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ScienceDirect
Journal of Nutritional Biochemistry 22 (2011) 820–827
Journal of Nutritional Biochemistry

# Ovariectomy and 17β-estradiol alter transcription of lipid metabolism genes and proportions of neo-formed n-3 and n-6 long-chain polyunsaturated fatty acids differently in brain and liver☆

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Received 15 January 2010; received in revised form 27 May 2010; accepted 2 July 2010

**Abstract**

Hormonal and nutritional factors regulate the metabolism of long-chain polyunsaturated fatty acids (LC-PUFA). We aimed to determine whether ovarian hormones influence the capacity of rats to synthesize the end-products 22:6n-3 (DHA) and 22:5n-6 (n-6DPA) from their respective dietary precursors (18:3n-3 and 18:2n-6), and can regulate PUFA conversion enzymes gene transcription in brain and/or liver. Females born with a low DHA status were fed from weaning to 8 weeks of age a diet providing both essential precursors, and were concurrently submitted to sham-operated control (SOC) or ovariectomy (OVX) in combination with or without 17β-estradiol (E2) dosed at 8 or 16 μg/day. Relative to SOC, OVX increased the hepatic Δ9-, Δ6- and Δ5-desaturase transcripts and cognate transcription factors (PPARα, PPARγ, RXRα, RARα), but it did not affect LC-PUFA contents in phospholipids. In comparison with SOC and OVX groups, both E2 doses prevented the increase of transcripts, while paradoxically augmenting DHA and n-6DPA in liver phospholipids. Thus, in the liver of rats undergoing ovariectomy, changes of LC-PUFA synthesizing enzyme transcripts and of LC-PUFA proportions were not correlated. In brain, ovariectomy did not modify the transcripts of lipid metabolism genes, but it decreased DHA (-15%) and n-6DPA (-28%). In comparison with SOC and OVX groups, ovariectomized females treated with E2 preserved their status of both LC-PUFA in brain and had increased transcripts of E2 receptor β, PPARδ, RARα and LC-PUFA synthesizing enzymes. In conclusion, E2 sustained the transcription of lipid metabolism genes and proportions of neo-formed DHA and n-6DPA differently in brain and liver.


*Keywords:* 17β-estradiol; Polyunsaturated fatty acids; Lipid metabolism genes; Liver; Brain

## 1. Introduction

Docosahexaenoic acid (DHA, 22:6n-3) and n-6 docosapentaenoic acid (n-6DPA, 22:5n-6) are the two respective end-products of the n-3 and n-6 polyunsaturated fatty acid (PUFA) series. When adequate amounts of n-3 PUFA are supply to maternal and infant diets, DHA accumulates in the fetal and infant brain, where it contributes to neuronal arborization, synaptic development and brain functions [1,2]. However, if n-3 PUFA are not provided in sufficient amounts, neo-formed n-6DPA, which is not a normal component of brain membranes, compensates for the collapse of DHA but does not support adequate development of brain functions [3]. DHA is mainly provided from fatty fishes and marine products or synthesized from its essential precursor α-linolenic acid (ALA; 18:3n-3) from vegetable oils. In animals fed low amounts of n-3 PUFA, the conversion of ALA into DHA in the liver is up-regulated at the transcriptional level [4,5]. However, the same enzyme pathway facing low n-3 substrates concurrently produces n-6DPA from linoleic acid (LA, 18:2n-6).

*Abbreviations:* AA, arachidonic acid [20:4n-6]; ALA, α-linolenic acid [18:3n-3]; AOX, acyl CoA- oxidase 1; DBP, D-bifunctional protein [or MFP2, multifunctional protein-2]; DHA, docosahexaenoic acid [22:6n-3]; n-6DPA, n-6 docosapentaenoic acid [22:5n-6]; Elovl, elongase of very long chain fatty acids; EPG, ethanolamine phosphoglycerolipids; E2, 17β-estradiol; ER, estrogen receptor; LA, linolenic acid [18:n-6]; LBP, L-bifunctional protein [or MFP1, multifunctional protein-1]; LC-PUFA, long-chain polyunsaturated fatty acids; OVX, ovariectomy; PPAR, peroxisome proliferators-activated receptors; PC, phosphatidylcholine; PS, phosphatidylserine; RIN, RNA Integrity Number; RAR, retinoic acid receptor; RXR, retinoid X receptor; TLDA, TaqMan Low-Density Array; Δ5-D, Δ5-desaturase (or FADS1, fatty acid desaturase 1); Δ6-D, Δ6-desaturase (or FADS2, fatty acid desaturase 2); Δ9-D, Δ9-desaturase (or SCD1, stearoyl-CoA desaturase 1).

☆ This work was financially supported by the Institut National de la Recherche Agronomique (INRA, Paris) and by the Organisation Nationale Interprofessionnelle des Graines et Fruits Oléagineux (ONIDOL, Paris). Audrey Extier was titular of a research grant from the French Ministère de l'Enseignement Supérieur et de la Recherche.

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doi:10.1016/j.jnutbio.2010.07.005

Nutritional and hormonal factors regulate the extent to which ALA is converted to DHA. Studies from different countries have shown that, under identical nutritional conditions, women have relatively higher DHA in their blood lipids and/or in subcutaneous adipose tissue than men [6–9]. Moreover, when a single dose tracer of ALA was given to non-deficient young adults, newly labelled upstream metabolite precursors of DHA, 20:5n-3 and 22:5n-3, and newly labelled DHA appeared in the blood lipids of women [10] while only 20:5n-3 and 22:5n-3 were labelled in men [11].

Importantly, it has been suggested that sex steroid hormones influence the partitioning of ALA towards oxidation for energy production and carbon recycling or towards conversion for DHA synthesis [12]. Sustained DHA synthesis in females would be related to high demands of the fetus and suckling infant's brain. On the other hand, the lowering effect of parous status on brain DHA level and its incidence on neurobiological alterations have been evidenced in adult female rats, supporting a role for DHA as a preventive factor against postpartum depression [13,14].

It has been shown in male rats that gene expression of long-chain PUFA (LC-PUFA) synthesizing enzymes is up-regulated in response to chronic n-3 deficiency [4,5]. In a recent study, males and females rats born with a low DHA status and fed at weaning a diet containing suboptimal ALA, were used to determine their capacity to synthesize DHA and n-6DPA from the respective dietary precursors ALA and LA [15]. Under these experimental conditions, in which young rats were submitted to an accentuated metabolic stimulation of n-3 LC-PUFA synthesis, females expressed hepatic Δ6- and Δ5-desaturase at a greater level and have a more sustained liver capacity of DHA replenishment than age-matched males [15]. It has been therefore suggested that ovarian hormones stimulate the conversion pathway at the transcriptional level, resulting in a more sustained DHA synthesis in females. We hypothesized that estrogenic action, actually the balance between estrogenic and androgenic effects, could underlie the sex-related difference of metabolic adaptation to diets.

