Effect of Sex Differences on Brain Mitochondrial Function and Its Suppression by Ovariectomy and in Aged Mice

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Sex steroids regulate brain function in both normal and pathological states. Mitochondria are an essential target of steroids, as demonstrated by the experimental administration of 17β-estradiol or progesterone (PROG) to ovariectomized female rodents, but the influence of endogenous sex steroids remains understudied. To address this issue, mitochondrial oxidative stress, the oxidative phosphorylation system, and brain steroid levels were analyzed under 3 different experimental sets of endocrine conditions. The first set was designed to study steroid-mediated sex differences in young male and female mice, intact and after gonadectomy. The second set concerned young female mice at 3 time points of the estrous cycle in order to analyze the influence of transient variations in steroid levels. The third set involved the evaluation of the effects of a permanent decrease in gonadal steroids in aged male and female mice. Our results show that young adult females have lower oxidative stress and a higher reduced nicotinamide adenine dinucleotide (NADH)-linked respiration rate, which is related to a higher pyruvate dehydrogenase complex activity as compared with young adult males. This sex difference did not depend on phases of the estrous cycle, was suppressed by ovariectomy but not by orchidectomy, and no longer existed in aged mice. Concomitant analysis of brain steroids showed that pregnenolone and PROG brain levels were higher in females during the reproductive period than in males and decreased with aging in females. These findings suggest that the major male/female differences in brain pregnenolone and PROG levels may contribute to the sex differences observed in brain mitochondrial function. **(*Endocrinology* 156: 2893–2904, 2015)**

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Mitochondria play a major role in bioenergetics and death/survival signaling in mammalian cells. Mitochondrial energy production is particularly critical for neuronal function, because cells of the central nervous system have a high metabolic rate and neurons need large amounts of ATP to maintain their membrane potential and for neurotransmission (1). Oxidative phosphorylation by the mitochondrial respiratory chain (RC) is the major pathway of ATP synthesis (Figure 1) (2). In addition to ATP production, mitochondria are the main cellular regulators of oxidative stress, with an elevated production of reactive oxygen species (ROS) by RC and several enzymatic or nonenzymatic antioxidative mitochondrial systems (3). Mitochondria also represent the site of the initial steps of steroidogenesis with 2 enzymes located in mitochondria: the cholesterol side-chain cleavage enzyme cytochrome P450 converting cholesterol to pregnenolone (PREG) and the 3β-hydroxysteroid dehydrogenase producing progesterone (PROG) from PREG, the latter is also located in the endoplasmic reticulum (4).

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Abbreviations: AKGDH, α-ketoglutarate dehydrogenase; C I, complex I (NADH-ubiquinone oxidoreductase); C II, complex II (succinate-ubiquinone oxidoreductase); C III, complex III (ubiquinone-cytochrome *c* oxidoreductase); C IV, complex IV (cytochrome *c* oxidase); CoA, coenzyme A; C V, complex V (ATP synthase); CX, orchidectomized (castrated); 5α-DH-PROG, 5α-dihydroprogesterone; $FADH^2$, reduced flavin adenine dinucleotide.

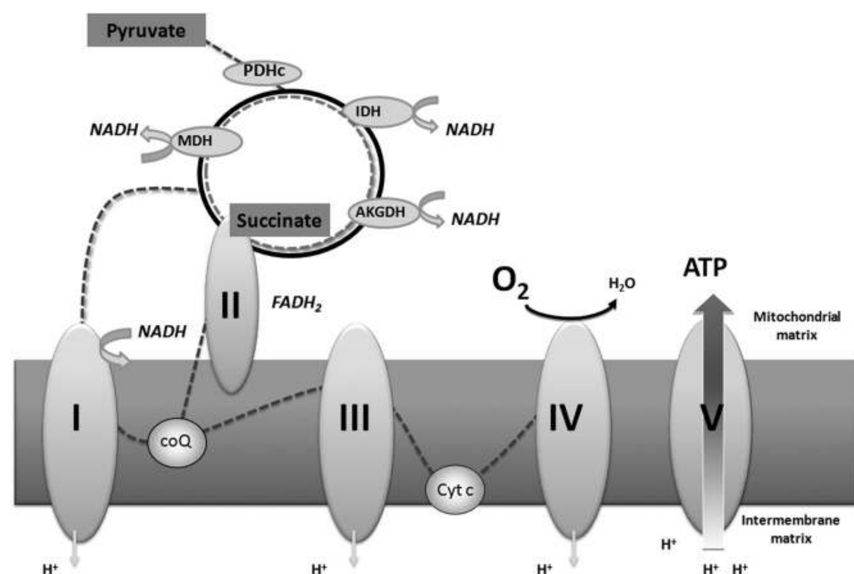

Figure 1. Schematic pathways of NADH and $FADH_2$-linked respirations. Pyruvate is converted to acetyl-CoA by the PDHc, and acetyl-CoA is a substrate for the TCA cycle. The TCA cycle provides reducing equivalents of NAD via the activity of IDH, AKGDH, and MDH. NADH electrons are carried to the ubiquinone pool by C I. C II couples the oxidation of succinate to fumarate with $FADH_2$ electron transfer directly to the ubiquinone pool. C III carries electrons from the ubiquinone pool to cytochrome *c*, and C IV catalyzes the transfer of reducing equivalents to molecular oxygen to produce water. The concomitant electrochemical gradient is used by C V to phosphorylate ADP to ATP. NADH-linked respiration depends on the activities of PDHc, TCA cycle enzymes, C I, C III, and C IV and of the phosphorylating apparatus efficiency. $FADH_2$-linked respiration depends on the activities of C II, C III, and C IV and of the phosphorylating apparatus efficiency.

Increasing evidence indicates that brain mitochondria are the targets of steroids action. Indeed, in neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, sex-dependent susceptibility, and mitochondrial dysfunction have been identified (5). Sex differences in mitochondrial responses have been described in experimental pathological models, such as oxidative stress, Parkinson's disease and traumatic brain injury; and the involvement of sex steroids was confirmed in some cases (6–8). Further, pharmacological studies with young adult female rodents showed that steroids influenced brain function: ovariectomy decreased brain mitochondrial oxidative phosphorylation and increased oxidative stress (9–13); exogenous administration of 17β-estradiol (9–12, 14–17) or PROG (14) rescued ovariectomy-induced modifications; PROG in the low physiological range reversed the decrease in mitochondrial respiration after traumatic brain injury (6). In gonadectomized male and female rats, estrogen treatment has been shown to suppress brain mitochondrial stress (12). These studies demonstrated the potential of 17β-estradiol and PROG as neuroactive therapeutic drugs. They also suggest that the effects of female sex steroids on mitochondrial function could contribute to the natural protection observed in females against neuronal damage. However, although administration of either 17β-estradiol or PROG to ovariectomized (OVX) rats enhanced brain mitochondrial respiration, coadministration of 17β-estradiol and PROG did not (14), suggesting possible antagonistic effects between the hormones. In the female rat hippocampus, continuous administration of 17β-estradiol associated with sequential addition of PROG reversed ovariectomy-induced alterations in gene expression whereas continuous coadministration of 17β-estradiol and PROG did not (18). Thus, the global effects of endogenous female steroids are difficult to fully appreciate from exogenous administration experiments. Studies in intact animals are necessary to take into account physiological endocrine variations and to evaluate the influence of endogenous female steroids on brain mitochondrial function. To address this issue, we analyzed the effects of 3 different endocrine conditions: 1) male and female mice during the reproductive period (intact and after gonadectomy), 2) females at 3 time points of the estrous cycle, and 3) aged males and females. For each set, we measured brain steroid concentrations by gas chromatography-mass spectrometry (GC/MS), and we evaluated 3 brain mitochondrial parameters: mitochondrial content (mitochondrial DNA [mtDNA] to nuclear DNA [nDNA] ratio, citrate synthase activity), mitochondrial oxidative phosphorylation function (oxygen consumption and enzymatic activities), and mitochondrial oxidative stress (aconitase to fumarase activity ratio, reduced glutathione [GSH] concentrations).

