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A sex difference in oxidative stress and behavioral suppression induced by ethanol withdrawal in rats

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Abstract

Ethanol withdrawal (EW) is referred to the abrupt termination of long-term heavy drinking, and provokes oxidative brain damage. Here, we investigated whether the cerebellum and hippocampus of female rats are less affected by prooxidant EW than male rats due to the antioxidant effect of 17 β -estradiol (E2). Female and male rats received a four-week ethanol diet and three-week withdrawal per cycle for two cycles. Some female rats were ovariectomized with E2 or antioxidant (Vitamin E+Co-Q10) treatment. Measurements were cerebellum (Rotarod) and hippocampus (water-maze)-related behaviors, oxidative markers (O₂^{•-}, malondialdehyde, protein carbonyls), mitochondrial membrane swelling, and a key mitochondrial enzyme, cytochrome c oxidase (CcO). Separately, HT22 (hippocampal) cells were subjected to ethanol-exposure and withdrawal for two cycles to assess the effect of a CcO inhibitor on E2's protection for mitochondrial respiration and cell viability. Ethanol-withdrawn female rats showed a smaller increase in oxidative markers in cerebellum and hippocampus than male rats, and E2 treatment decreased the oxidative markers. Compared to male counterparts, ethanol-withdrawn female rats showed better Rotarod but poorer water-maze performance, accompanied by more severe mitochondrial membrane swelling and CcO suppression in hippocampus. E2 or antioxidant treatment improved Rotarod but not water-maze performance. In the presence of a CcO inhibitor, E2 treatment failed to protect mitochondrial respiration and cell viability from EW. These data suggest that antioxidant E2 contributes to smaller oxidative stress in ethanol-withdrawn female than male rats. They also suggest that EW-induced severe mitochondrial damage in hippocampus may blunt E2's antioxidant protection for hippocampus-related behavior.

Keywords

Antioxidant; Cytochrome c oxidase; 17 β -Estradiol; Ethanol withdrawal; Cerebellum; Hippocampus

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1. Introduction

Most individuals with alcohol (ethanol) dependence display ethanol withdrawal (EW) syndromes such as anxiety, tremor, and seizure when they abruptly stop drinking. EW is clinically significant because ethanol dependent individuals must withdraw from ethanol for the detoxification process. EW is hyperexcitatory in nature by upregulating excitatory glutamate neurotransmission. EW is also a prooxidant stimulus [1, 2] which causes oxidative organ damage. Compared to normal individuals, the serum of alcoholic patients during detoxification contained an elevated level of malondialdehyde (MDA), the end product of lipid peroxidation, and a reduced activity of antioxidant superoxide dismutase [3–5]. A higher level of lipid peroxide and a lower activity of superoxide dismutase were also found in the cerebral spinal fluid of alcohol withdrawn patients compared to normal individuals [2]. In agreement with these human studies, ethanol-withdrawn rats showed an increase in the plasma levels of reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS), a byproduct of oxidative lipid damage [1]. The excessive amount of ROS oxidizes cellular components, thereby impeding the normal function of cells, a phenomenon known as oxidative stress. The physiological significance of EW-induced oxidative stress is inferred from a study where a higher level of MDA in alcoholic patients during EW suffered from more severe EW syndromes [3]. Even after an extended period of detoxification, alcoholics showed a decrease in the serum level of antioxidant vitamin E and vitamin A, suggesting that EW provokes persistent oxidative damage.

EW-induced oxidative organ damage can be problematic to not only men but also women. In 2014, 10.6 million men and 5.7 million women aged 18 or older were reportedly heavy drinkers [6]. These epidemiological data indicate that drinking problems and consequent EW syndromes prevail in the population of both men and women. Sex differences in EW have been shown in human and animals studies which yielded somewhat controversial results. The period between the onset of alcohol abuse and the first occurrence of EW syndromes was shorter in women than men [7], indicating that women are more vulnerable to EW. In agreement, an animal study reported that female mice showed more severe cell death in the forebrain compared to male mice [8]. On the other hand, more men than women displayed withdrawal syndromes such as seizure, tremor, anxiety, and insomnia [7, 9]. The greater vulnerability of male than female subjects has been shown in an animal study where EW-induced anxiety was more severe in male rats than female rats [10]. Devaud et al. [11] have demonstrated that female rats are less susceptible to EW-induced seizure than male rats. Female rats were recovered from seizure more quickly and more protected by a neuroprotective steroid, pregnanolone than male rats [12]. These studies imply a complexity of a sex difference in EW, and raise a question of whether a sex difference in EW depends on a specific effect or a target area of EW.

17 β -estradiol (E2) is a steroidal hormone that is synthesized from cholesterol by ovary, and to a lesser extent, from testosterone by the catalytic action of aromatase. In addition to its well-known effect on reproductive functions, E2 exerts a variety of other effects including the modulation of neuronal signals and mitochondrial energy metabolism. Recently, E2 has been recognized as a conditional neuroprotectant [13], meaning that E2 protects neurons under a specific condition rather than any condition. In fact, E2 has shown a protective,

detrimental, or no effect on brain under different pathological conditions or brain areas [13–15]. Among many actions of E2, an unequivocal effect of E2 is an antioxidant activity through which the phenolic moiety of E2's structure scavenges free radicals [16, 17]. This antioxidant activity of E2 has been reported to play a role in a sex difference in oxidative stress. Oxidative damage to lipid and DNA are less in the liver mitochondria and synapsis of young adult female rats than male rats [18]. This sex difference was decreased by ovariectomy and reinstated by E2 replacement [18], suggesting that E2 may protect females from oxidative damage.

In the current study, we were interested in determining whether female and male rats respond to EW-induced oxidative stress differently, and if so, whether E2 plays a role in that difference. We focused on the cerebellum and hippocampus because these brain areas are particularly vulnerable to the damaging effect of EW. We report that EW increases oxidative stress less severely in the cerebellum and hippocampus of female than male rats through E2's antioxidant activity. We also report that while the antioxidant effect of E2 contributes to the improvement of cerebellum-related behavior, it fails to improve hippocampus-related behavior of ethanol-withdrawn female rats.