We aimed, therefore, to determine whether ovariectomy (OVX) and estradiol replacement alters the metabolic adaptation of females to diets. We examined the effects of steroid hormone alterations induced by bilateral OVX and E2 treatment on LC-PUFA synthesis. Our hypothesis is that OVX attenuates, whereas E2 treatment accentuates, the capacity of n-3 deficient females to benefit from the introduction of suboptimal amounts of ALA in diet. Young females were placed under the same nutritional conditions of supplementation with suboptimal ALA in which DHA synthesis was found to be more sustained in females than in males. Females born with a low DHA status were fed from weaning to 8 weeks of age a diet providing ALA and LA, and were concurrently submitted to sham-operated controls (SOC) or ovariectomy (OVX) in combination with or without E2 dosed at 8 or 16 μg/day. We show that E2 alters the transcription of lipid metabolism genes and proportions of neo-formed DHA and n-6DPA differently in cerebral cortex and liver.

## 2. Materials and methods

### 2.1. Animals and pre-experimental diet

Animals were treated in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC). Parent female Wistar rats were fed from mating to pregnancy and lactation a diet deficient in n-3 PUFA. This pre-experimental semi- synthetic diet contained 1.2 g LA/100 g and only 4.5 mg ALA/100 g from sunflower oil (6.2% by weight of dry matter) as the sole lipid source (Table 1). We used a variety of sunflower oil containing 21.8% by weight of total fatty acids as LA and less than 0.1% as ALA. There was no other n-3 PUFA in the diet. This type of low ALA diet, with a ratio of LA to ALA equals to 270, produces 75% deficits of DHA in the pup brain compared to adequate diets [16]. At birth, 32 females with homogeneous weight were chosen among 8 litters (of males and females) born from 8 pre-experimental dams fed the n-3 deficient diet, and were randomly redistributed under 4 pre-experimental lactating mothers to reconstitute 4 new litters of 8 females each. After being fed milk during 3 weeks, the 32 females were separated from dams and assigned to one of the 4 experimental groups (n=8 individuals/group). All groups were then fed the same post-weaning diet that contained 4-fold more ALA than the pre–experimental n-3 deficient diet (see Table 1).

Table 1
Composition of pre-experimental and post-weaning diets

| | n-3 deficient diet (pregnancy and lactation) | Post-weaning diet (experimental groups) |
|---|---|---|
| Vegetable oil (g/100 g of diet) | | |
| Sunflower oil (0.08% 18:3) | 6.2 | 4.8 |
| Rapeseed oil (8.4% 18:3) | 0 | 0.2 |
| main fatty acids * (mg/100 g) | | |
| 16:0 | 245 | 200 |
| 18:0 | 135 | 105 |
| 18:1n-9 | 3795 | 3070 |
| 18:1n-7 | 185 | 150 |
| 18:2n-6 | 1215 | 980 |
| 18:3n-3 | 4.5 | 20 |
| 18:2n-6/18:3n-3 | 270 | 49 |

Each diet contained (g/100g): casein (22), DL-methionine (0.16), cellulose (2), mineral mix (4), vitamin mix (1), corn starch (43.5), sucrose (21.7).
* Long-chain PUFA were not detectable.

### 2.2. Experimental design and surgery

All procedures relating to animals surgery were approved by the French Ministry of Agriculture according to the French regulation for animal use and experimentation (authorization n° 78–34). After one night of fasting, the females (mean weight of 64 g) were assigned to bilateral OVX (group OVX), OVX and one (OVX+1E2) or two (OVX+2E2) E2 treatments, or to SOC. Anaesthesia was induced and maintained throughout surgery by inhalation of isoflurane. The ovaries were removed through a small abdominal incision. In SOC animals, the ovaries were exposed, manipulated and returned to the abdominal cavity. At the time of surgery, groups OVX+1E2 and OVX+2E2 received one or two E2-pellets, each contained 0.5 mg E2 and released 8 μg E2/day for 60 days (Innovative Research of America, Sarasota, FL, USA). All groups were reared for 5 weeks on an n-3 repletion diet containing 4.8% sunflower oil and 0.2% rapeseed oil (Table 1), which supplied a suboptimal amount of 20 mg ALA/100 g of diet. This procedure of partial repletion diet was the same as that used previously to compare the capacity for DHA synthesis from ALA and the expression of related enzymes in the liver and brain between male and female rats [15].

At 8-weeks of age, rats were killed by decapitation. Cerebral cortex and livers were removed and separated into two pools: one was immediately frozen in liquid nitrogen, and stored at -80°C until lipid extraction, isolation of phospholipid classes and analysis of fatty acids; the other was stored at -20°C in 2 ml of RNAlater (RNA Stabilization Reagent, Qiagen, France) until extraction and quantification of mRNA. Uteri were removed and weighed as a measure for the effect of ovarian hormones removal by OVX or in response to E2 treatments.

### 2.3. Fatty acid analysis

Total lipids were extracted from dilacerated samples of cerebral cortex and liver with 4 ml of chloroform-methanol (2:1, vol:vol). The three main phospholipid classes, phosphatidylserine (PS), phosphatidylcholine (PC), and ethanolamine phosphoglycerolipids (EPG) were isolated by solid-phase extraction on a 500 mg aminopropylbonded silica cartridge [17]. The cartridges were equilibrated beforehand with eluent I (isopropanol/chloroform, 1:2, by vol). Each sample of total lipids was dried under nitrogen, resolubilized in 250 μl of eluent I and deposited onto a single-use cartridge, which was immediately eluted successively with 3 ml of eluent I, 3 ml of diethylether/acetic acid (98:2, by vol), 1 ml of acetonitrile, 8 ml of acetonitrile/n- propanol (3:1, by vol) to recover the PC fraction, and then with 2 ml of acetonitrile/n- propanol (1:1, by vol) and 3 ml of methanol to recover the EPG fraction (the latter consisted in a mixture of phosphatidylethanolamine and 1-O-alkylenyl-2-acyl glycerophosphoethanolamine). The PS fraction was recovered at the end of the procedure by eluting with isopropanol/ methanolic HCl 3N (4:1, vol/vol). The three phospholipid fractions were dried under a nitrogen flux and transmethylated by methanol at 90°C in the presence of 10% $BF_3$. The fatty acids were transmethylated, then separated by gas chromatography and quantified using flame ionization detection. The resulting peaks were identified by comparison of their retention times to those of a mix of standard compounds (mix GLC-68A from Nu-Chek-Prep, MN). The proportion of each fatty acid was automatically computed from the ratio of its peak area to the sum of total areas and expressed as a percent by weight of total fatty acids.