Materials and Methods

Animals

Female and male C57BL6J mice were purchased from Janvier Labs and were housed in a temperature-controlled room on a 12-hour light, 12-hour dark cycle (lights on from 8 AM to 8 PM). Mice were included in experimental sets after 2 weeks of habituation to the local facilities. All procedures concerning animal care and use were carried out in strict accordance with national

GC/MS, gas chromatography-mass spectrometry; GSH, reduced glutathione; GSSG, oxidized glutathione; IDH, isocitrate dehydrogenase; m-Cl-CCP, carbonyl cyanide m-chlorophenylhydrazone; MDH, malate dehydrogenase; mtDNA, mitochondrial DNA; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; nDNA, nuclear DNA; OVX, ovariectomized; PDHc, pyruvate dehydrogenase complex; PREG, pregnenolone; PROG, progesterone; RC, respiratory chain; ROS, reactive oxygen species; TCA, tricarboxylic acid; 3α,5α-THPROG, 3α,5α-tetrahydroprogesterone; 3β,5α-THPROG, 3β,5α-tetrahydroprogesterone.

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guidelines (authorization 94-345 to R.G., animal facility approval 94-043-13) and with French ethical laws (Act 87-848 and Act 2013-11, Ministère de l'Agriculture et de la Forêt, European Communities Council Directives of November 24, 1986 86/609/EEC). For the 3 experimental sets, a total of 43 animals were used.

To check cycling regularity, the estrous cycle was monitored daily, between 10 and 11 AM (2–3 h after lights on), over 10 consecutive days by examination of vaginal smears in a large group of intact young adult females. The adopted nomenclature was proestrus, estrus, metestrus, and diestrus according to the percentage of different cell types present: proestrus (a majority of nucleated epithelial cells), estrus (a majority of cornified epithelial cells), metestrus (cornified epithelial cells and leukocytes), and diestrus (a majority of leukocytes) (19). After 10 consecutive days, the estrous cycle stage was checked in all females, and those in the desired stages were selected to constitute the different experimental groups of that day.

The first experimental set was composed of 4 groups of young adult mice (3-month-old, n = 5 per group): intact males, intact females (diestrus stage), orchidectomized (castrated) (CX) males, OVX females. The second experimental set was composed of 3 groups of young adult female mice (3-month-old, n = 4 per group) corresponding to 3 different time points of the estrous cycle (diestrus, estrus, and proestrus stages). The third experimental set was composed of intact aged male and female mice (n = 5–6 per group). Mice aged 20 months were chosen to ensure that the females were reproductively senescent (20).

For the second experimental set, the gonadectomy procedure was performed by Janvier Labs 3 weeks before the day of the experiment. Briefly, surgery was performed under general anesthesia: ketamine (100 mg/kg) + xylazine (10 mg/kg). Additional analgesia was achieved with carprofen (5 mg/kg). The anesthetized animals were placed on a heating pad to maintain body temperature. After incision of the skin at the level of the scrotum for the orchidectomy and of the skin and muscle layers at each side for the ovariectomy, testis or ovaries were isolated, ligated, and sectioned. The incision was then closed with sutures. On the day of the experiment, the complete absence of testis in CX males and ovaries in OVX females was verified visually after dissection of each animal.

Sample preparation

Mice were handled gently to minimize stress and were quickly decapitated by rapid cervical dislocation without anesthesia. Mice were killed between 11 and 12 AM (3–4 h after lights on) for all experiments to avoid potential circadian effects. The whole brain minus the cerebellum was rapidly dissected on ice. The left hemisphere was immediately frozen on dry ice and stored at −80°C for steroid analysis. The right hemisphere was used for mitochondrial analysis. It was transferred to an ice-cold isolation buffer (20mM Tris, 0.25M sucrose, 40mM KCl, 2mM EGTA, and 1 mg/mL BSA; pH 7.2) and homogenized on ice by 5 strokes with a glass-Teflon potter homogenizer.

mtDNA quantification

Total DNA was extracted from the right hemisphere homogenate using a standard procedure. The mtDNA copy number per cell was measured by quantitative PCR based on the ratio of mtDNA to nDNA as described (21). Quantitative PCR was performed to amplify a 155-nt region of the mitochondrial *MTCO2* gene (reference sequence NC_005089.1, with primers F1 AACCGAGTCGTTCTGCCAAT; R1 AACCCTGGTCGGTTTGATGTT) (22) and a 189-nt region of the nuclear *PPIB* gene (reference sequence NC_000075.6, with primers F2 CTCTACC AAGGCCCCTGTGA; R2 ACTGTGTGGGGTTAGCCACT).

Isolation of a mitochondria-enriched fraction

The right hemisphere homogenate was centrifuged at 330*g* for 7 minutes at 4°C to eliminate debris and nuclei. The supernatant was then centrifuged at 11 400*g* for 10 minutes at 4°C. The pellet was resuspended in isolation buffer supplemented with Percoll 5%, rehomogenized, and centrifuged again at 11 400*g* for 10 minutes at 4°C. The resulting pellet constituted the mitochondria-enriched fraction (23). Based on the cytochrome *c* oxidase activity pelleted in the mitochondrial fraction, the yield was 47 ± 11% of total mitochondria. The integrity of the mitochondrial outer membrane was checked by measuring cytochrome *c* oxidase activity: only 1.4 ± 0.6% of the total activity in the presence of 2.5mM laurylmaltoside was measured in the absence of the detergent.

Mitochondrial oxygen consumption

A part of the freshly prepared mitochondria-enriched fraction was suspended at a concentration of 0.25 mg of protein/mL in a respiratory medium (0.3M mannitol, 10mM KCl, 5mM $MgCl_2$, 10mM KH_2PO_4, and 1 mg/mL BSA; pH 7.4) and mitochondrial oxygen consumption was measured polarographically using a Clarke oxygen electrode (Hansatech Instruments) in a magnetically stirred chamber maintained at 37°C as described (23). reduced nicotinamide adenine dinucleotide (NADH)-linked respiration was initiated by the addition of pyruvate (8mM)/malate (0.2mM). In another chamber, reduced flavin adenine dinucleotide ($FADH_2$)-linked respiration was started by the addition of succinate (10mM) in presence of ATP (0.4mM) and rotenone (4μM). Mitochondrial oxygen consumption was then stimulated by the addition of ADP (0.16mM) in both chambers. Complex V (ATP synthase) (C V) was inhibited by the addition of oligomycin (5μM), to evaluate proton leakage. Uncoupled respiration was measured by the addition of carbonyl cyanide m-chlorophenylhydrazone (m-Cl-CCP) (2.4μM). Finally, nonrespiratory oxygen consumption was evaluated by the addition of KCN (1.3mM). The rate of oxygen consumption was based on the slope of the response of mitochondria to the successive administration of substrates and expressed as nanomoles of O_2 per minute and per milligram of protein.