2. Material and methods

2.1. Materials

Major analytic reagents were purchased from Qiagen (Valencia, CA), Sigma Aldrich (St. Louis, MO), Abcam (Cambridge, MA), EMD/Millipore (Billerica, MA), and Cell Signaling (Danvers, MA). Diet ingredients were purchased from Research Organics (Cleveland, OH) or MP Biomedicals (Irvine, CA). HT22 cells, a murine hippocampal cell line, were the generous gift of Dr. David Schubert (Salk Institute, San Diego, CA).

2.2. Animals

Female and male Sprague-Dawley rats were three months old at the beginning of an ethanol diet. All animals were housed at 22–25°C and 55% humidity, with ad libitum access to water and a 12-hour light/dark cycle. Animal experimentation was conducted in accordance with the Guide to the Care and Use of Laboratory Animals [DHHS/NIH 85-23, 1996, Office of Science and Health Reports, DRR/NIH] and was approved by the University of North Texas Health Science Center Animal Care and Use Committee.

2.3. Ovariectomy and E2 implantation

The procedure of ovariectomy and E2 implantation was described in detail in our previous publication (Jung et al., 2002) [19]. Briefly, ovariectomy was performed on some female rats under isoflurane (2% v/v) anesthesia such that a small incision was made in the abdominal cavity directly above the ovary. The ovaries were removed bilaterally, and the incisions were closed with stainless steel wound clips. Subsequently, silastic pellets containing E2 or oil were subcutaneously implanted on the dorsal part of the rat, and thereafter replaced every three weeks [19]. Incisions were closed with Prolene (Ethicon, Somerville, NJ) suture. Two weeks were allowed for recovery from the surgery and for ovarian hormone clearance before an ethanol diet began.

2.4. In vivo EW paradigm and animal groups

We used the model of repeated EW (multiple episodes of EW) because it mimics the drinking pattern of human alcoholics [20–23] and increases the risk of brain damage [24]. Rats were assigned to 8 groups (6–10 rats/group). Four groups were gonad intact female and male rats that were assigned to the EW or the control (dextrin) group. Another 4 groups were ovariectomized rats that were divided into dextrin, EW, EW+E2, and EW+Vitamin E +Coenzyme-Q10 groups. For an antioxidant treatment, Coenzyme-Q10 (123 mg/kg body weight) and vitamin E (α -tocopherol acetate, 200 mg/kg body weight) (Sigma Chemical Co.) were daily administered by a gavage method throughout the diet program. We chose this combination (called Vt.E+Co-Q10 herein) based on a previous study where Vt.E+Co-Q10 more effectively improved psychomotor performance of mice than either drug alone [25]. Rats received a nutritionally balanced liquid diet containing 7.5% (v/v) of ethanol for four weeks followed by withdrawal for three weeks per cycle for two cycles. Dextrin (complex carbohydrate) isocalorically substituted for ethanol for a control diet. The concentration of ethanol was gradually increased to 7.5% during the first week of the ethanol diet. The physical appearance and body weights were monitored daily. Animals were fed chow pellets during withdrawal periods and were sacrificed on the last day of the EW paradigm to collect cerebellum and hippocampus.

2.5. Blood ethanol concentrations

Three hours after placement of fresh diet bottles on the last day of an ethanol diet, the rats were secured in a Plexiglas restraint device and a syringe fitted with a 25-gauge needle was inserted to tail vein. Blood (200 μ l) was withdrawn and immediately mixed with 90 μ l of ice-cold 0.55 M HClO₄. Samples were centrifuged at $1,500 \times g$ for 10 minutes to sediment protein precipitate. Supernatants were adjusted to ~pH 5 with 200 μ l of a solution containing 0.6 M KOH and 50 mM acetic acid and then centrifuged to sediment KClO₄ precipitate. Ethanol in the supernatant was measured by colorimetric assay [26] in which NAD⁺ reduction to NADH is coupled to ethanol oxidation by alcohol dehydrogenase (extinction coefficient $\epsilon = 6.2 \text{ mM}^{-1}\cdot\text{cm}^{-1}$) in a Beckman DU 640 spectrophotometer.

2.6. Behavioral tests and brain collection

2.6.1. Accelerating Rotarod—Rotarod method measures the ability of movement and balance, and these functions are controlled by the cerebellum [27]. This test was initiated at the end of a diet program (at the end of the second EW). The motor driven treadmill (Omnitech Electronics, Columbus, OH) records how quickly rats fall from an accelerating rod such that a shorter latency indicates poorer motor performance. The rotor consists of four cylinders that are mounted 35.5 cm above a padded surface. Rats were placed on the cylinder and a timer switch was simultaneously activated to rotate the cylinders. Acceleration continued until 44 rpm for maximum 90 seconds or animals fell to the padded surface, which simultaneously stopped the timer. Rats were tested for 3 sessions/day for 5 days with a 20-minutes resting period between sessions [28].

2.6.2. Morris water-maze—The Morris water-maze measures the ability of spatial memory and learning related to hippocampal functions [29]. Water-maze task was

performed according to a modified procedure that was originally designed by Morris [29], and was initiated at the end of the diet program. A dark circular pool, 140 cm in diameter and 60 cm in height was filled with water ($21 \pm 1^\circ\text{C}$). A transparent platform (10 cm $\times$ 10 cm) was located in the southeast quadrant of the pool. The pool was located in a room with a constant environment and visual cues around the pool. A video camera connected to a tracking system (Ethovision XT, Noldus Inc.) was mounted above the pool to analyze and record latency to reach the platform. Rats were first subjected to a pretraining phase of the water-maze task for 5 days to learn swimming and climbing to the platform. During this phase, the platform was located in a way that was visible above the surface of the water. Rats were then subjected to the acquisition test for 4 days to assess spacial learning. This test measures how well rats remember the same location of the platform that is now hidden 1 cm below the surface of the water. After three days of rest, rats were subjected to the retention memory test with the same procedure to that of the acquisition test. This test measures how well the memory of the platform location is retained without water-maze task for 3 days. At the beginning of each trial, an animal started at one of four different starting points in a random order (designated north, east, south, and west). The rat was carefully placed in the water and positioned to face the wall of the pool and allowed to locate the platform, which was situated in the southeast quadrant during all trials. A maximum time of 90 seconds was allowed for each trial. If the rat did not locate the platform within the maximum time, it was guided to the platform and allowed to remain there for 20 seconds. Three trials were conducted each day.