### 2.4. Real-time RT-PCR using low density array

Total RNA was isolated from 50 mg liver or cerebral cortex using an RNeasy Lipid Tissue Midi Kit with DNase digestion (Qiagen, France). RNA concentration and purity

Table 2
Gene names and GenBank accession numbers

| | Usual name | GenBank NM- |
|---|---|---|
| Fatty acid conversion | | |
| Fatty acid desaturase 1 | Δ5-D | 53445 |
| Fatty acid desaturase 2 | Δ6-D | 31344 |
| Stearoyl-Coa desaturase 1 | Δ9-D | 139192 |
| Acyl-CoA oxidase 1 | AOX | 17340 |
| Multi-functional protein 1 | LBP | 17008 |
| Multi-functional protein 2 | DBP | 24392 |
| Elongase of very long-chain FA 2 | Elovl 2 | 1109118 |
| Nuclear receptors | | |
| Peroxisome proliferator-activated receptors | PPARα | 13196 |
| | PPARγ | 13124 |
| | PPARδ | 13141 |
| Retinoic acid receptor α | RARα | 31528 |
| Retinoid X receptor α | RXRα | 12805 |
| Estrogen receptors | ERα | 12689 |
| | ERβ | 12754 |

were verified by A260/A280 and A260/A230 ratios (Biophotometer Eppendorf, France). The quality and integrity of RNA were then checked by microcapillary electrophoresis measurements on an Agilent 2100 bioanalyseur (Agilent Technologies, France) with an RNA 6000 Nano LabChip Kit (Agilent Technologies). The Agilent 2100 expert software calculates the 28S:18S ratio and the RNA Integrity Number (RIN). Only samples displaying a RIN≥8.0 and a 260/280>1.8 were retained. The cDNA were prepared from 1 μg total RNA using a high-capacity cDNA Archive Kit (Applied Biosystems, France). We quantified mRNA levels of key genes by creating as previously described [15] a rat PUFA metabolism TaqMan Low-Density Array (TLDA) (Applied Biosystems) (Table 2) in which each well contains specific primers and probe capable of detecting one single gene. The TaqMan probe and primers sets for each gene were selected from predesigned TaqMan Gene Expression Assay (Applied Biosystems). The cDNA samples were diluted with nuclease-free water (1:4, vol/vol) and mixed with 55 μl of TaqMan Universal PCR Master Mix. The sample- specific PCR mixture (100 μl) was loaded on a sample port and distributed into wells by centrifugation of the plates at 1200 rpm for 1 minute. The PCR amplification was performed with an Applied Biosystems Prism 7900 HT sequence detection system. The thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 30 s at 97°C and 1 min at 59.7°C. TLDA cards were analysed with RQ documents and the RQ Manager Software for automated data analysis. In each sample, the threshold cycle (Ct) of each target gene was compared to that of 18S rRNA. The differences of Ct between one given target gene and 18S generated the Δ Ct values which were automatically averaged from 4 individual determinations (each repeated twice). On the basis that each cycle theoretically produces a 2-fold amplification, the abundance of each mRNA in each sample was automatically normalized to that of 18S according to the equation:

$$R = 2^{-\Delta Ct} \text{ with } \Delta Ct = Ct(\text{target}) - Ct(18S).$$

The Ct values of all target genes being greater than that of 18S (the higher the Ct, the lower the abundance), the same multiplicative factor of $10^7$ was applied on the R values to scale all relative expressions with the same arbitrary units.

### 2.5. Statistical analysis

Data were analysed for by one-way ANOVA (SigmaStat 3.1 software, advisory statistics for scientists) with treatment (SOC, OVX, OVX+1E2 or OVX+2E2) as factor. When the normality test of ANOVA was passed, post hoc comparisons between means within each group were performed using Bonferroni's test. Data are expressed as the mean±SD. The acceptable level of significance was set at $P<.01$ for fatty acid proportions and $P<.05$ for transcript levels.

## 3. Results

### 3.1. Effect of ovariectomy and E2 treatments on uterus growth

Atrophy of the uterus associated with bilateral ovariectomy is expected owing to the well-known roles of follicular estrogens on the uterus [18]. In the present study, OVX resulted in uterine atrophy, whereas treatment of ovariectomized animals with E2 consistently prevented this atrophic response (Fig. 1a). Mean uterine weight was significantly reduced ($P<.01$) in only the OVX females compared to that of SOC animals or those receiving one or two E2 pellets (Fig. 1b).

### 3.2. Proportions of long-chain PUFA in phospholipid classes

The proportions of DHA and n-6DPA in the three main phospholipid classes of the cerebral cortex and liver are indicated in Table 3. The higher proportion of n-6DPA compared to DHA in all groups confirmed that n-3 repletion was not completed after 5 weeks of feeding with the 20 mg ALA diet. Precisely, the comparison of DHA and n-6DPA between groups gave indications that the synthesis of both end-products was concurrently altered upon ovariectomy and E2 treatments, although differently in liver and brain.

In the cortex, both end-products were lowered in group OVX compared to SOC. Ovariectomy decreased ($P<.01$) cerebral DHA by 15% in EPG, and n-6DPA by 28% and 26% in EPG and PS, respectively. There was no significant impact of OVX on 20:4n-6 and 22:4n-6 (data not shown). In the ovariectomized females implanted with E2 pellets, cerebral DHA and n-6DPA were maintained at proportions similar to