Enzymatic activities

A part of the mitochondria-enriched fraction was resuspended in isolation buffer to a concentration of 2 mg of protein/mL. Citrate synthase, complex I (NADH-ubiquinone oxidoreductase) (C I), complex II (succinate-ubiquinone oxidoreductase) (C II), complex III (ubiquinone-cytochrome *c* oxidoreductase) (C III), complex IV (cytochrome *c* oxidase) (C IV), isocitrate dehydrogenase (IDH), α-ketoglutarate dehydrogenase (AKGDH) and malate dehydrogenase (MDH) activities were spectrophotometrically measured at 37°C as described (23, 24). Pyruvate dehydrogenase complex (PDHc) activity was measured by monitoring NADH formation at 340 nm according to the method described by Hinman and Blass with some minor modifications (25). Mitochondria were

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placed in a reaction mixture at 37°C containing buffer pH 7.5 (50mM K_2HPO_4, 0.01% CHAPS, 6mM pyruvate, 0.2mM thiamin pyrophosphate, 0.25mM coenzyme A [CoA], 5mM $MgCl_2$, 0.3mM KCN, and 0.08mM rotenone). The reaction was initiated by the addition of 2.5mM nicotinamide adenine dinucleotide (NAD). All enzymatic activities were expressed as nanomoles per minute and per milligram of protein.

Oxidative stress markers

Brain mitochondrial oxidative stress was evaluated on the basis of the aconitase to fumarase activity ratio and by mitochondrial glutathione concentration. For aconitase and fumarase activities, a part of the mitochondria-enriched fraction was resuspended in isolation buffer to a concentration of 2 mg of protein/mL. Activities were spectrophotometrically measured at 37°C and the ratio of the activities of aconitase to fumarase was calculated (24). The tricarboxylic acid (TCA) cycle enzyme aconitase contains an iron-sulfur cluster in its catalytic site that makes it very susceptible to inactivation by ROS, whereas the activity of fumarase, another TCA cycle enzyme, is ROS insensitive (26). Decrease in the ratio of aconitase to fumarase activity is a functional indicator of ROS production. For glutathione determination, a part of brain homogenate and a part of mitochondria-enriched fraction were immediately frozen on dry ice and stored at −80°C. GSH and oxidized glutathione (GSSG) concentrations were measured by reverse phase HPLC coupled with electrochemical detection as previously described (27). Limits of quantification were 0.6μM and 0.9μM for GSH and GSSG, respectively.

Measurement of brain steroid levels by GC/MS

PREG, PROG, 5α-dihydroprogesterone (5α-DHPROG), 3α,5α-tetrahydroprogesterone (3α,5α-THPROG), 3β,5α-tetrahydroprogesterone (3β,5α-THPROG), and 17β-estradiol levels were determined by GC/MS according to the validated protocol described by Liere et al (28, 29) with minor modifications. Briefly, steroids were extracted from individual left brain hemispheres by adding 10 volumes of methanol. The internal standards epietiocholanolone (for PREG), $^2H_5$-17β-estradiol (for 17β-estradiol), 19 Nor-PROG (for PROG) and $^2H_6$-5α-DHPROG (for 5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG) were introduced into the extract for steroid quantification. Samples were purified and fractionated by solid-phase extraction with the recycling procedure and HPLC. Two fractions were collected: 5α-DHPROG and $^2H_6$-5α-DHPROG were eluted in the first HPLC fraction (3–10 min) and were silylated with N-methyl-N-trimethylsilyltrifluoroacetamide/NH_4I/dithioerythritol (1000:2:5 vol/vol/vol) for 15 minutes at 70°C. The second fraction (10–31 min) contained PREG, PROG, 3α,5α-THPROG, 3β,5α-THPROG, 17β-estradiol, epietiocholanolone, and 19-nor PROG. This fraction was derivatized with heptafluorobutyric anhydride in anhydrous acetone for 1 hour at 20°C. Both fractions were dried under a stream of N_2 and resuspended in hexane. Calibration and biological samples were injected with an AS 2000 autosampler (Thermo Scientific) and analyzed by GC/MS. The Focus GC gas chromatograph is coupled with a DSQII mass spectrometer (Thermo Scientific). Injection was performed in the splitless mode at 250°C (1 min of splitless time), and the temperature of the gas chromatograph oven was ramped between 50°C and 350°C. The helium carrier gas flow was maintained constant at 1 mL/min. The transfer line and ionization chamber temperatures were 300°C and 180°C, respectively. Electron impact ionization was used for mass spectrometry with ionization energy of 70 eV. Identification of each steroid was supported by its retention time and its 2 diagnostic ions (Supplemental Table 1). Quantification was performed in single ion monitoring mode according to the major diagnostic ion, called the quantification ion, and to the retention time of each derivatized steroid. The limit of detection of each steroid in brain tissues (200 mg) is indicated in Supplemental Table 1.

Statistical analysis

Data were expressed as mean ± SEM and were analyzed by a commercially available program (GraphPad Prism 4.1; GraphPad, Inc). Differences between intact and gonadectomized males and females were analyzed by two-way ANOVA (sex × gonadal status) followed by Bonferroni post hoc test. Differences between the 3 groups at different time points of the estrous cycle were analyzed by one-way ANOVA followed by Bonferroni post hoc test. For 2 groups' analysis, Student's *t* test was used. $P < .05$ was considered statistically significant.

Results

Sex-dependent differences in brain mitochondrial function of young adult mice and effect of gonadectomy

Brain mitochondrial content

Mitochondrial content was estimated by mtDNA quantification and citrate synthase activity: two indicators of mitochondrial number. The ratio of mtDNA to nDNA and the citrate synthase activity were similar in intact male and female mice and in gonadectomized mice (Supplemental Table 2).

Brain mitochondrial oxidative function

Mitochondrial oxygen consumption using different substrates (pyruvate for NADH-linked respiration and succinate for $FADH_2$-linked respiration) and the activities of the principal enzymes involved were determined (Figure 1).

NADH-linked respiration

Pyruvate+ADP oxidation, initiated by pyruvate+malate and ADP additions, depends on pyruvate uptake and metabolism, on the activities of C I, C III, and C IV, and on the phosphorylating apparatus efficiency. Two-way ANOVA analysis showed a very significant effect of sex ($F_{1,15} = 13.12$; $P = .002$), a very significant effect of gonadal status ($F_{1,15} = 10.87$; $P = .005$) and a trend of interaction between sex and gonadal status ($F_{1,15} = 4.22$; $P = .06$) on pyruvate+ADP oxidation rate. Bonferroni post hoc test revealed that pyruvate+ADP oxidation rate

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was significantly higher in the brain of female mice when compared with males ($P < .01$) and decreased after ovariectomy ($P < .01$). Orchidectomy did not modify male pyruvate+ADP oxidation rate. No difference was observed between intact or gonadectomized males and females, for the pyruvate+oligomycin oxidation rate, which principally reflects proton leak. Two-way ANOVA analysis showed a very significant interaction between sex and gonadal status on the pyruvate+m-Cl-CCP oxidation rate ($F_{1,15} = 9.54$; $P = .007$). This rate, which displays substrate oxidation exclusively, was significantly higher in females than in males (post hoc test $P < .01$) and decreased after ovariectomy (post hoc test $P < .05$) but not orchidectomy (Figure 2A).