2.6.3. Collection of brain tissues—At the end of behavioral tests, rats were humanely sacrificed under anesthesia (20 mg/kg of xylazine, and 100 mg/kg of ketamine, intraperitoneally), and cerebellum and hippocampus were collected for biochemical assays. We chose cerebellum and hippocampus because these brain areas are vulnerable to the damaging effect of EW [28, 30].

2.7. Isolation of mitochondria

Mitochondria were isolated by conventional differential centrifugation with slight modifications [31]. Cerebelli or hippocampi were dissected, rinsed, and rapidly transferred to a homogenizer containing ice-cold isolation buffer (320 mM sucrose, 1 mM K_2EDTA , 10 mM Tris-HCl). A homogenate was prepared and centrifuged at $1,330 \times g$ for 5 minutes at 4°C and the supernatant was saved. The pellet was resuspended in half the volume of the original isolation buffer and centrifuged again under the same conditions. The two supernatants were combined and centrifuged further at $21,200 \times g$ for 5 minutes. The resulting pellet was resuspended in 12% Percoll solution (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and centrifuged at $6,900 \times g$ for 10 minutes. The resulting soft pellet was washed once with mitochondrial isolation buffer and centrifuged again at $6,900 \times g$ for 10 minutes. The pellet contained mitochondria and was used in this study. Using isolated mitochondrial fraction, Bradford assay was conducted to analyze mitochondrial protein concentrations, according to manufacturer's instructions (Bio-Rad, Hercules, CA).

2.8. Assessment of oxidative markers

Superoxide ($O_2^{\bullet-}$), malondialdehyde (MDA), and protein carbonyls were measured to determine the extent to which EW provokes ROS generation, and oxidative damage to membrane lipid, and proteins, respectively.

2.8.1. $O_2^{\bullet-}$ — $O_2^{\bullet-}$ is the primary ROS species produced in mitochondria [32, 33]. $O_2^{\bullet-}$ was measured by recording the superoxide dismutase-inhibitable reduction of acetylated ferricytochrome c [34, 35]. The mitochondrial pellet was suspended in a hypotonic buffer (30 mM potassium phosphate, pH 7.4) and sonicated using a Branson Sonifier 250 equipped with a microtip (Danbury, CT). The sonicated sample was then centrifuged at $5,000 \times g$ for 10 minutes. The supernatant was centrifuged at $100,000 \times g$ for 40 minutes and the resulting pellet was resuspended in 30 mM potassium phosphate, pH 7.4. Ferricytochrome c reduction was monitored at 550 nm in a Beckman DU 640 spectrophotometer after addition of 7.5 mM succinate in the absence and presence of 100 U/ml superoxide dismutase. Ferricytochrome c concentration was obtained by applying an extinction coefficient of $27.7 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ to the absorbance values [34].

2.8.2. MDA—MDA is the end product of lipid peroxidation and often measured as a marker of oxidative damage to membrane lipid [36]. Brain samples were homogenized in ice-cold PBS and centrifuged at $4,000 \times g$ for 10 minutes at 4°C . Supernatants were acidified with trichloroacetic acid. Fifteen minutes later, thiobarbituric acid solution (0.75 ml) was added. The sample was incubated in a 100°C water bath for 45 minutes, cooled under running water, and 2 ml of n-butanol was added. After thorough mixing, the mixture was centrifuged at $4,000 \times g$ for 10 minutes. The absorbance of the upper phase was read at 532 nm using a microplate reader (Epoch Microplate Spectrophotometer, BioTek) [37].

2.8.3. Protein carbonyls—ROS modify proteins [38] by the carbonylation of protein residues, functionally inactivating the proteins [39]. Protein carbonyls were measured [40] using 2, 4-dinitrophenylhydrazine (DNPH), which combines with aldehyde or ketone moieties in proteins to form DNP-protein adducts. Briefly, brain tissues were homogenized in 50 mM HEPES buffer (pH 7.2) containing 10 mM KCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Calbiochem, San Diego, CA). DNPH (0.2 ml of 10 mM) in 2 N HCl was added to the 1 ml of homogenate, and 0.2 ml of 2 N HCl was added to another 1 ml of homogenate to provide a blank. Mixtures were incubated for 60 minutes at room temperature. The protein was precipitated with an equal volume of 20% trichloroacetic acid and was washed with ethanol-ethyl acetate (1:1 v/v). The final precipitate was dissolved in 2 ml of 6 M guanidine hydrochloride (pH 2.3), and insoluble debris was removed by centrifugation. Absorbance of DNP-protein adducts was measured at 360 nm ($\epsilon = 22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) [40] in a Beckman DU 640 spectrophotometer.

2.9. Mitochondrial membrane swelling

Mitochondrial membrane swelling was measured by monitoring absorbance decline at 540 nm [41]. Intact mitochondria scatter light at 540-nm wavelength. The prolonged or excessive opening of mitochondrial permeability transition pore provokes the swelling and rupture of mitochondrial membranes, which reduces mitochondrial light scattering and the absorbance.

Mitochondria were isolated as described above and the precipitant mitochondrial pellets were suspended in medium containing phosphate, which induces mitochondrial membrane swelling. The medium contained 250 mM sucrose, 10 mM Tris-MOPS, 0.05 mM EGTA, 5 mM pyruvate, 5 mM malate, and 1 mM phosphate, pH 7.4. Absorbance was measured at 540 nm in a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA).