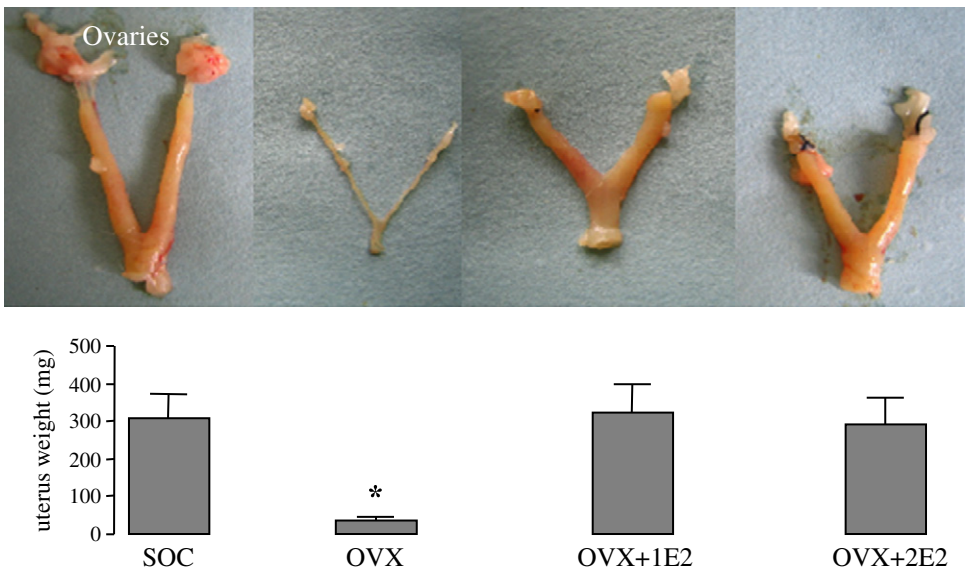


Fig. 1. Effect of ovariectomy (OVX) at 3 weeks of age and of E2 treatment for 5 weeks on the uterus growth, in sham-operated controls (SOC), ovariectomized females (OVX), and E2-implanted females (OVX+1E2 or OVX+2E2). * Different weight (mean±sd; n=8) at the age of 8 weeks ($P<.01$).

Table 3
Proportions of long-chain PUFA in cerebral cortex phospholipids at the age of 8 weeks

| | SOC | OVX | OVX+1E2 | OVX+2E2 |
|---|---|---|---|---|
| Cortex 22:5n-6 | | | | |
| PC | 2.5±0.3 | 2.3±0.2 | 2.3±0.6 | 2.6±0.5 |
| EPG | 16.9±0.7[a] | 12.2±2.3[b] | 16.3±0.6[a] | 16.8±1.5[a] |
| PS | 17.8±1.5[a] | 13.1±1.5[b] | 16.2±2.1[a] | 17.5±0.8[a] |
| Cortex 22:6n-3 | | | | |
| PC | 1.7±0.3 | 2.1±0.3 | 1.6±0.5 | 2.0±0.2 |
| EPG | 14.2±0.6[a] | 12.1±0.8[b] | 13.1±0.4[ab] | 14.3±1.2[a] |
| PS | 10.8±0.7 | 10.8±1.8 | 10.1±0.8 | 11.3±0.8 |
| Liver 22:5n-6 | | | | |
| PC | 3.2±0.7[a] | 3.0±0.5[a] | 4.5±0.7[ab] | 5.4±1.6[b] |
| EPG | 5.6±0.7[a] | 5.6±0.7[a] | 8.8±1.2[ab] | 11.7±3.8[b] |
| PS | 3.8±0.4[a] | 4.3±0.5[a] | 6.3±0.5[b] | 7.9±1.1[b] |
| Liver 22:6n-3 | | | | |
| PC | 2.7±0.1[a] | 2.6±0.1[a] | 3.3±0.4[b] | 3.0±0.2[ab] |
| EPG | 6.3±0.7[a] | 5.8±0.3[a] | 8.6±0.6[b] | 7.9±0.6[b] |
| PS | 2.6±0.4[a] | 2.4±0.3[a] | 3.7±0.6[ab] | 4.7±1.7[b] |

Superscript letters (a,b) indicate significant difference between groups (P<.01). (mean±sd,% by weight of total FA in PC, EPG or PS).

control values. Thus, OVX altered the proportions of end-products from both series in the cerebral cortex, a reduction which was prevented with E2 treatment.

In contrast, OVX did not modify the proportions of DHA and n-6DPA in the liver phospholipids, while both end-products were increased upon E2 treatment: compared to group SOC, DHA was increased by 36% and 25% in the EPG fractions of groups OVX+1E2 and OVX+2E2, respectively (Table 3). DHA also increased in PS by 42% in group OVX+1E2 (not significant) and by 81% in group OVX+2E2. Concurrently, n-6DPA increased by 108% in both the EPG and PS fractions of group OVX+2E2 compared to group SOC. Besides, OVX increased the proportion of AA in PS by 25% in comparison with the three other groups, while AA was not altered in the different EPG and PC fractions (data not shown).

In summary, changes in the hormonal status altered the end-products in a tissue- specific manner: in the cerebral cortex, E2 dosed at 8 or 16 μg/d prevented the ovariectomy-induced decrease of DHA and n-6DPA, while in liver their proportions were not altered by ovariectomy but significantly increased with E2 treatments.

### 3.3. Gene expression of LC-PUFA synthesizing enzymes

In an unexpected manner, the relative quantities of the Δ6- and Δ5-D transcripts expressed in liver were about 2-times greater in OVX than in SOC females (Fig. 2). Furthermore, OVX also resulted in a 6.7-times increase of the Δ9-D expression compared to SOC. In contrast, the