$FADH_2$-linked respiration

Mitochondrial oxygen consumption using succinate, an electron donor to FAD in C II, was also measured to explore oxidative functions mediated by C II, C III, and C IV and the phosphorylating apparatus. Two-way ANOVA showed no significant effect of sex or gonadal status on succinate-oxidation rates in presence of ADP (stimulation), oligomycin (ATP synthase inhibitor), and m-Cl-CCP (uncoupler) (Figure 2B).

RC complexes, TCA cycle enzymes, and PDHc activities

To further investigate mitochondrial oxidative function, activities of RC complexes, TCA cycle enzymes, and PDHc were measured (Figure 3). No significant differences of C I, C II, C III, and C IV activities were observed between the 4 groups (Figure 3A). The activities of the 3 TCA cycle enzymes involved in NADH/NAD recycling (IDH, AKGDH, and MDH) were also determined and were similar in the brains of intact or gonadectomized male and female mice (Figure 3B). Two-way ANOVA analysis showed a significant interaction between sex and

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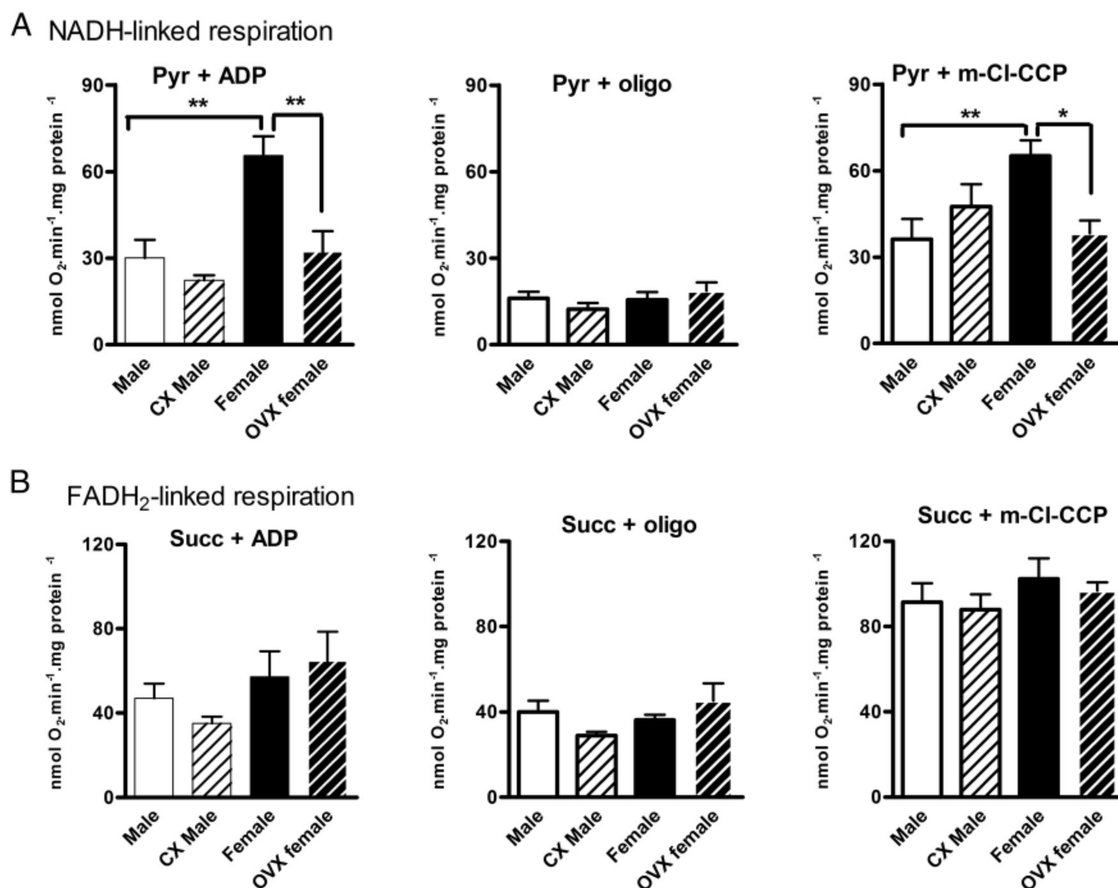

Figure 2. Sex differences in brain mitochondrial oxygen consumption function in 3-month-old mice: respiration rates that depend on the pyruvate oxidation pathway are higher in females than in males and decrease after ovariectomy. A, NADH-linked respiration rates. Oxygen consumption was measured polarographically in freshly isolated mitochondria by successive additions of pyruvate (8mM)-malate (2mM) + ADP (0.16mM) (pyr+ADP), ATP synthase inhibitor oligomycin (5μM) (pyr+oligo), and the uncoupler m-Cl-CCP (2.4μM) (pyr+m-Cl-CCP). B, $FADH_2$-linked respiration rates. Oxygen consumption was measured polarographically in freshly isolated mitochondria by successive additions of succinate (10mM) + ADP (0.16mM) (succ+ADP), ATP synthase inhibitor oligomycin (5μM) (succ+oligo), and the uncoupler m-Cl-CCP (2.4μM) (succ+m-Cl-CCP). Intact females were in diestrus. Estrous cycle classification was determined by daily vaginal smears examination. Gonadectomy was carried out 3 weeks before the experiment. Data represent mean ± SEM of 5 mice. Statistical analysis: two-way ANOVA (sex × gonadal status) followed by Bonferroni post hoc test. Significance: *, $P < .05$; **, $P < .01$.