2.10. CcO activity

Cytochrome c oxidase (CcO) is the terminal enzyme of electron transport chains in mitochondria. The activity of CcO in cerebellum and hippocampus was measured spectrophotometrically using a previously described method [42]. Briefly, reduced cytochrome c was prepared by mixing 100 mg of cytochrome c and 4 mg of ascorbic acid in 10 mL of potassium phosphate buffer (10 mmol/L, pH 7.0). Mitochondria solubilized with 0.2% sodium deoxycholate were added to the solution to initiate an enzymatic activity. The excess ascorbic acid was removed by dialysis for 24 hours. The CcO activity assay was carried out by the oxidation of exogenously reduced cytochrome c. The activity was monitored by recording the change in absorbance at 550 nm and calculated with the molar extinction coefficient of $29.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

2.11. In vitro procedures

2.11.1. In vitro EW—HT22 cells were used for an in vitro model of EW. The HT22 cell line is a good model to assess excitatory stress to hippocampal cells as the HT22 line was originally derived from mouse hippocampus, and selected from HT4 cells based on glutamate sensitivity [43]. In addition, an in vitro system allows more focused mechanistic manipulation. An in vitro model of EW has been used in others' studies in which EW induced the excitatory synaptic responses in cultured hippocampal cells [44]. HT22 cells were cultured in flasks until they reached 70% confluency, according to a method established by Perez et al. [45]. The cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and gentamicin (50 $\mu\text{g/mL}$), at 37°C in an atmosphere containing 5% CO_2 . HT22 cells were then exposed to ethanol (0 or 100 mM) for 20 hours followed by the removal of ethanol solution to create withdrawal stress for four hours. This cycle was repeated once more. The treatment with cyclosporin A (0.5 μM , the inhibitor of mitochondrial permeability transition pore) or NaN_3 (1 μM , CcO inhibitor) was restricted to EW phases to focus on EW stress. Separately, ethanol-free HT22 cells were treated with glutamate (3 mM) for 24 hours with or without NaN_3 . This was to mimic the property of EW that upregulates glutamate and inhibits CcO.

2.11.2. Mitochondrial membrane potential (Ψm)—The effect of EW on Ψm was assessed using a fluorescence microscope in HT22 cells. HT22 cells (2×10^4 cells/ml) were cultured in 12-well culture plates. Cells were exposed to ethanol (100 mM) for 20 hours and withdrawn from ethanol for 4 hours, repeating once more. The cells were then washed with PBS twice. JC-1 solution (0.5 ml; 2.5 $\mu\text{g/ml}$) was transferred into each well. The cells were incubated at 37°C in a 5% CO_2 incubator for 20 minutes. Subsequently, the cells were digested with trypsin. Precipitates obtained from the suspension after centrifugation (3 minutes, room temperature, $800 \times g$) were resuspended in 2 ml of PBS followed by centrifugation. JC-1-aggregates in healthy mitochondria emit red fluorescence at 590 nm.

JC-1-monomers that were leaked from stressed mitochondria emit green fluorescence at 530 nm. The cells were then observed with a fluorescence microscope using a dual-band pass filter designed to simultaneously detect fluorescein and rhodamine or fluorescein and Texas Red.

2.11.3. Mitochondrial respiration—Mitochondrial respiratory function was assessed by measuring the mitochondrial O₂ consumption rate according to a method provided by the XF respirometry manufacturer (Seahorse Bioscience, San Diego, CA). Briefly, HT22 cells (600 cells/well) were seeded into 24-well microplates, cultured, and exposed to the aforementioned in vitro EW paradigm [46]. At the end of the EW paradigm, the cell plate was placed on an O₂ sensor cartridge and inserted to the XF respirometer. E2's protection against EW-induced mitochondrial respiratory suppression was tested by treating cells with E2 (1 μ M) during the first and second phases of EW. Whether CcO inhibition blunts the protective effect of E2 was tested using a CcO inhibitor (NaN₃, 1 μ M). NaN₃ was preloaded in the reagent injection ports of the O₂ sensor cartridge and injected into the wells after the XF respirometry read the basal O₂ consumption rate. O₂ consumption rates (pmoles/minutes) were obtained approximately every 5 minutes.

2.11.4. Calcein-acetoxymethyl (Calcein-AM) ester viability assay—This assay was conducted to determine the cellular consequence of CcO inhibition under a hyperexcitatory milieu (glutamate or EW), and to determine whether CcO inhibition blunts the protective effect of E2 on cells. The membrane-permeant Calcein-AM ester dye (Invitrogen, Carlsbad, CA) was used to measure cell viability. Briefly, HT22 cells received the aforementioned EW with or without treatment with NaN₃ (1 μ M) and/or E2 (1 μ M) during EW phases. Separately, HT22 cells were exposed to glutamate (3 mM) for 24 hours with or without NaN₃ (1 μ M) cotreatment. After the removal of the medium from the 96-well cell plates, the cells were rinsed once with PBS, and incubated in PBS solution containing 2.5 μ M Calcein-AM. Twenty minutes later, fluorescence was determined using a BioTek FL600 microplate reader (BioTek Instruments, Winooski, VT) with an excitation/emission filter set at 495/515 nm. Wells treated with methanol served as blanks.

2.12. Data and statistical analysis

All numerical data are expressed as mean $\pm$ standard error of mean (SEM). Two-way ANOVA was used to analyze data of oxidative markers (diet $\times$ sex), Rotarod (diet/sex $\times$ day), water-maze (diet/sex $\times$ test-phase), CcO (diet $\times$ sex/brain area), and mitochondrial respiration (EW condition $\times$ CcO inhibitor treatment). Data of mitochondrial membrane swelling were analyzed using paired t-test or using the area-under-curve of sigmoidal curves. Data of cell viability were analyzed using one-way ANOVA (by a factor of treatment). One-way or two-way ANOVA was followed by post hoc Tukey's test to determine an individual group difference. p value was set less than 0.05 to indicate a statistically significant difference between groups.