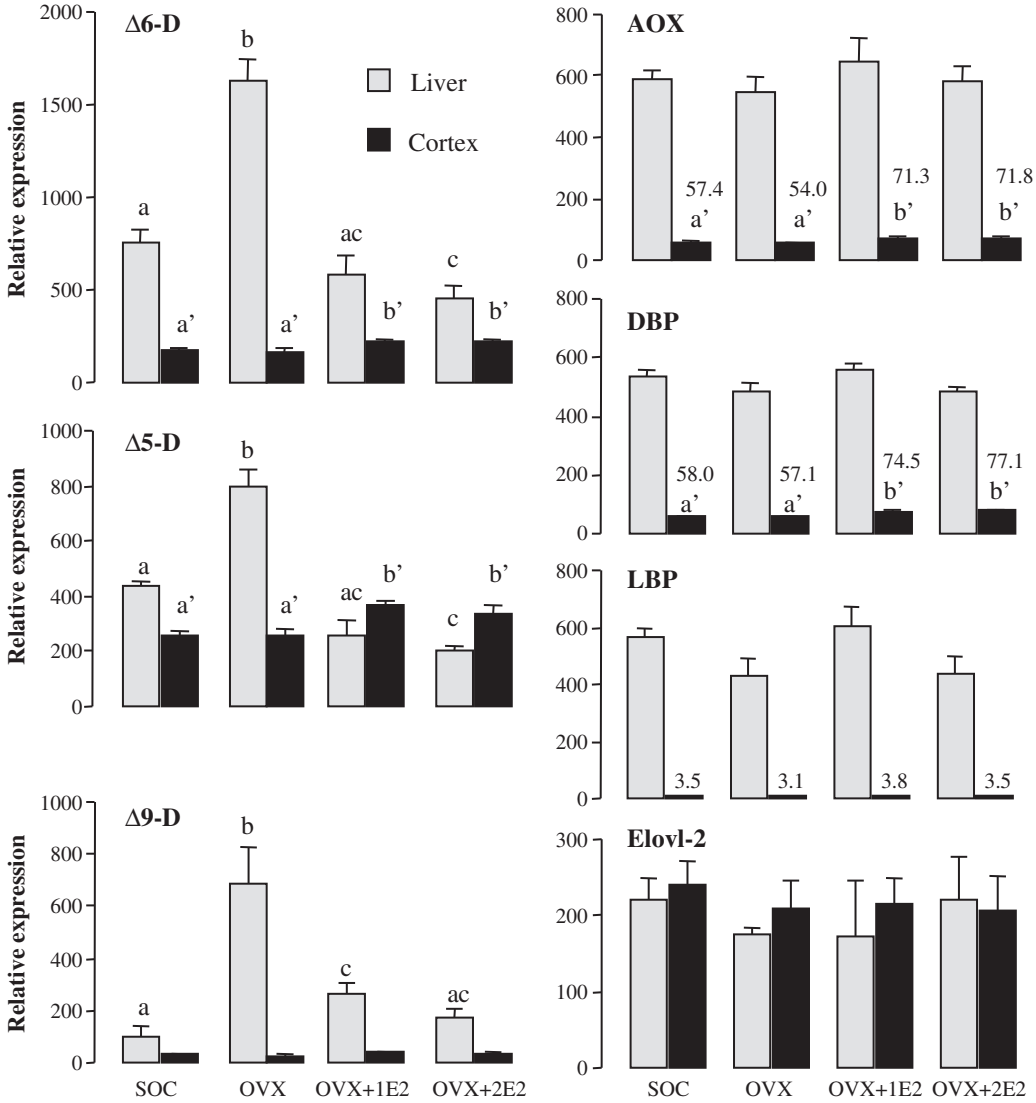


Fig. 2. Relative expression of lipid metabolism genes in the liver and cerebral cortex of sham-operated controls (SOC), ovariectomized females (OVX), and E2-implanted females (OVX+1E2 or OVX+2E2). Different superscripts indicate significant difference of mean values (±SD) between groups (P<.05) in liver (a, b, c) or in cortex (a', b', c').

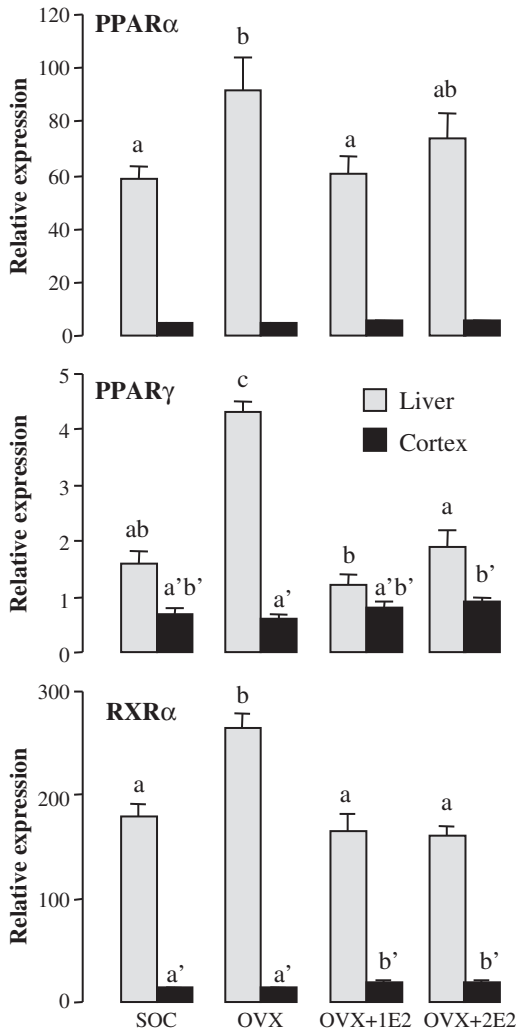


Fig. 3. Relative expression (mean±SD) of PPARα, PPARγ and RXRα in the liver and cerebral cortex of sham-operated controls (SOC), ovariectomized females (OVX), and E2-implanted females (OVX+1E2 or OVX+2E2). Different superscripts indicate significant difference between groups in liver (a, b, c) or in cortex (a', b', c') ($P$<.05).

transcripts of AOX, DBP, LBP or Elovl2 were not altered by ovariectomy. The E2 treatments prevented the OVX-induced increases of the desaturase transcripts, even more it significantly lowered the Δ6- and Δ5-D expression in group OVX+2E2 compared to SOC. These changes were unexpected inasmuch as they were at the opposite of those observed in the liver phospholipids for end-products of the desaturation-elongation pathway. Thus, in the liver of females submitted to hormone alterations, the changes of desaturase expression and of end-product proportions were not positively correlated.

In the cerebral cortex, none of the transcripts of Δ6-D, Δ5-D, Δ9-D, AOX, DBP, LBP and Elovl2, was altered by ovariectomy. However, the E2 treatment moderately increased the cerebral transcripts of Δ6-D (x 1.25±0.1), Δ5-D (x 1.4±0.1), Δ9-D (x 1.3±0.1), AOX (x 1.25±0.1) and DBP (x 1.3±0.1). In both groups of ovariectomized females treated with E2, the relative expression of Δ5-D became even greater in the cerebral cortex compared with its counterpart in liver (Fig. 2). These increases of gene expression were coherent with the increased proportions of DHA and n-6DPA in the cerebral cortex phospholipids of both groups treated with E2 (Table 3). Thus, treatment with E2 sustained the transcript levels of conversion enzymes and preserved the LC-PUFA status in the cerebral cortex of ovariectomized females.

