 2005). The absence of ERE in the promoter region of CYP19a gene do not support an ER-mediated effect. It is interesting to note that similar concentration of E2 leads to an alteration of oogenesis in females, the ovaries of exposed fish being composed mainly of immature oocytes (Brion *et al.*, 2000). It can be suggested that the effects seen at histological level are mediated, at least in part, by inhibition of ovarian aromatase.

Exposure of female zebrafish to the steroidal aromatase inhibitor ATD resulted in dramatic inhibition of AA in both brain and ovary. ATD is known to inhibit vertebrate aromatase by binding irreversibly to the active site of the enzyme (Yue and Brodie, 1997). We previously showed that ATD exhibited a high efficiency at inhibiting *in vitro* brain and ovarian microsomal AA in rainbow trout (Hinfray et al., 2004). The *in vivo* effects of ATD on AA are thus consistent with the *in vitro* inhibitory effect of this molecule. In contrast to the

effect seen at the enzymatic level, exposure to ATD had no effect neither on the transcriptional activity of the CYP19b gene nor the CYP19a gene. As previously stated, CYP19b transcription in fish brain is up-regulated by E2 through a positive feedback loop (Callard *et al.*, 2001). Thus, it could be expected that the local deprivation of E2 due to aromatase inhibition in the brain could lead to a decreasing transcript abundance of the CYP19b gene. The absence of effect might be due to the short-term exposure duration of fish to ATD and it is likely that a prolonged exposure time would result in a significant effect, as shown in letrozole-exposed zebrafish larvae for 30 days (Kazeto *et al*., 2004).

## CONCLUSION

This study provides relevant data on aromatase expression at the gene and enzymatic levels in brain and in ovary of adult female fish. We showed that CYP19b expression was predominant in brain whereas CYP19a expression was predominant in ovary and that AA were higher in brain than in ovary. Our results indicate that E2 and ATD exposures deeply affect aromatase expression and/or activities in the zebrafish. The observed effects were dependent on the sub-cellular level at which aromatase function was assessed (gene expression, AA), the target tissue (brain, ovary) and the life stage of development (larvae, adult). From these results it appears that measurements of both CYP19 gene expression and AA in the zebrafish are relevant and promising molecular and biochemical markers of EDCs in fish.

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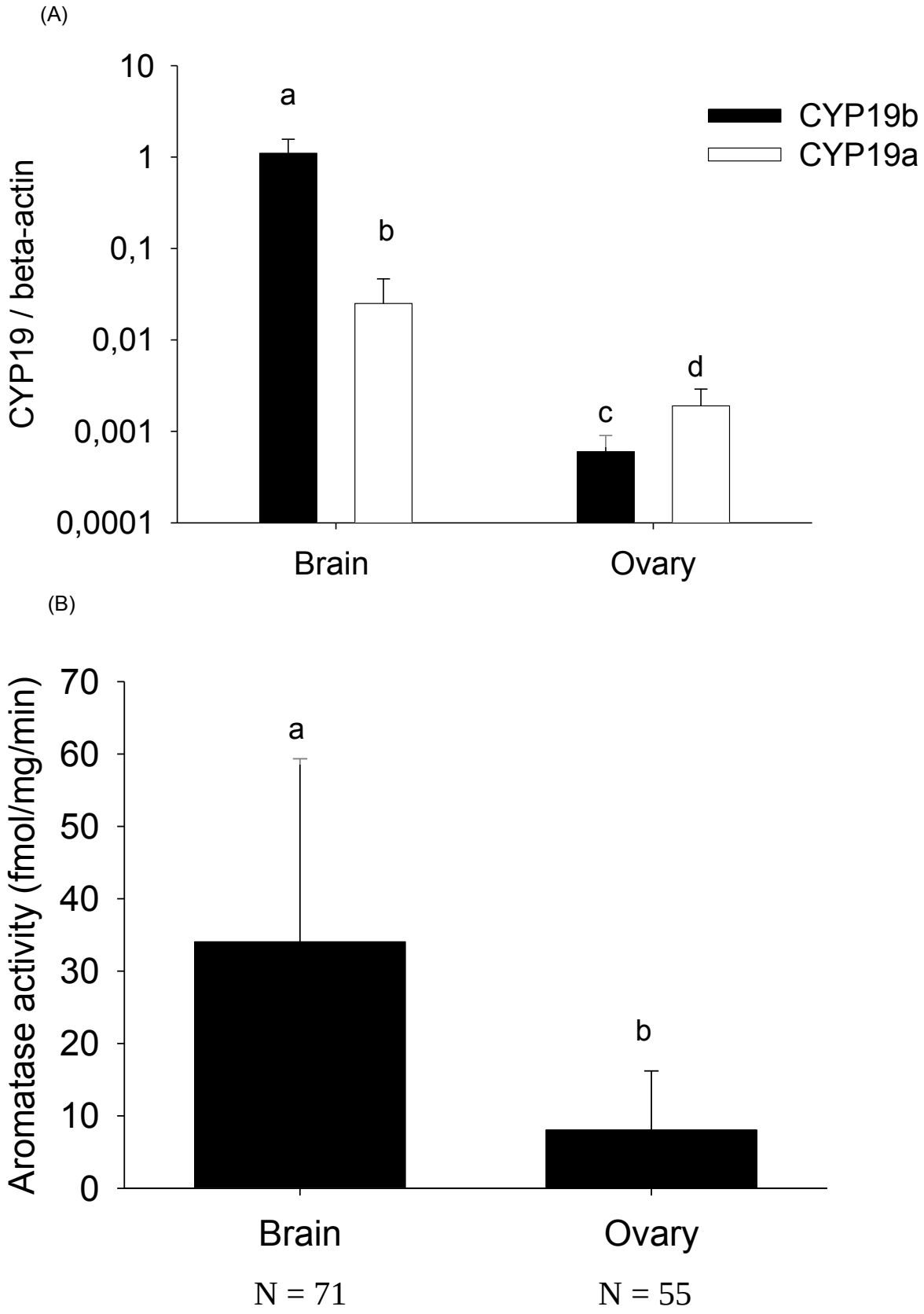
FIGURE LEGENDS