3. Results

3.1. Cerebellum

3.1.1. EW increases oxidative stress less severely in the cerebellum of female rats than male rats—

Compared to control dextrin-diet rats, the cerebellum of ethanol-withdrawn female and male rats showed an increase in $O_2^{\bullet-}$, MDA, and protein carbonyls (Figure 1) [F (1, 22) = 281, $p < 0.001$ for $O_2^{\bullet-}$; F (1, 23) = 28, $p < 0.001$ for MDA; F (1, 22) = 92, $p < 0.001$ for protein carbonyls by a factor of a diet]. The prooxidant effect of EW on $O_2^{\bullet-}$, MDA, and protein carbonyls was less severe in the cerebellum of female rats than male rats [F (1, 22) = 108, $p < 0.0001$ for $O_2^{\bullet-}$; F (1, 23) = 14, $p = 0.001$ for MDA; F (1, 22) = 13, $p = 0.0016$ for protein carbonyls by a factor of sex]. Although the magnitude of a sex difference was moderate under a dextrin-diet condition, the smaller amount of $O_2^{\bullet-}$ ($p = 0.017$) and protein carbonyls ($p < 0.001$) were observed in female cerebelli than male cerebelli. There was a tendency of a similar sex difference in MDA, but it did not reach a statistical significance. Since E2 possesses an antioxidant property, we tested whether E2 contributes to the sex difference in EW-induced oxidative stress. We used a model of ovariectomy with or without E2 replacement to exclude the potential effect of ovarian hormones other than E2. The levels of $O_2^{\bullet-}$ ($p < 0.001$), MDA ($p < 0.0001$), and protein carbonyls were lower in E2 treated, ovariectomized rats than vehicle-treated ovariectomized rats [F (2, 15) = 685.0, $p < 0.0001$ for $O_2^{\bullet-}$; F (2, 15) = 34.9, $p < 0.0001$ for MDA; F (2, 21) = 20.3, $p < 0.0001$ for protein carbonyls by a factor of a diet/treatment]. These results suggest that the lower level of oxidative markers in the cerebellum of ethanol-withdrawn female rats than ethanol-withdrawn male rats is in part attributed to the antioxidant effect of E2.

3.1.2. EW provokes mitochondrial membrane swelling less severely in the cerebellum of female rats than male rats—

Mitochondrial membranes contain electron transport chains, the major source of ROS generation. The excessive generation of ROS deteriorates the integrity of mitochondrial membranes, leading to mitochondrial membrane swelling and the opening of mitochondrial permeability transition pore [47]. For example, the addition of ROS generator (KO_2) provoked brain mitochondrial swelling [48]. Our observation of lesser oxidative stress in the cerebellum of ethanol-withdrawn female than male rats led us to test whether a similar sex difference favoring female rats occurs in the effect of EW on the mitochondrial membrane integrity. The more rapid decline in the absorbance indicates the more severe mitochondrial membrane swelling. Compared with dextrin-diet rats, more rapid mitochondrial membrane swelling occurred in the ethanol-withdrawn female [$t = 2.82$, $df = 12$, $p = 0.016$] and male [$t = 5.91$, $df = 12$, $p = 0.015$] rats (Figure 2). To compare mitochondrial membrane swelling of female and male rats, we measured the area-under-curve of the sigmoidal curve of dextrin and EW group. A bigger difference in the area between the two groups (marked as a gray area) indicates more severe mitochondrial membrane swelling induced by EW [area-under-curve = 3.94 for female cerebellum; area-under-curve = 4.7 for male cerebellum]. This comparison revealed that EW-induced mitochondrial membrane swelling is less severe in female cerebellum than male cerebellum. These data indicate that EW perturbs the integrity of cerebellar mitochondrial membranes, a phenomenon that is less severe in female than male rats. The

mitochondrial membranes of dextrin-diet female rats also showed less swelling than male counterparts [$t = 3.2$, $df = 12$, $p = 0.008$], but the magnitude of the sex difference was less prominent than EW [a difference in area-under-curve = 1.6 between dextrin-diet female and male rats].

3.1.3. E2 attenuates EW-induced mitochondrial membrane swelling through its antioxidant activity—Our observation of less severe mitochondrial membrane swelling in the cerebellum of ethanol-withdrawn female than male rats raised a question of whether E2 plays a role in this sex difference (Figure 3). To test this possibility, we compared the mitochondrial membrane swelling of ovariectomized rats with or without E2 replacement. Ethanol-withdrawn ovariectomized rats treated with E2 showed a slower decline in the absorbance than vehicle-treated ovariectomized rats [$t = 3.49$, $df = 12$, $p = 0.0044$], indicating that E2 protects the mitochondrial membrane integrity of female cerebellum from EW. Should E2 protect mitochondrial membranes through its antioxidant activity, an antioxidant treatment alone would show a similar protection as did E2. We confirmed this presumption by the observation that Vt.E+Co-Q10 attenuated the effect of EW on mitochondrial membrane swelling [$t = 6.0$, $df = 12$, $p < 0.001$]. We then tested whether the absorbance decline in this assay reflects the opening of the mitochondrial permeability transition pore using the pore inhibitor (cyclosporin A). We observed that cyclosporine A treatment attenuated EW-induced the absorbance decline compared to vehicle-treated EW rats [$t = 6$, $df = 3.2$, $p = 0.007$].

3.1.4. EW impedes cerebellum-related behavior less severely in female than male rats—So far, we have observed a smaller prooxidant effect of EW on the cerebellum of female than male rats, and the antioxidant effect of E2. Given this, we hypothesized that EW impedes a cerebellar-related behavior (Rotarod) of female rats less severely than male rats through the antioxidant effect of E2. When the averages of 5-days-data were compared between treatment groups, ethanol-withdrawn rats fell from Rotarod quicker than dextrin rats, an indication of poor Rotarod performance of EW rats [$F(1, 56) = 5.68$, $p = 0.0205$ by diet] (Figure 4A). This effect of EW was less severe in female rats than male rats [$F(1, 56) = 13.93$, $p = 0.0004$, by sex]. Like the sex difference in EW, the Rotarod performance of dextrin-diet female rats was better (longer latency) than that of dextrin-diet male rats ($p < 0.0079$). The EW-induced suppression of Rotarod performance was attenuated by E2 ($p < 0.01$) or Vt.E+Co-Q10 ($p < 0.01$) treatment to ovariectomized rats [$F(3, 33) = 9.6$, $p < 0.01$] (Figure 4B). These data suggest that the antioxidant property of E2 contributes to a better Rotarod performance of ethanol-withdrawn female than male rats. We also examined whether EW impedes the learning of Rotarod performance by analyzing data by days of testing. We conducted Two-way ANOVA by a factor of treatment (dextrin-diet female rats, dextrin-diet male rats, EW female rats, and EW male rats) and a factor of days. Results indicated that both factors significantly altered Rotarod performance, but there was no interaction between two factors [$F(3, 132) = 4.9$, $p = 0.0032$ by treatment; $F(4, 132) = 9.6$, $p < 0.0001$ by day; $F(12, 119) = 0.57$, $p = 0.86$ by interaction] (Figure 4C). A similar phenomenon was observed in ovariectomized rats with or without E2 or antioxidant treatment. Treatment (dextrin-diet, EW, EW+E2, or EW+ Vt.E+Co-Q10) and days of testing altered Rotarod latency, but no interaction was found between two factors [$F(3, 122) = 4.8$,