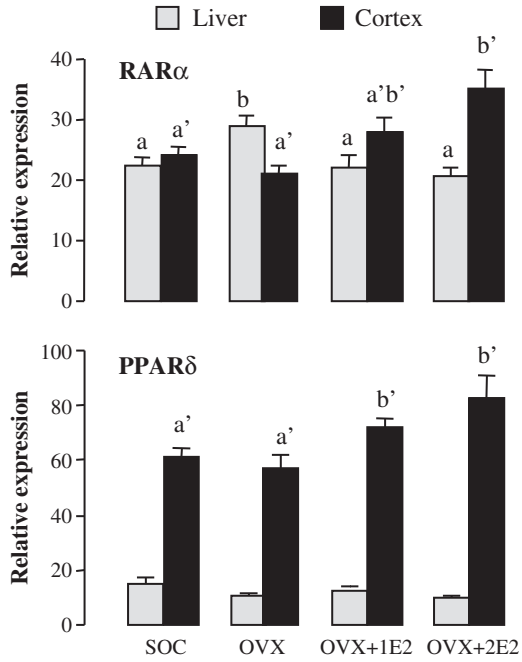


Fig. 4. Relative expression (mean±SD) of RARα and PPARδ in the liver and cerebral cortex of sham-operated controls (SOC), ovariectomized females (OVX), and E2-implanted females (OVX+1E2 or OVX+2E2). Different superscripts indicate significant difference between groups in liver (a, b, c) or in cortex (a', b', c') ($P$<.05).

### 3.4. Gene expression of transcription factors

In an attempt to identify the pathway of transcriptional regulations which could be recruited upon hormone alterations, we compared the transcript levels of lipid metabolism cognate transcription factors in the cerebral cortex and liver. The results again showed that alterations of ovarian hormones produced tissue-specific changes.

In liver, OVX resulted in a significant increase of PPARα (x 1.6±0.2 compared to SOC), PPARγ (x 2.7±0.1), and RXRα (x 1.5±0.1) which was prevented with E2 dosed at 8 or 16 μg/d (Fig. 3). In cortex, OVX had no impact on the expression of these transcription factors (Fig. 3), but the E2 treatment significantly increased the expression of RARα (x 1.5±0.1) and PPARδ (x 1.3±0.1) (Fig. 4).

### 3.5. Gene expression of estrogen receptors

In liver, the ERα transcripts, but not those of ERβ, were found to be quantifiable, their levels being not altered by OVX with or without E2 treatments (Fig. 5A). In the cerebral cortex, ERα and ERβ were both expressed at very low levels (about 40-fold less than that of ERα in liver). OVX decreased the ERα transcripts by 33% in cerebral cortex compared to SOC, a reduction prevented by E2 (Fig. 5B). There was also a trend for ERβ transcripts to decrease in the cerebral cortex of OVX females compared to SOC, although not significantly, and to increase upon E2 treatment (+57% in group OVX+2E2 compared to group OVX).

### 3.6. Comparing the transcript levels of PPARδ, desaturases, transcription factors and estrogen receptors in the cerebral cortex

PPARδ being the most abundant PPAR isoform in brain, we examined whether the transcript levels of cerebral cortex PPARδ were correlated to those of target genes or cognate transcription factors. The transcript levels of Δ6-D, RARα, RXRα, PPARα, PPARγ, ERα and ERβ in the cerebral cortex of each group were plotted against those of

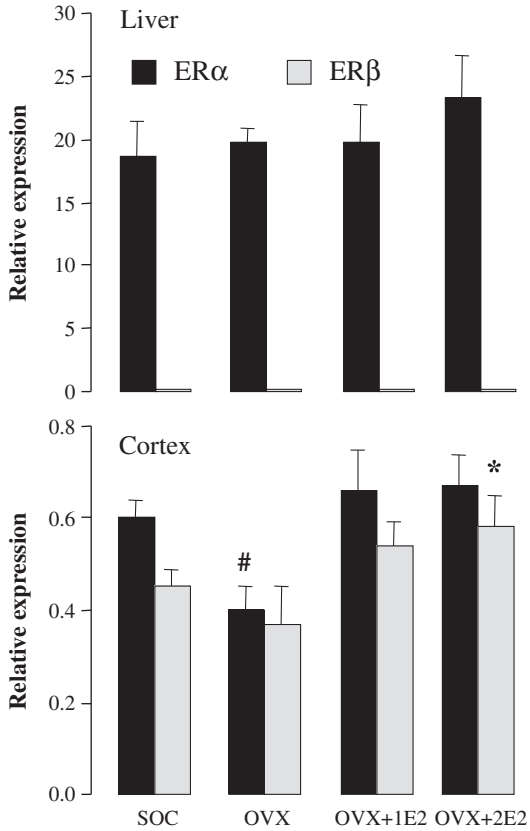


Fig. 5. Expression of E2 receptors in liver and cerebral cortex. ERβ transcripts were not detectable in liver (note that a different scale is used for liver and cortex). #ERα, different mean value in OVX compared to other groups ($P<.05$). *ERβ, different mean value in group OVX+2E2 compared to group OVX ($P<.05$).

PPARδ (Fig. 6). The data plot shows that changes of Δ6-D and PPARδ levels were concurrent, the lowest expression of both genes being found in group OVX, the highest in group OVX+2E2 (Fig. 6A). For both transcripts, there was no significant difference between groups OVX and SOC on the one hand, and between groups OVX+1E2 and OVX+2E2 on the other hand. The same pattern of concurrent variations was drawn from the transcript levels of Δ5-D and PPARδ (not shown). Moreover, data plot of the transcription factors showed that the transcripts of RARα and PPARδ in the different groups were consistently commensurate (Fig. 6B). The transcript levels of the two estrogen receptors and of PPARδ also varied concurrently, although not linearly (Fig. 6C).

## 4. Discussion

We recently showed that female rats, born with a low DHA status and submitted to an n-3 repletion diet from weaning to sexual maturity, expressed hepatic Δ6- and Δ5-desaturase at a greater level and have a more sustained liver capacity of DHA synthesis than males [15]. In addition, comparing the effects of estradiol and testosterone in a neuroblastoma cell model of n-3 conversion indicated that E2 increased, whereas 5-dihydrotestosterone decreased, both the gene expression of Δ5-D and the production of ALA-long chain derivatives [19]. Nevertheless, the underlying mechanisms of regulation by sex hormones have not been identified. The present study aimed to examine whether alterations of the ovarian hormone status change the metabolic capacity of LC-PUFA synthesis. We focused on liver owing to its pivotal role in lipid metabolism, and brain whose membranes need the end-products of the conversion pathway, especially DHA if dietary n-3 fatty acids are adequate, or DHA and