Fig. 1 : Relative transcript abundance of CYP19a and CYP19b gene (normalised to beta-actin transcript abundance in the same samples) (A) and aromatase activity (B) measured in brain and ovary of adult female zebrafish. Results are expressed as means and SD. Different letters indicate statistically different values (Student's t-test, $p < 0.05$)

Fig. 2 : Effect of E2 on CYP19a and CYP19b in ovary and brain respectively (A) and on aromatase activity (B). Results are expressed as means and SD. SC = solvent control. Different letters indicate statistically different values (Student's t-test, $p < 0.05$).

Fig. 3 : Effect of E2 alone or in combination with ICI 182-780 on CYP19a and CYP19b transcript abundance in zebrafish larvae after a 72 hours *in vivo* exposure between 17 and 20 dpf. SC = solvent control. N = 2 replicate for each group tested (one replicate correspond to 10 larvae). Different letters indicate statistically different values (Student's t-test, $p < 0.05$).

Fig. 4 : Effect of ATD on CYP19a and CYP19b transcript abundance in ovary and brain respectively (A) and on aromatase activity (B). Results are expressed as means and SD. SC = solvent control.Different letters indicate statistically different values (Student's t-test, $p < 0.05$).


(A)
CYP19 / beta-actin
10
1
0,1
0,01
0,001
0,0001
a
b
c
d
CYP19b
CYP19a
Brain
Ovary
(B)
Aromatase activity (fmol/mg/min)
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60
50
40
30
20
10
0
a
b
Brain
Ovary
N = 71
N = 55

Fig. 1.