$p = 0.0033$ by treatment; $F(4, 122) = 4.7$, $p = 0.0012$ by day; $F(12, 122) = 1.3$, $p = 0.24$ by interaction] (Figure 4D). These data suggest that while animal learn Rotarod task as they repeat the task for 5 days, neither EW nor sex significantly alters the learning process of this motoric performance.

3.2. Hippocampus

3.2.1. EW increases oxidative stress less severely in the hippocampus of female than male rats—As was the case for cerebellum, the hippocampus of ethanol-withdrawn female and male rats showed an increase in $O_2^{\bullet-}$, MDA, and protein carbonyls levels compared to dextrin-diet rats (Figure 5) [$F(1, 22) = 12$, $p < 0.0001$ for $O_2^{\bullet-}$; $F(1, 22) = 85$, $p < 0.0001$ for MDA; $F(1, 22) = 174$, $p < 0.0001$ for protein carbonyls by diet]. The prooxidant effect of EW on $O_2^{\bullet-}$, MDA, and protein carbonyls was less severe in the hippocampus of female rats than male rats [$F(1, 22) = 280$, $p < 0.0001$ for $O_2^{\bullet-}$; $F(1, 22) = 15$, $p = 0.0008$ for MDA; $F(1, 23) = 48$, $p < 0.0001$ for protein carbonyls by sex]. Unlike cerebellum, none of $O_2^{\bullet-}$, MDA, and protein carbonyl levels showed a significant sex difference under a dextrin-diet condition. Since we observed the antioxidant effect of E2 on cerebellum, we tested whether E2 also contributes to the lower level of oxidative stress in the hippocampus of female than male rats. The levels of $O_2^{\bullet-}$, MDA, and protein carbonyls were lower in E2 treated ovariectomized rats than vehicle-treated ovariectomized rats [$F(2, 15) = 296.5$, $p < 0.0001$ for $O_2^{\bullet-}$; $F(2, 16) = 22.22$, $p < 0.0001$ for MDA; $F(2, 21) = 31.73$, $p < 0.0001$ for protein carbonyls by diet or treatment]. These results indicate that the lower level of oxidative markers in the hippocampus of female rats than male rats is in part attributed to the antioxidant effect of E2.

3.2.2. EW provokes mitochondrial membrane swelling more severely in the hippocampus of female rats than male rats—Like cerebellum, ethanol-withdrawn female [$t = 7.44$, $df = 298$, $p < 0.0001$] and male rats [$t = 10.17$, $df = 149$, $p < 0.0001$] showed rapid mitochondrial membrane swelling compared with dextrin-diet rats. Unlike cerebellum, this effect of EW was more severe in female hippocampus (larger gray area-under-curve = 4.7) than male (area-under-curve = 2.9) hippocampus (Figure 6). Among dextrin-diet rats, no significant sex difference was found in hippocampal mitochondrial swelling. Neither E2 nor Vt.E+Co-Q10 treatment attenuated EW-induced mitochondrial membrane swelling of ovariectomized rats, indicating that the antioxidant effect is not sufficient to protect the mitochondrial membranes of hippocampus from EW.

3.2.3. EW impedes hippocampus-related behavior in female rats but not in male rats—In contrast to the sex difference in Rotarod performance in ethanol-withdrawn rats, EW suppressed the water-maze performance of female rats but not male rats [$F(3, 119) = 3.41$, $p = 0.02$, by diet $\times$ test phases]. Specifically, the time to reach platform was longer in ethanol-withdrawn female rats compared to dextrin-diet female rats ($p = 0.02$) or ethanol-withdrawn male rats ($p < 0.01$), but did not differ between ethanol-withdrawn male rats and dextrin-diet male rats (Figure 7A). After three days of rest, ethanol-withdrawn female rats took a longer time to find a platform during the retention phase compared to the last day (day 4) of an acquisition phase ($p = 0.01$). We analyzed the average of all data from the acquisition phase and separately from the retention phase to better compare a sex difference

in water-maze performance. Among dextrin-diet rats, female rats took a longer time to find a platform during the acquisition ($p < 0.01$) and the retention phase ($p < 0.01$) than male rats (Figure 7C). However, when the average data across testing days were compared between groups, it did not yield a difference between the acquisition and the retention phases. Ovariectomy blunted the sex specific effect of EW on the water-maze performance. Specifically, the time to find a platform did not differ between ovariectomized dextrin-diet and ethanol-withdrawn rats. Neither E2 nor Vt.E+Co-Q10 treatment improved the water-maze performance of ovariectomized rats (Figure 7B), indicating that the antioxidant effect is not sufficient to protect hippocampal behaviors of female rats from EW.