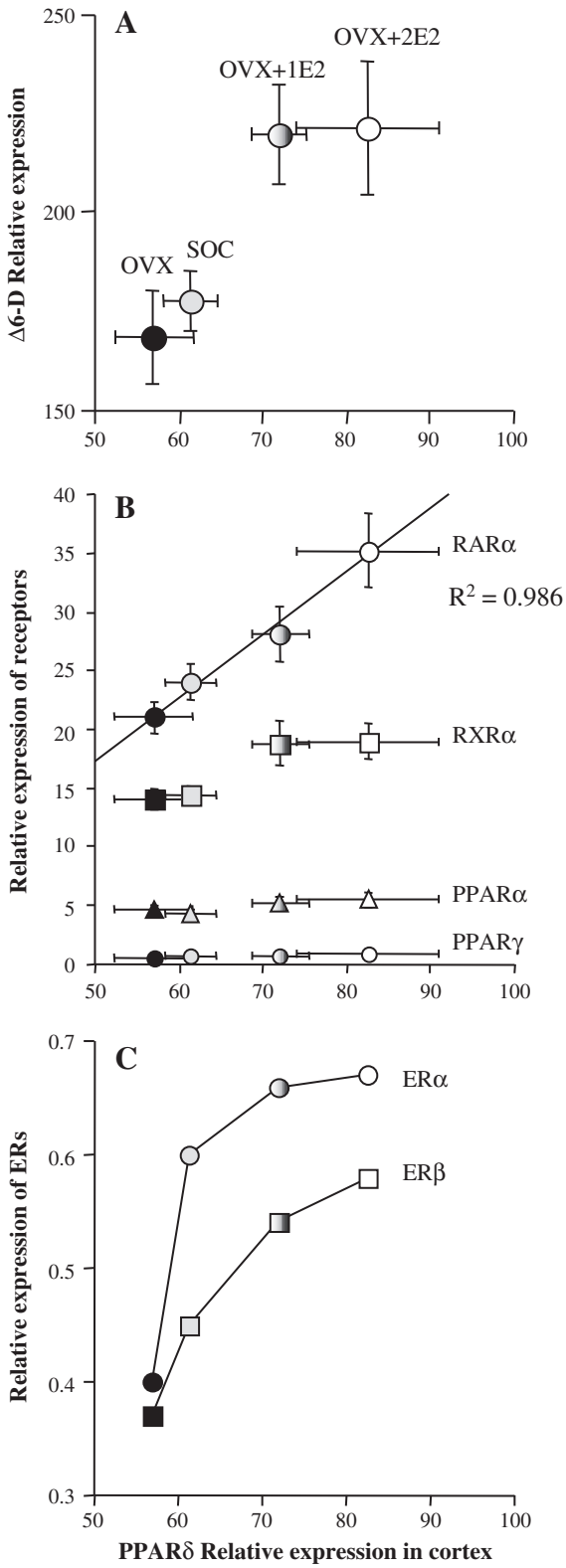


Fig. 6. Data plot of Δ6-D (A), transcription factors (B) and estrogen receptors (C) against PPARδ. In each panel, the black, grey, shadowed and open symbols correspond to the mean value (±SD) of groups OVX, SOC, OVX+1E2 and OVX+2E2, respectively. A: E2-implanted groups had the highest Δ6-D and PPARδ expression levels, both significantly different from groups SOC and OVX. B: Data plot of RARα (circles), RXRα (squares), PPARα (triangles) and PPARγ (small circles). The PPARδ and RARα mean values from the 4 groups fitted linearly. C: Data plot of ERα (circles) and ERβ (squares) (SD are not presented for legibility). Note that this pattern is about similar to that of Δ6-D against PPARδ.

n-6DPA when n-3 fatty acids are deficient or suboptimal. Differences appeared between our two rat studies, the comparison between males and females being not transposable to that of ovariectomized and intact females, nor to ovariectomized females and those implanted with E2 pellets.

### 4.1. E2 treatments sustain proportions of end-products in the cerebral cortex

Our finding that ovariectomy decreased DHA in the EPG fraction from cerebral cortex phospholipids and n-6DPA in EPG and PS, and that E2 treatment prevented these alterations provides evidence for the role of E2 in the homeostasis of membrane LC-PUFA from both series. A recent study has shown that ovariectomy decreased DHA in the total phospholipids of hippocampus, but not in the prefrontal cortex or midbrain, and that E2 treatment did not prevent the lowering of hippocampal DHA [20]. It should be emphasized that we did not find difference for cortex DHA between groups in the PC and PS fractions but only in EPG. Furthermore, we found that under partial n-3 replenishment, ovariectomy is also susceptible to alter n-6DPA in EPG and PS (n-6DPA contents have not been reported in [20]). Therefore, alterations in brain fatty acid composition due to ovariectomy appeared to be specific for different phospholipids classes as well as for different brain areas (shown in [20]), but not for n-3 or n-6 series. Besides, several differences in the experimental design may explain discrepancies between the two studies on the effect of exogenous E2, notably regarding the pre- experimental and experimental nutritional status (ALA contents in diets and ratio of lipid energy), the age of females at ovariectomy, 21 days herein (before sexual maturity) vs 56 days in [20] (achievement of sexual maturity) and thus their pre-exposure to ovarian hormones, the administration mode and the concentration of E2 (8 or 16 μg release/day for 5 weeks vs 2 μg every 4 days for 10 weeks in [20]). The impact of ovariectomy and treatment with E2 on brain lipids may be more marked before the achievement of sexual maturity. Actually, several distinct critical periods or "windows in time" when estrogen influences tissue morphogenesis have been observed for many organs, including the brain [21].

### 4.2. Lipogenic response of liver to ovariectomy

The question remains whether changes in the status of ovarian hormones produce alterations in brain fatty acid composition through topic action or if they impact primarily in liver. We analyzed the fatty acid composition of hepatic phospholipid classes and found that the response of liver to ovariectomy and to E2 treatments was totally different from that of cortex. The whole data are summarized in Table 4. In the liver of OVX animals, DHA and n-6DPA were not altered while E2 treatments induced both fatty acids to increase in EPG and PS. Therefore, our study clearly shows that in a nutritional situation of partial n-3 replenishment, exogenous E2 sustains or enhances the production of the end-products of both series, not only that of DHA.

However, ovariectomy by itself had no impact on liver DHA and n-6DPA, suggesting that the hepatic conversion pathway might be not responsive to early removal of ovaries. In an attempt to explain this effect at the transcriptional level, we quantified the gene expression of conversion key enzymes. The results were paradoxical since OVX females had the highest transcript levels of Δ6- and Δ5-D in their liver, while those receiving the greatest dose of E2 had the lowest ones (Table 4). Thus, there was no correlation in OVX females between transcript levels of hepatic desaturases (which were increased) and end-products in their liver phospholipids (unchanged). The increased expression of Δ6- and Δ5-D could be interpreted as being a compensatory response, preserving the synthesis of end-products in the liver of OVX animals.