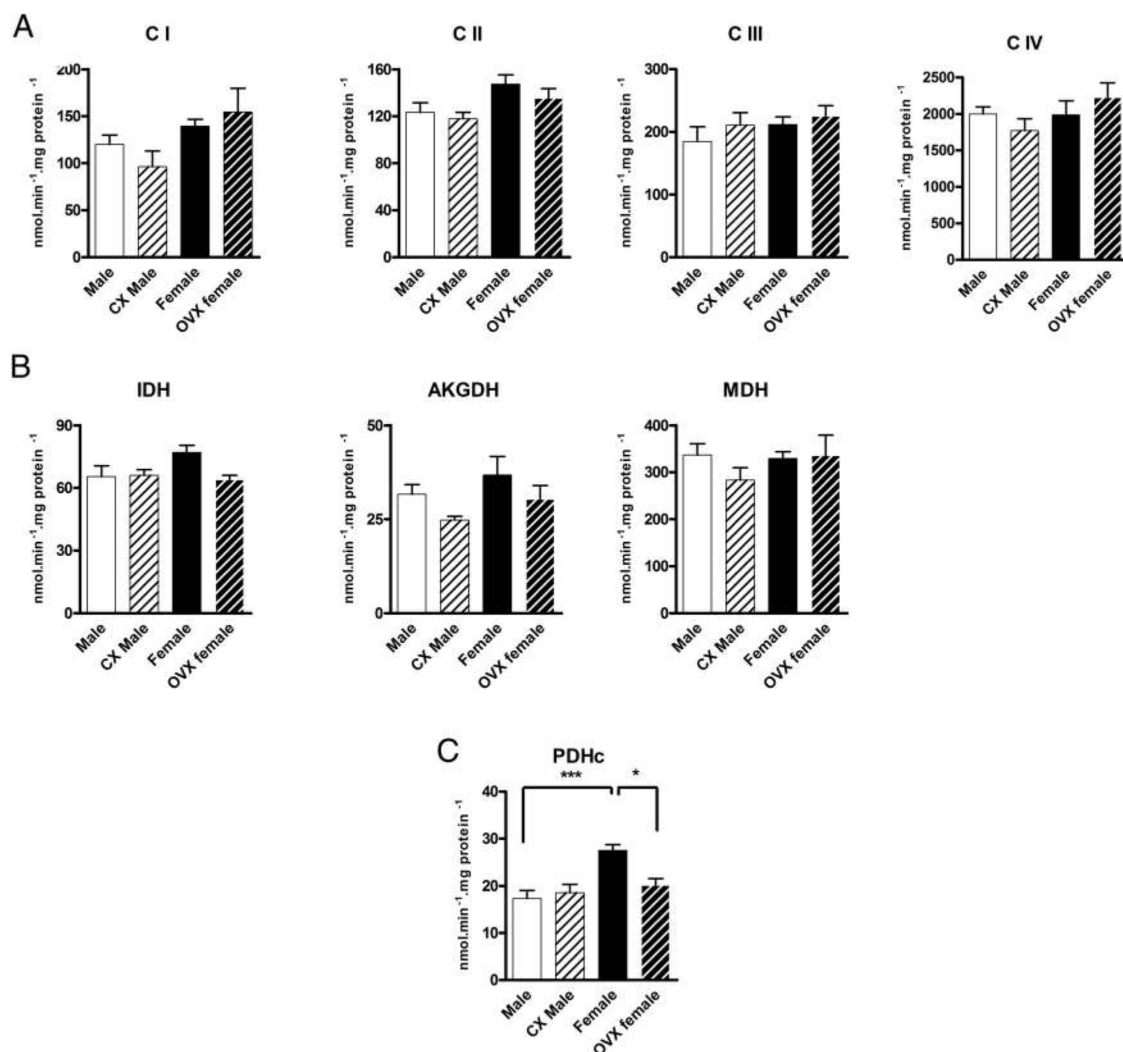

Figure 3. Analysis of brain mitochondrial enzymatic activities of RC complexes, TCA cycle enzymes, and PDHc in 3-month-old mice: the sex difference is in PDHc activity. A, Activities of electron transport chain enzymes. B, Activity of TCA cycle enzymes involved in NADH/NAD recycling. C, Activity of PDHc. Maximal enzyme activities were measured at 37°C in detergent-solubilized mitochondria. Intact females were in diestrus. Estrous cycle classification was determined by daily vaginal smears examination. Gonadectomy was carried out 3 weeks before the experiment. Data represent mean ± SEM of 5 mice. Statistical analysis: two-way ANOVA (sex × gonadal status) followed by Bonferroni post hoc test. Significance: *, $P < .05$; ***, $P < .001$.

gonadal status on the activity of PDHc ($F_{1,15} = 7.57$; $P = .01$). Bonferroni post hoc test revealed that the PDHc activity was significantly higher in the brains of female mice as compared with those of males (post hoc test $P < .001$) and decreased after ovariectomy (post hoc test $P < .05$). Orchidectomy did not modify PDHc activity (Figure 3C).

Brain mitochondrial oxidative stress

Two-way ANOVA analysis showed a very significant interaction between sex and gonadal status on the aconitase to fumarase activity ratio ($F_{1,16} = 9.10$; $P = .008$). This ratio was higher in intact females compared with intact males (post hoc test $P < .05$), reflecting lower mitochondrial ROS production in females and decreased after ovariectomy (post hoc test $P < .05$), whereas orchidectomy had no effect (Figure 4A).

Two-way ANOVA analysis showed a very significant interaction between sex and gonadal status on the GSH mitochondrial concentration ($F_{1,16} = 8.73$; $P = .009$). Compared with males, females presented a higher GSH concentration in brain mitochondria (post hoc test $P < .05$). This concentration decreased after ovariectomy (post hoc test $P < .01$) but not after orchidectomy (Figure 4B). The levels of GSSG were below the limit of quantification for all samples. GSH was also measured in brain homogenates (the cytosolic pool) and was equivalent in the 4 groups: 17.4 ± 2.0 nmol · mg protein^{-1} in intact males, 13.3 ± 0.1 nmol · mg protein^{-1} in CX males, 15.2 ± 1.5 nmol · mg protein^{-1} in intact females, and 13.1 ± 0.5 nmol · mg protein^{-1} in OVX females.

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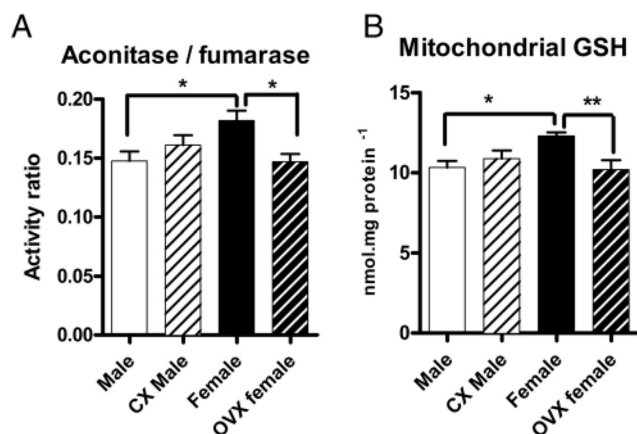

Figure 4. Sex differences in the markers of oxidative stress in brains of 3-month-old mice and their suppression by ovariectomy. A, Aconitase to fumarase activity ratio. Maximal enzyme activities were measured at 37°C in detergent-solubilized mitochondria. B, Mitochondrial GSH concentration. GSH concentrations were measured by reverse phase HPLC coupled with electrochemical detection. Intact females were in diestrus. Estrous cycle classification was determined by daily vaginal smears examination. Gonadectomy was carried out 3 weeks before the experiment. Data represent mean ± SEM of 5 mice. Statistical analysis: two-way ANOVA (sex × gonadal status) followed by Bonferroni post hoc test. Significance: *, $P < .05$; **, $P < .01$.