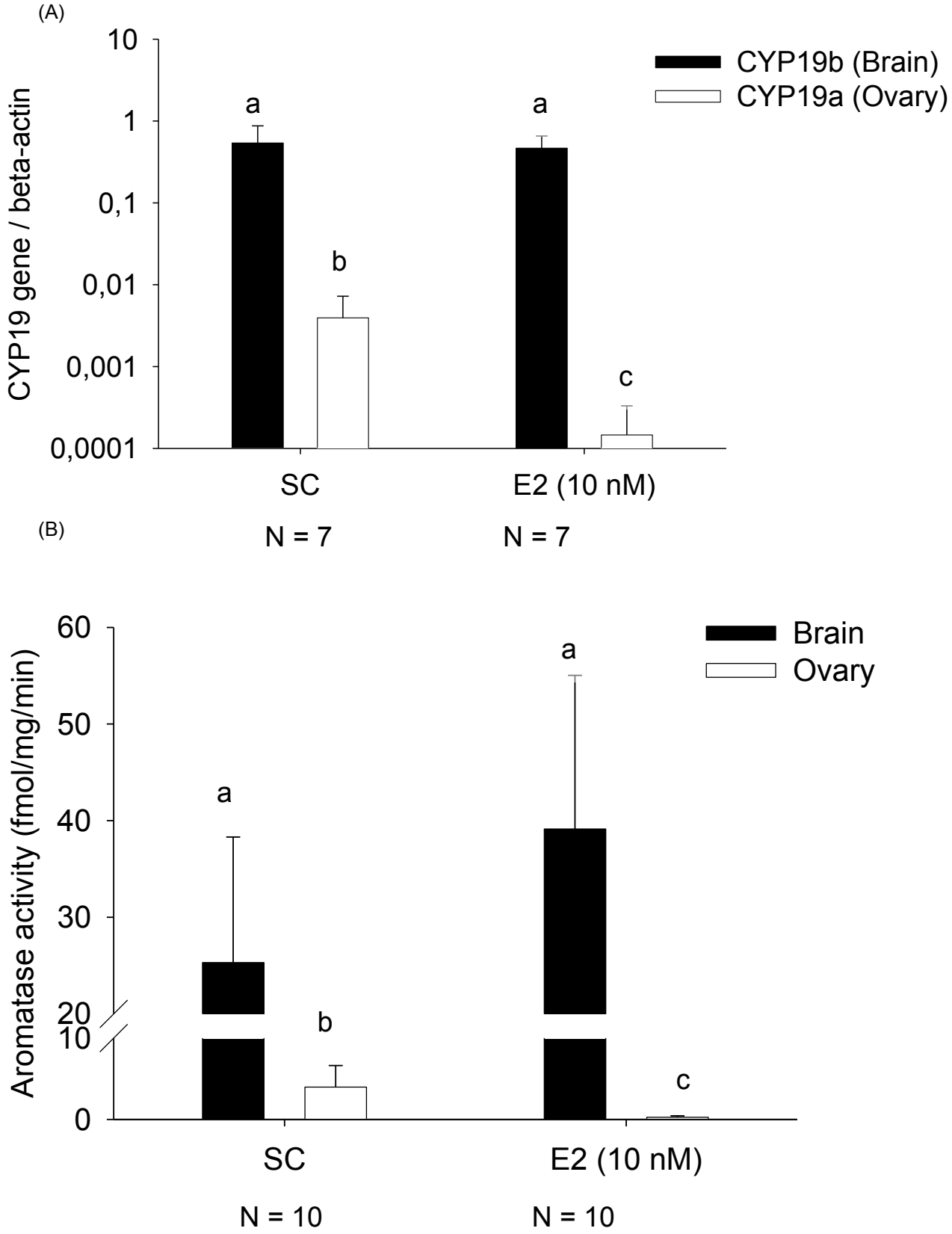
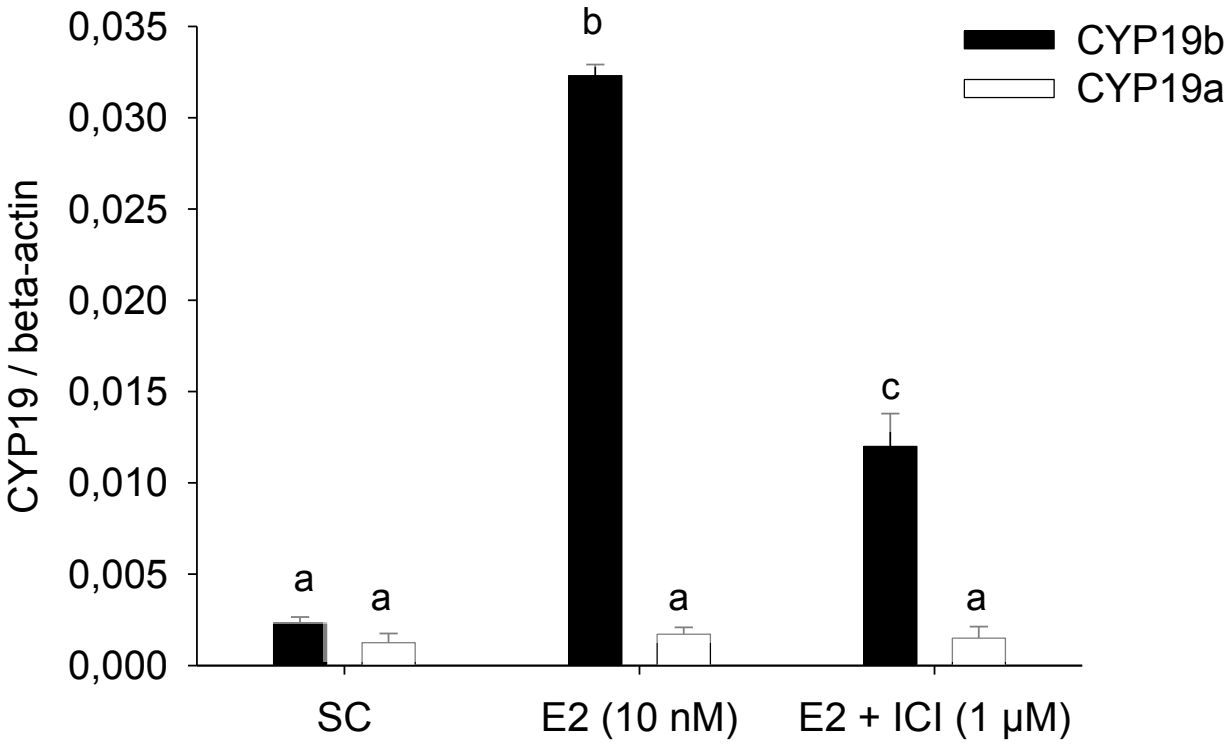