Table 4
Summary of changes in transcript levels and fatty acid contents in the liver and cerebral cortex phospholipids in OVX and OVX+2E2 groups

| | Liver | | Cortex | |
|---|---|---|---|---|
| | OVX | OVX+2E2 | OVX | OVX+2E2 |
| Δ9-D | ⇑⇑⇑⇑ | ~ | ~ | ~ |
| Δ6-D, Δ5-D | ⇑⇑⇑ | ⇓⇓ | ~ | ⇑ |
| AOX, DBP | ~ | ~ | ~ | ⇑ |
| PPARα | ⇑⇑ | ~ | ~ | ~ |
| PPARγ | ⇑⇑⇑ | ~ | ~ | ~ |
| PPARδ | ~ | ~ | ~ | ⇑⇑ |
| RXRα | ⇑⇑ | ~ | ~ | ⇑ |
| RARα | ⇑ | ~ | ~ | ⇑⇑ |
| ERs | ~ | ~ | ⇓(α) | ⇑(β) |
| AA* | ⇑ (PS) | ~ | ~ | ~ |
| DHA | ~ | ⇑ (EPG, PS) | ⇓ (EPG, PS) | ~ |
| n-6DPA | ~ | ⇑⇑⇑ | ⇓ (EPG) | ~ |

* Data not shown.

Actually, ovariectomy has been shown to produce pleiotropic effects, resulting in a global lipogenic response of liver as evidenced by increased transcription of the lipogenic transcription factor SREBP1c and its main target genes, Fatty acid synthase, AcylCoA-carboxylase and Stearoyl-CoA desaturase (Δ9-D) [22, 23]. Ovariectomy combined to high-fat diet (42% of fats) has been shown to produce adiposity and lipid infiltrations in liver, and E2 to prevent these effects [24]. Antilipogenic effects of E2 have been also shown in intact obese mice [25]. The lipogenic response of liver to ovariectomy, and its prevention with E2 treatment, is also clearly evidenced in our study with the dramatic increase of Δ9-D transcripts, which enzyme having saturated fatty acids as substrate is not involved in the synthesis of LC-PUFA. There was in parallel a marked increase of PPARγ suggesting that this lipid sensor nuclear receptor, known for its pivotal role in adipocyte differentiation [26], may be involved in the lipogenic response of liver. Therefore, the increased transcription of Δ6-D and Δ5-D in the liver of OVX females should be considered as being non-specific but involved in the global lipogenic response. This response in mass had no specific effect on the proportions of DHA and n-6DPA relatively to those of the whole fatty acids concurrently synthesized in liver. It is clear that E2 treatment prevented the lipogenic response of liver since it standardized the relative expression of desaturases and of lipid metabolism cognate nuclear receptors, PPARα, PPARγ, RXRα and RARα. On the other hand, ovariectomized females treated with E2 had increased DHA and n-6DPA in their liver phospholipids, indicating that the antilipogenic effect of E2 specifically preserved the end-products, an effect that cannot be accounted for by transcriptional up-regulation of hepatic Δ6-D, Δ5-D, AOX, DBP, LBP or Elovl2 (Table 4). Other genes or other mechanisms could be implemented upon E2 treatment, possibly at traductional or post-traductional levels. Further studies are needed to elucidate this point.

### 4.3. Transcriptional effects of E2 in the cerebral cortex

In contrast with what happened in liver, ovariectomy did not increase in the cerebral cortex the desaturase transcripts. Furthermore, these transcripts and those of AOX and DBP were enhanced (+20–30%) after E2 treatment (Table 4). Finally, the increases of end-products in the cerebral cortex phospholipids of E2-treated females (relative to OVX females without E2 treatment) occurred concurrently with those of transcript levels, suggesting a topic action of E2. Moreover, the concurrent changes of transcript levels of conversion enzymes, on the one hand, and of PPARδ and RARα on the other hand, gave clues that these two nuclear receptors could play a dual role in the E2-dependent transcriptional response of the cerebral cortex. It

has been shown in cell models that retinoic acid is a high affinity ligand of both PPARδ and RARα (both functioning as a heterodimer with RXR) suggesting that, in some tissues, retinoic acids may activate transcription of target genes not only through RARα but also through PPARδ [27–29]. In cortex, the transcripts of PPARδ and RARα varied commensurately in the different groups, suggesting that the expression of these two receptors could be concurrently regulated under hormone stimulation. It is known that both ERα and ERβ are expressed in the adult rat brain with ERβ mRNA-containing cells more widely dispersed throughout the brain than those expressing ERα mRNA [30]. Whether the increased expression of RARα and PPARδ in the cerebral cortex of E2-treated females was mediated through the primary recruitment of ERα and/or ERβ was not addressed in this study. However, we noted that there was a trend for ovariectomy to decrease, and for E2 to increase, the transcripts of both receptors in the cerebral cortex, suggesting a possible loop of regulation. In summary, the transcripts of PPARδ, RARα, ERα and ERβ increased in the cerebral cortex of ovariectomized females treated with E2, a primary effect that could underlie that on Δ5- and Δ6-D, and *in fine* on the proportions of end-products in the cerebral cortex. None of these apparent relationships was observed in liver (Table 4).

In conclusion, our study shows that E2 treatment in ovariectomized females concurrently sustained the end-products of the n-3 and n-6 conversion pathway both in liver and cerebral cortex. Tissue-specific mechanisms probably involve the recruitment of distinct nuclear receptors and transcription factors. In liver, the effects of ovariectomy were essentially of lipogenic nature (with the probable implication of PPARγ and RXRα) and were not specific for the LC-PUFA synthesis pathway. In particular, there was no correlation between transcript levels in liver and proportions of end-products in liver phospholipids. In brain, our data raise the hypothesis of a possible role of PPARδ and/or RARα in the transcriptional regulation of conversion enzymes, a process that would be particularly responsive to steroid hormones.

## Acknowledgments

We are very grateful to Patrice Dahirel and Claire Maudet for animal care, Alain Linard and Marie-Sylvie Lallemand for gas chromatography analysis, and Mélanie Jouin for her help in dissection of rats. We also thank Claudia Bevilacqua from the PICT Plateform (INRA, Jouy-en-Josas) for her advices in using the PCR device, Christine Heberden for helpful discussions, and Georges Veermersch and Noëmie Simon (ONIDOL, Paris) for their constant support.

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