Brain steroid levels in young adult male and female mice

The above results for one brain hemisphere showed that sex differences in brain mitochondrial function were abolished by ovariectomy whereas orchidectomy was without effect. These observations pointed to a role of endogenous female brain steroid levels in mitochondrial function. We therefore measured the levels of PREG, the precursor of all steroids, PROG and its metabolites (5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG) and 17β-estradiol by GC/MS in the other brain hemisphere. The results are shown in Table 1. Brain PREG levels were about twice higher in female during the diestrus stage than in male mice ($P < .001$). PROG brain concentration was 13-times higher in females than in males ($P < .001$). The 5α-DHPROG/PROG ratio was 18.0 ± 4.0 in males and 0.64 ± 0.11 in females ($P < .01$), 5α-DHPROG levels being similar in males and females. Levels of 3α,5α-THPROG (also named allopregnanolone), and 3β,5α-THPROG (isoallopregnanolone) were not significantly different between both sexes. Brain levels of 17β-estradiol are very low in mice during the diestrus stage and they were below the limit of detection of our GC/MS assay (20 pg/g). Brain levels of PROG and its reduced metabolites were markedly reduced after gonadectomy in both sexes.

Brain steroid levels and mitochondrial function in female mice at 3 time points of the estrous cycle

The potential influence of endogenous female steroids on mitochondrial function raised the question of putative

Table 1. Brain Levels of PREG, PROG, and Its 5α-Reduced Metabolites in Intact and Gonadectomized Young Adult Male and Female Mice, in Young Adult Female Mice at 3 Time Points of the Estrus Cycle, and in Aged Male and Female Mice: GC/MS Analysis

	PREG	PROG	5α-DHPROG	3α,5α-THPROG	3β,5α-THPROG
	ng/g				
3-month-old mice: sex differences					
Intact males	5.59 ± 0.71[a]	2.64 ± 1.10[a]	19.22 ± 2.13	1.05 ± 0.46	0.21 ± 0.05
Intact females (diestrus)	11.56 ± 1.06	35.08 ± 5.79	20.39 ± 2.24	2.39 ± 0.42	0.29 ± 0.04
3-month-old mice: gonadectomy effect					
CX males	2.54 ± 0.15	0.52 ± 0.11	2.20 ± 0.26	0.30 ± 0.10	0.02 ± 0.01
OVX females	6.78 ± 0.89	0.61 ± 0.33	1.70 ± 0.43	0.11 ± 0.02	0.03 ± 0.01
3-month-old female mice: levels at 3 time points of the estrus cycle (samples collected 3–4 h after lights on)					
Proestrus	10.88 ± 0.83	9.01 ± 4.63[b]	7.52 ± 2.20[c]	3.29 ± 1.24	0.27 ± 0.12
Estrus	13.47 ± 1.89	4.24 ± 2.74[c]	3.63 ± 1.31[c]	2.10 ± 1.02	0.15 ± 0.05
Diestrus	12.02 ± 1.23	30.14 ± 3.87	20.90 ± 2.82	2.53 ± 0.50	0.25 ± 0.02
20-month-old mice: sex differences					
Aged males	4.89 ± 0.28	0.28 ± 0.10	2.85 ± 0.43	0.63 ± 0.25	0.12 ± 0.04
Aged females	3.46 ± 0.50	1.30 ± 0.50	3.79 ± 0.40	1.10 ± 0.47	0.05 ± 0.02

Brain levels of steroids were determined in one hemisphere and are expressed as nanograms per gram of brain. Gonadectomy was carried out 3 weeks before the experiment. Estrus cycle classification was determined by daily vaginal smears examination, and samples were collected 3–4 h after lights on.

[a] $P < .001$ intact males vs intact females in diestrus stage (Student's t test).

[b] $P < .05$ estrus or proestrus stage vs diestrus stage (one-way ANOVA followed by Bonferroni post hoc test). Mean ± SEM of 4–6 mice.

[c] $P < .01$ estrus or proestrus stage vs diestrus stage (one-way ANOVA followed by Bonferroni post hoc test). Mean ± SEM of 4–6 mice.

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variations in brain mitochondrial efficiency in cycling females. To address this issue, we measured brain steroid levels and analyzed mitochondrial parameters in young female mice at 3 time points of the estrous cycle.

Brain steroid levels

Mice were assigned to 3 groups (proestrus, estrus, and diestrus groups according to the cells composition of vaginal smears), brain samples were collected 1–2 hours after smears collection, ie, 3–4 hours after lights on.

The levels of the precursor PREG were not significantly different in the 3 groups studied. PROG and 5α-DHPROG differed significantly (ANOVA $F_{2,8} = 14.91$; $P = .002$ for PROG; ANOVA $F_{2,9} = 16.96$; $P = .001$ for 5α-DHPROG). Brain levels were lower in estrus and proestrus as compared with those in diestrus groups (post hoc test $P < .01$ estrus vs diestrus; post hoc test $P < .05$ proestrus vs diestrus). However, the 5α-DHPROG to PROG ratios were not significantly different (0.8 ± 0.3 for proestrus group, 1.5 ± 0.3 for estrus group, and 0.7 ± 0.1 for diestrus group). Levels of 3α,5α-THPROG and 3β,5α-THPROG were not significantly different (Table 1). 17β-estradiol concentrations were below the limit of detection for 8 out of 12 samples (20 pg/g).

Brain mitochondrial function

Markers of mitochondrial content (Supplemental Table 3), mitochondrial respiration rates (Supplemental Figure 1), and oxidative stress indicators (Supplemental Figure 2) were measured at the 3 time points of the estrous cycle studied. Total GSH was also determined in brain homogenates: 12.5 ± 0.3 nmol · mg protein^{-1} for proestrus group, 17.3 ± 1.6 nmol · mg protein^{-1} for estrus group, and 13.7 ± 0.6 nmol · mg protein^{-1} for diestrus group. No significant differences in all these parameters were detected in this physiological model of short-term variations of female steroid levels.

Brain steroid levels and mitochondrial function in aged male and female mice

To further evaluate the effect of female steroids on brain mitochondrial function, aged male and female mice were studied, as a model of permanent decrease in gonadal steroids.

Brain steroid levels of aged male and female mice

Brain PREG levels were quite similar between aged males and aged females. Brain PROG levels were strongly decreased in aged females compared with young females, but they still tended to be higher in aged females than in aged males (1.3 ± 0.5 vs 0.3 ± 0.1 ng/g), without, however, reaching statistical significance ($P = .10$). Levels of the reduced metabolites of PROG decreased in aged mice and were similar in both sexes as was observed in young adult mice (Table 1). Brain concentrations of 17β-estradiol were below the limit of detection for both sexes.

Brain mitochondrial function in aged male and female mice

Mitochondrial content was not significantly different between aged male and female mice (Supplemental Table 4). Unlike what was observed for young adult mice (Figure 2A), the NADH-linked respiration rates did not statistically differ between aged male and female mice (Figure 5A) and nor did $FADH_2$-linked respiration rates (Figure 5B). No sex differences in markers of mitochondrial oxidative stress were observed between aged male and female mice (Figure 6). GSH in brain homogenates were also similar (15.6 ± 1.4 nmol · mg protein^{-1} in aged males and 14.5 ± 0.6 nmol · mg protein^{-1} in aged females).

Discussion

The aim of the present study was to evaluate the global influence of endogenous gonadal steroids on brain mitochondrial function. Our results show a sex difference in brain mitochondrial respiration and oxidative stress that is suppressed with aging and ovariectomy but not orchidectomy, pointing to a role of ovarian steroids. A decline in ovarian steroids levels was concomitant with decreased PDHc activity but had no effect on mtDNA content nor on enzymatic activities of the RC complexes or the TCA cycle. It is worthy to note that the higher mitochondrial respiration and the lower oxidative stress were observed in mice which had the higher brain levels of PREG and PROG.