(A)
CYP19 gene / beta-actin
10
1
0,1
0,01
0,001
0,0001
a
b
a
c
CYP19b (Brain)
CYP19a (Ovary)
SC
E2 (10 nM)
N = 7
N = 7
(B)
Aromatase activity (fmol/mg/min)
60
50
40
30
20
10
0
a
b
a
c
Brain
Ovary
SC
E2 (10 nM)
N = 10
N = 10

Fig. 2.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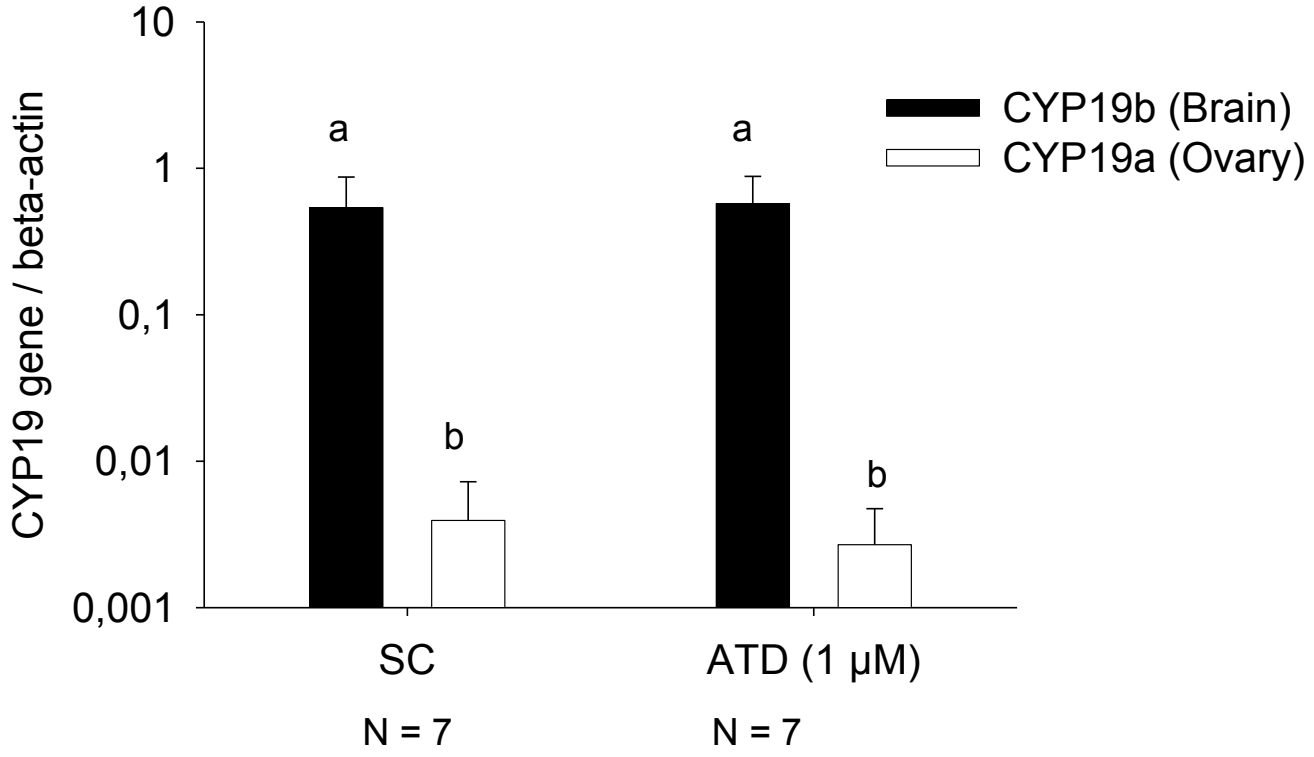
0,020
0,015
0,010
0,005
0,000
CYP19 / beta-actin
CYP19b
CYP19a
a
a
b
a
c
a
SC
E2 (10 nM)
E2 + ICI (1 µM)