3.3. CcO in the cerebellum and hippocampus of female rats is less and more vulnerable to EW, respectively than male counterparts

Our results of mitochondrial membrane swelling in hippocampus and water-maze showed a similar sex difference in EW with a greater vulnerability of female hippocampus than male hippocampus to EW. To better understand this sex difference, we approached CcO located in mitochondrial membranes and essential for mitochondrial integrity. EW significantly suppressed the activity of CcO [$F(1, 20) = 1115$, $p < 0.0001$] (Figure 8). This effect of EW was less severe in female cerebellum than male cerebellum [$F(1, 20) = 22.82$, $p = 0.0001$], but more severe in female hippocampus than male hippocampus [$F(1, 20) = 4$, $p < 0.01$] (Figure 8A). Quite different phenomena from EW rats were observed in dextrin-diet rats. Compared to male cerebellum, female cerebellum showed a lower ($p < 0.0005$) activity of CcO. CcO activity in the hippocampus did not significantly differ between dextrin-diet female and male rats. E2 attenuated the EW-induced CcO inhibition in the cerebellum and hippocampus of ovariectomized rats [$F(2, 22) = 450.4$, $p < 0.0001$], but this effect of E2 was significantly smaller in the hippocampus than cerebellum of ovariectomized rats [$F(1, 22) = 5.74$, $p = 0.0255$] (Figure 8B). These data suggest that the vulnerability of mitochondrial membranes varies depending upon the nature of cellular milieu (control or EW) and brain area.

3.4. E2 is unable to protect mitochondrial respiration from EW in the presence of a CcO inhibitor

Our observation of the greater vulnerability of CcO in female hippocampus than male hippocampus raised a question of whether the severe inhibition of CcO counteracts the protective effect of E2 on mitochondrial respiration. We first measured the effect of EW on Ψ_m because damaged mitochondrial membranes would have low Ψ_m . Healthy mitochondria have high Ψ_m , and JC-1-aggregates emit red fluorescence. Damaged mitochondrial membranes have low Ψ_m , and JC-1-monomers emit green fluorescence. When cells were observed with a fluorescence microscope, ethanol-withdrawn cells exhibited more green fluorescence and less red fluorescence than control cells, indicating that EW results in more depolarized mitochondria (Figure 9A). This was accompanied by mitochondrial respiratory suppression in ethanol-withdrawn cells ($p < 0.001$), a phenomenon that was attenuated by E2 ($p = 0.002$) but exacerbated by NaN_3 ($p < 0.05$) (Figure 9B) [$F(2, 30) = 26.2$, $p < 0.0001$]. Notice that in the presence of a CcO inhibitor, the mitochondrial respiration of EW+E2 cells is not higher than that of vehicle-treated, ethanol-withdrawn cells. This suggests that E2 is unable to increase mitochondrial respiration in the presence of

a CcO inhibitor. The severe inhibition of CcO in hippocampus may hence blunt E2's protection for hippocampus.

3.5. CcO inhibition mediates the cytotoxicity of EW and blunts E2's cytoprotection

Based on our observation that EW suppresses CcO, we assessed the effect of glutamate on cell viability with or without NaN₃ treatment (Figure 10). This approach is to mimic the property of EW that upregulates glutamate and inhibits CcO. NaN₃ ($p < 0.02$) or glutamate ($p < 0.001$) treatment decreased cell viability, a phenotype that became more severe ($p < 0.0001$) by the combination of glutamate and NaN₃ [$F(3, 24) = 28.46$, $p < 0.0001$]. E2 treatment attenuated the cytotoxicity of EW ($p < 0.01$) but failed to do so in the presence of NaN₃ treatment [$F(3, 20) = 22.54$, $p < 0.0001$]. These data raise a possibility that EW-induced severe suppression of CcO may hamper the protective effect of E2 on hippocampus.

3.6. Blood ethanol concentrations do not differ between female and male rats

Blood ethanol concentrations (BEC) were measured to determine whether blood ethanol concentrations correlates with the sex difference in parameters measured in this study. Blood ethanol concentrations were 0.99 ± 0.03 mg/ml in female rats, and 1.02 ± 0.06 in male rats, showing no significant sex difference in BEC. This suggests that BEC unlikely contributes a sex difference in EW-induced oxidative stress and behavioral suppression.

3.7. The result summary of sex specific effects of EW on oxidative, mitochondrial, and behavioral markers

The comparisons between the responses of female and male rats to EW described so far in the Result section are listed in the table 1.

4. Discussion

The primary finding of this study is that EW-induced oxidative stress and impairment in the cerebellar-related behavior are less severe in female rats than male rats. The sex difference appears to be attributed in part to the antioxidant effect of E2. By comparison with cerebellum, EW impairs the hippocampus-related behavior only in female rats. While E2 treatment attenuates oxidative stress to the hippocampus of ethanol-withdrawn rats, it is unable to improve EW-induced suppression of hippocampal behaviors. These findings are the first report that EW-induced oxidative stress and E2 protection depend on a sex or a brain region.

Our observation of a smaller increase in oxidative markers in ethanol-withdrawn female than male rats agrees with a previously reported sex difference in oxidative stress, favoring females. The plasma level of TBARS and O₂^{•-} were increased in male mice but not female mice in response to hypertension-inducing agent [49]. Compared to ovary intact rats, ovariectomized rats showed an increase in the basal oxidation level of membrane lipid [18]. These studies are corroborating with our current finding showing that ethanol-withdrawn female rats display a smaller level of oxidative molecules in the cerebellum than ethanol-withdrawn male rats (Figure 1). A similar sex difference in oxidative stress was observed in dextrin-diet rats although the level of MDA did not reach a statistically significant sex

difference. We found a high variability in MDA data obtained from dextrin-diet male rats largely due to a couple of rats, which might have contributed to the statistical results. Nevertheless, it seems clear that female rats are resistant to oxidative stress, leading to the question of whether E2 mediates this sex difference. E2 is a potent antioxidant by directly scavenging ROS [16, 17]. E2 promotes the antioxidant defense system by increasing the expression and/or activity of antioxidant molecules such as superoxide dismutase [50]. It is thus reasonable to speculate that the smaller oxidative stress to female cerebellum than male cerebellum is attributed to an antioxidant effect of E2. E2 treatment indeed lowered oxidative markers in the cerebellum of ethanol-withdrawn female rats, accompanied by a coherent sex difference in the effect of EW on cerebellar behaviors (Rotarod). These results suggest that the antioxidant effect of E2 contributes to a better Rotarod performance in female rats than male rats upon EW insult. Should the antioxidant effect of E2 mediate the sex difference in cerebellar behaviors, antioxidant Vt.E+Co-Q10 treatment alone would show a similar effect to that of E2. As expected, Vt.E+Co-Q10 treatment improved Rotarod performance of female rats as did E2, an indicator of antioxidant protection against EW. Our analysis of Rotarod data by days of testing revealed that rats learned Rotarod task as they repeated the task for 5 days. There was a tendency of slower learning of ethanol-withdrawn rats than dextrin-diet rats, and male rats than female rats, but the difference did not reach a statistical significance. The suppressing effect of prooxidant EW on cerebellar behaviors may not necessarily suppress the learning ability of motoric tasks.