Brain levels of PROG and its reduced metabolites 5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG were measured by GC/MS, the reference method for the precise analysis and profiling of steroids (29, 30). However, despite its accuracy and specificity, the limit of sensitivity of GC/MS (20 pg/g) was above the threshold for the detection of low quantities of 17β-estradiol in the mice brain. This was unfortunate, as estrogens have a major influence on mitochondrial bioenergetics (31). However, the analysis of PREG, PROG, and its 5α-reduced metabolites as markers for endocrine changes still provides significant new information. The first major finding was the very elevated ratio of brain 5α-DHPROG to PROG in the male brains as compared with female brains, suggesting that 5α-reductase activity was higher in males. Interestingly, the 5α-DHPROG to PROG ratio remained low in females throughout the 3 studied time points of the estrous cycle. As a consequence, despite the much lower levels of PROG

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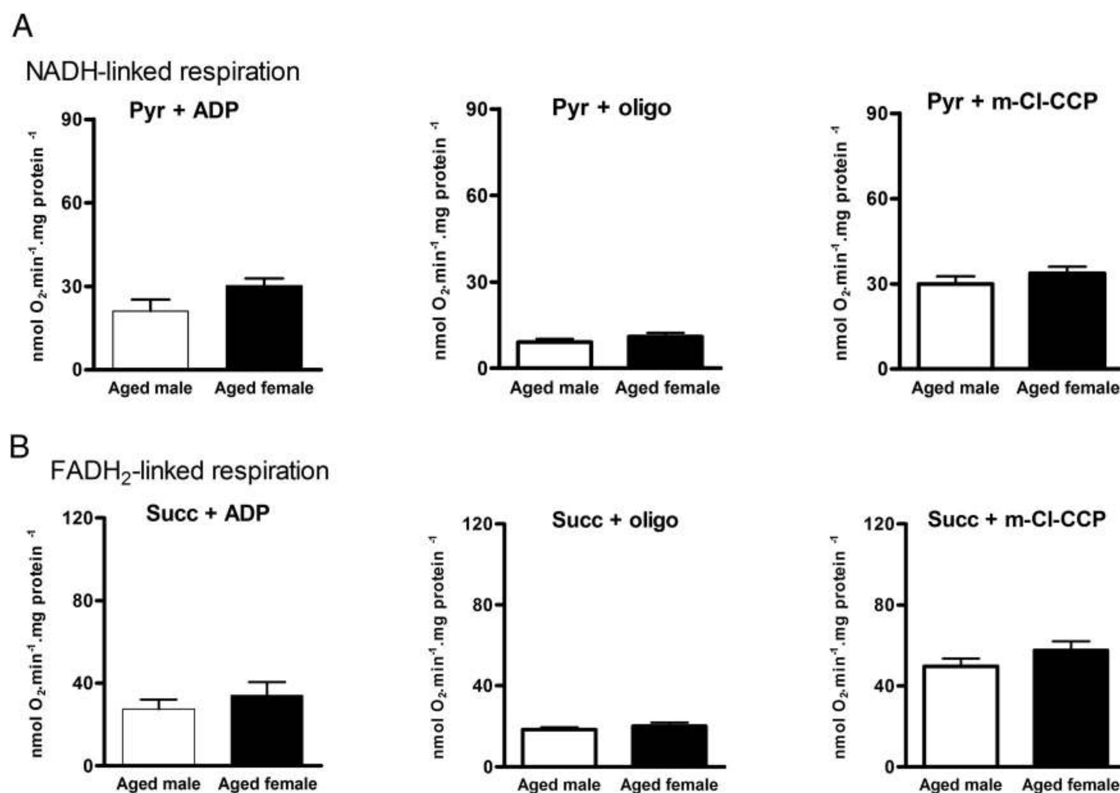

Figure 5. Brain mitochondrial oxygen consumption in 20-month-old male and female mice: sex differences are suppressed in aged mice. A, NADH-linked respiration rates. Oxygen consumption was measured polarographically in freshly isolated mitochondria by successive additions of pyruvate (8mM)-malate (2mM) + ADP (0.16mM) (pyr+ADP), ATP synthase inhibitor oligomycin (5μM) (pyr+oligo), and the uncoupler m-Cl-CCP (2.4μM) (pyr+m-Cl-CCP). B, $FADH_2$-linked respiration rates. Oxygen consumption was measured polarographically in freshly isolated mitochondria by successive additions of succinate (10mM) + ADP (0.16mM) (succ+ADP), ATP synthase inhibitor oligomycin (5μM) (succ+oligo), and the uncoupler m-Cl-CCP (2.4μM) (succ+m-Cl-CCP). Data represent mean ± SEM of 5–6 mice. Statistical analysis: Student's *t* test.

in the male brain, levels of 5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG were similar in both sexes. The 5α-reduction is indeed a rate-limiting step in the metabolism of both PROG and testosterone, PROG being the preferred substrate (32). It has been reported that the brain 5α-reductase may not be regulated by sex steroids (33). In the light of the higher brain 5α-DHPROG to PROG ratio in intact males than in females, this interpretation may have to be reconsidered. A second important observation concerns the pattern of the decrease of brain steroids in aged mice. Although PROG and 5α-DHPROG brain levels were very low in aged males and females, PREG brain levels were relatively preserved.

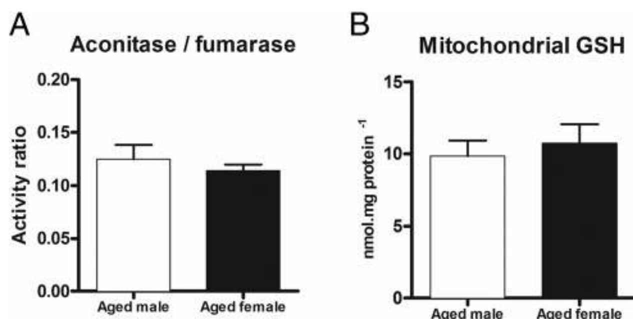

Figure 6. Markers of oxidative stress in brains of 20-month-old male and female mice: sex differences are suppressed in aged mice. A, Aconitase to fumarase activity ratio. Maximal enzyme activities were measured at 37°C in detergent-solubilized mitochondria. B, Mitochondrial reduced GSH concentration. GSH concentrations were measured by reverse phase HPLC coupled with electrochemical detection. Data represent mean ± SEM of 5–6 mice. Statistical analysis: Student's *t* test.

A question deserving particular attention is the role of PROG and its metabolites in the regulation of mitochondrial function. Both PROG and 5α-DHPROG bind to intracellular PROG receptors and can activate gene transcription in neural cells. A recent study has reported a major role of PROG signaling via its intracellular receptors for the regulation of mitochondrial bioenergetics (34). On the other hand, allopregnanolone does not bind to PROG receptor, but is a potent positive modulator of $GABA_A$ receptors (35, 36) and it also regulates mitochondrial functions (34, 37). Our results show that the brain levels of 5α-reduced PROG metabolites were similar in intact male and female mice, whereas levels of PROG were higher in females. This could suggest that it is PROG and not its metabolites which is important for the observed sex differences in brain mitochondrial function.