Fig. 3.


10
1
0,1
0,01
0,001
CYP19 gene / beta-actin
a
a
b
b
CYP19b (Brain)
CYP19a (Ovary)
SC
ATD (1 µM)
N = 7
N = 7

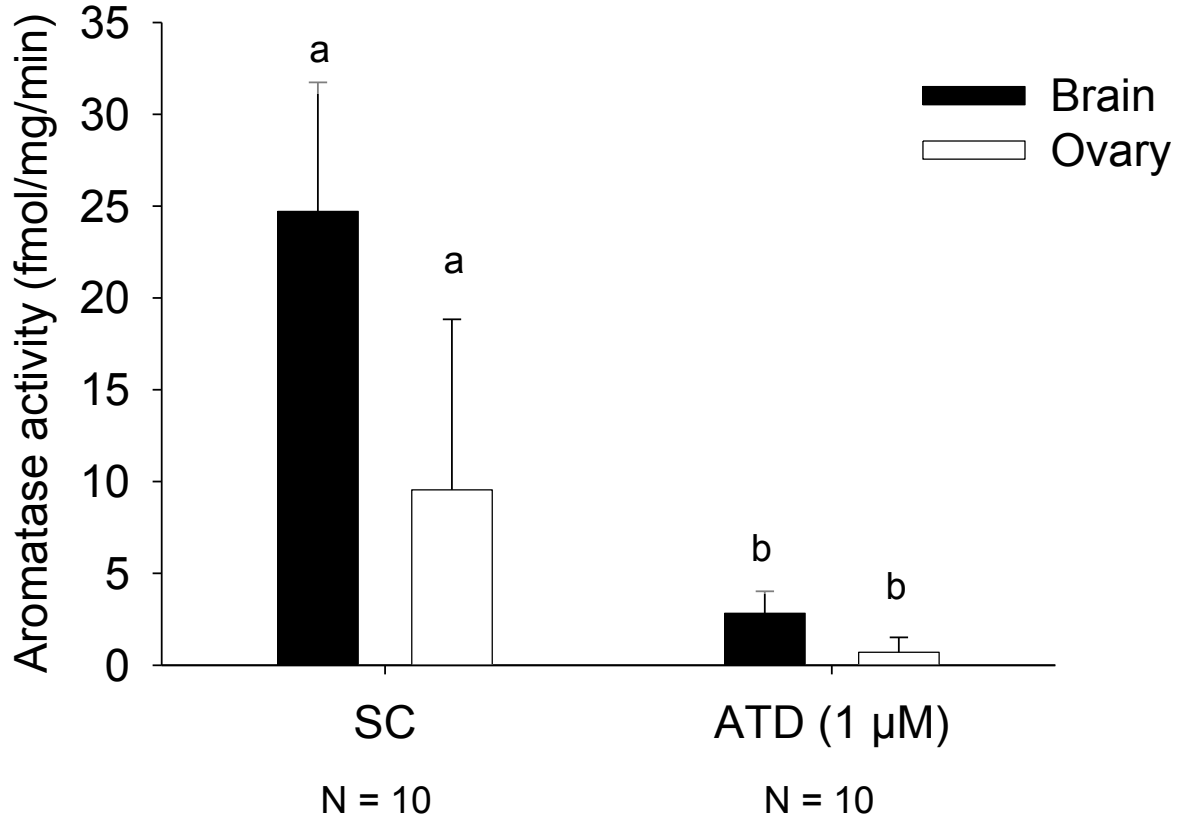

35
30
25
20
15
10
5
0
Aromatase activity (fmol/mg/min)
a
a
b
b
Brain
Ovary
SC
ATD (1 µM)
N = 10
N = 10

Fig. 4.