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Our results demonstrate that young female mice have higher concentrations of mitochondrial GSH, a major antioxidant in brain (38), and a higher aconitase/fumarase activity ratio, a functional indicator of ROS production (26), compared with young males. This suggests that, during the reproductive period, females may have better protection against damage induced by ROS. Interestingly, GSH contents in brain homogenates (mainly the cytosolic pool of GSH) were similar in males and females, as described in other studies (39, 40). The depletion of female steroids by ovariectomy potentially enhanced oxidative damage with decreased mitochondrial GSH, but not cytosolic GSH. In contrast, male orchidectomy did not modify the oxidative stress parameters. These results show that endogenous female steroids at physiological levels regulate brain mitochondrial oxidative stress and especially the mitochondrial GSH pool, which is distinct from the cytosolic one and whose regulation is particularly critical for neuronal function (41).

Our study showed another important sex difference in mitochondrial function: the NADH-linked respiration rate was higher in young females than in young males. Gonadectomy experiments indicated that endogenous female sex steroids were also involved in these sex differences. We explored several hypotheses in order to determine more precisely the associated mechanisms. We first checked if this could be due to a difference in the mitochondrial content of cells, as steroids have been reported to modulate mitochondrial biogenesis (42, 43). However, mitochondrial content was comparable between young males and females as attested by mtDNA to nDNA ratios and citrate synthase activity. Brain mitochondrial respiration was higher in females only when the respiratory donor substrate pyruvate was used, whereas it was similar when succinate was used. This indicates that only NADH-linked but not $FADH_2$-linked respiration was higher in females than in males. In addition, both pyruvate+ADP and pyruvate+m-Cl-CCP oxidation rates were higher in young females compared with males. Taken together, these results exclude a difference in phosphorylating activity and rather point to a difference in the capacity for pyruvate oxidation, depending either on pyruvate uptake or on the activities of pyruvate carboxylase, PDHc, TCA cycle enzymes, or C I, C III, and C IV. We first explored activities of the RC complexes, because several studies have shown that they can be regulated by female steroids (9–11, 14, 16). We did not detect any significant differences in the activities of C I, C III, and C IV between males and females. Moreover, 21 days after gonadectomy, activities of the RC complexes were similar between intact and gonadectomized mice. However, the effect of ovariectomy on the activities of the RC complexes may be brain region-dependent and/or time-dependent. Indeed, decreases of C I+III, C II, and C IV activities have been observed 28 days after ovariectomy in mitochondria isolated from female mice cortices (13). Another study reported a decrease of C IV activity but not C I activity in mitochondria isolated from whole brain 90 days after ovariectomy (9). As the higher pyruvate oxidation rate in young female mice observed in our study could not be explained by greater activities of RC complexes, we then explored a role of TCA cycle enzymes responsible for NADH/NAD recycling. It has indeed been reported that some of TCA cycle enzymes can be influenced by female steroids (44). The lack of changes in IDH, AKGDH, and MDH activities dismissed our hypothesis. Next, we studied PDHc activity. This enzyme complex constitutes the link between the glycolytic pathway and oxidative metabolism, catalyzing pyruvate oxidative decarboxylation to acetyl-CoA. We showed that PDHc activity was higher in young females than in young males and decreased after ovariectomy but not after orchidectomy. These findings indicate that endogenous female steroids regulate PDHc activity and are consistent with previous observations showing that ovarian hormones deprivation decreased the activity of PDHc (9). Taken together, our results suggest that the impact of the endogenous female gonadal hormones is primarily on glycolysis rather than on the oxidative phosphorylation system itself. It may also be noted that exogenous administration of 17β-estradiol or agonists to OVX female rats increased the expression of PDHc subunits and/or modified PDHc phosphorylation (10, 16, 17). Further analyses are now necessary to determine how endogenous female steroids act on PDHc activity.

To further understand the influence of ovarian hormones on brain mitochondrial function, we analyzed females at 3 time points of the estrous cycle, a model of transient physiological variations of gonadal steroids. We showed that brain PROG and 5α-DHPROG varied, with the highest levels for the samples from the diestrus group and the lowest levels for those collected from the estrus group, whereas levels of the steroid precursor PREG did not change. It is important to underline that our measurements are snapshots of the cycle and do not represent the overall dynamic changing levels of brain steroids. In particular, the expected proestrus PROG peak has occurred probably out of our collection period. No differences were observed in brain mitochondrial content, brain mitochondria respiration and oxidative stress despite the differences in PROG and 5α-DHPROG brain levels measured at the 3 time points studied. These results could seem inconsistent with the demonstration of endogenous female steroid effects on brain mitochondrial function. However, PROG levels fluctuate over a few hours in cycling females and

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levels of PROG in the female brain remain higher than in males whatever the time of analysis over the cycle. In addition, levels of the steroid precursor PREG in females are twice those in males at the 3 times studied. These observations, together with the distinct impact of gonadal hormones deprivation in males and females, suggest that the specific higher brain mitochondrial respiration rate and antioxidant protection observed in young females depends on the strong and permanent impregnation of the brain by endogenous female steroids over the reproductive period. Results from aged male and female mice are consistent with this hypothesis, as brain steroid levels decreased with aging and no subsequent sex differences in mitochondrial function were observed. In the aged mice, the difference between male and female brain PROG levels was strongly attenuated and the difference in PREG levels was abrogated. Under these endocrine conditions, females develop the same mitochondrial phenotype as males.

A strong divergence in the effects of ovariectomy and orchidectomy was observed, suggesting that brain mitochondrial function is impacted to a greater degree by loss of female hormones than by male hormones. However, we could not exclude subtle brain region-specific changes and/or effects of steroids on other parameters not analyzed in our study. After orchidectomy of Sprague-Dawley rats, the expression of the transcriptional factor peroxisome proliferator-activated receptor γ coactivator 1, a key regulator of mitochondrial function, was decreased in the hippocampus but increased in the cortex (45). The use of the whole hemisphere for the isolation of mitochondria in our study could conceal opposite effects in different brain regions. Further analysis of specific brain regions could be useful to determine whether endogenous steroids effects are brain region-dependent. In whole-brain mitochondria, an effect of orchidectomy has been shown for aconitase activity with Fisher 344 rats. However, the administration of testosterone or dihydrotestosterone failed to reverse the orchidectomy-induced oxidative damage in rats whereas estrogen administration reversed it (12). These observations, along with our owns, suggest a predominant role of female hormones.

To summarize, our results show that young adult females have a lower oxidative stress and a higher NADH-linked respiration rate as compared with young adult males. These observations and data from measurements of steroid brain levels suggest that the higher endogenous brain levels of PREG and PROG in young females compared with males rather than hormonal fluctuation over the cycle may be key variables in regulating brain mitochondrial function. The demonstration of a sex difference in brain mitochondrial function may contribute to explaining sex influence on neurodegenerative diseases. Moreover, these findings emphasize the necessity to include both males and females in experimental studies, and in particular in treatment strategies which target mitochondria. Indeed, in many cases, the results may differ depending on sex. Understanding these differences is critical in reaching the correct conclusion and sex-based considerations and sex-specific observations will clearly strengthen the findings.

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