Mitochondrial membranes are the major source of ROS generation, and thus, damage to mitochondrial membranes increases ROS production. Given this, smaller oxidative stress to the cerebellum of ethanol-withdrawn female rats than male rats may be associated with a sex specific effect of EW on mitochondrial membranes. We tested this possibility by assessing mitochondrial membrane swelling, an index of the opening of the mitochondrial permeability transition pore. This pore regulates the passage of molecules across mitochondrial membranes for mitochondrial and cell survival [51]. Its prolonged opening leads to ROS overproduction in vivo [51, 52]. We observed that EW-induced mitochondrial membrane swelling was smaller in the cerebellum of female rats than male rats. These observations suggest that stronger mitochondrial membranes and the antioxidant property of E2 protect female cerebellum from EW insult. The resistance of female cerebellum to EW stress may also be associated with an innate sex difference in cerebellum, favoring females. Healthy adult women showed a higher rate of metabolism in the cerebellum than men, suggesting a higher basal neuronal activity in female cerebellum than male cerebellum [53]. Female rats under a normal condition also showed a higher basal level of the mRNA of nerve growth factor in the cerebellum than male rats [54]. If female cerebellum is naturally stronger than male cerebellum, it is not surprising that female cerebellum better copes with stressful insults such as EW than male cerebellum. Supportive of this view is a study using female mice with a neurodegenerative disorder (Angelman syndrome); they outperformed male counterparts on the Rotarod task [55]. This sex difference may not be due to a different structural volume of cerebellum between sexes. Brain magnetic resonance imaging has revealed that the volume of female cerebellum is 8% smaller than that of males [56]. There is no evidence that smaller cerebellum is responsible for a greater strength of cerebellum. Since Purkinje cells are the major cerebellar neurons, some studies on sex difference in

cerebellum have approached Purkinje cells. Male mice but not female mice with impaired aromatase (E2 synthesizing enzyme) lose Purkinje cells as they age in a manner that is attenuated by E2 treatment [58]. Purkinje cells are essential for the conditioning animals to blink eyes in response to air puff [57]. E2-treated ovariectomized female rats performed the eye-blink test better than vehicle-treated ovariectomized rats during EW (our unpublished observation). E2 treatment significantly improved Rotarod performance of ischemic rats [59], suggesting that E2 exerts a protective role in cerebellar neurons.

Our observation of the sex difference in EW-induced oxidative stress and cerebellar behavioral suppression led us to the question of whether hippocampus, a brain area for memory functions, shows a similar sex difference in EW. Like cerebellum, the hippocampus of ethanol-withdrawn female rats showed a smaller increase in the level of $O_2^{\bullet-}$, MDA, and protein carbonyls than male counterparts. E2 treatment decreased the oxidative markers, indicating that E2 exerts an antioxidant effect on hippocampus as does on cerebellum. However, opposite to Rotarod performance, EW impeded the water-maze performances of only female rats, a phenomenon that was not improved by E2 or an antioxidant treatment. These results suggest that the antioxidant effect of E2 is not sufficient to protect hippocampal behaviors from EW. The inability of E2 to improve hippocampal behavior has been previously shown in studies where E2 treatment had no impact on some hippocampal measurements. E2 treatment failed to protect against hippocampal apoptosis and DNA damage, and poor water-maze tasks in traumatized female rats [14, 15]. Directly relevant to the greater susceptibility of female hippocampus to EW than male hippocampus is a study done by Walls et al. [30]. They found that repeated EW elicited a greater toxicity in hippocampal neurons of female rats than male rats. In that study, the susceptibility of female hippocampus to EW was enhanced by stress hormone corticosterone treatment [30], suggesting that the stress hormone may be involved in the susceptibility of female hippocampus to EW.

In contrast to cerebellum, an innate sex difference in hippocampus appears to favor male hippocampus. Notice that dextrin-diet male rats significantly outperformed dextrin-diet female rats on water-maze performance (Figure 7). Compared to female hippocampus, male hippocampus shows a greater neuronal density [60] and generates more new cells than females in rats [61]. Hippocampal slices from male rats exhibited higher excitatory postsynaptic potential (a measure of a neuronal activity) than those from female rats, a sex difference that was decreased by E2 treatment [62]. Men showed a better performance in computerized version of the water-maze task than women [63]. The innate strength of male hippocampus may help maintain the proper level of basal oxidative markers. If this is the case, it explains our results showing that the basal level of oxidative markers in dextrin-diet male rats is not necessarily higher than female rats in hippocampus (Figure 5). Unfortunately, the ability to maintain a proper basal redox status seems insufficient for preventing prooxidant EW; the hippocampus of ethanol-withdrawn male rat shows a higher level of oxidative markers than female hippocampus. Some mechanisms other than or additional to oxidative mechanisms must mediate a sex specific response to EW. A potential explanation for a greater vulnerability of female hippocampus to EW is inferred from the relationship between E2 and glutamate. The upregulation of glutamatergic neurotransmissions has been recognized as a hallmark of EW stress in humans and animals

[2, 64–67]. The failure of E2 to protect against EW-induced impairment in water-maze tasks may be associated with the effect of E2 on glutamate in hippocampus. For example, chronic E2 treatment enhanced the stimulating effect of glutamate on hippocampal neurons [68]. This effect of E2 on glutamate may be exacerbated under the condition of EW with already upregulated glutamate levels. Subsequently, excitotoxic molecular activities are triggered in hippocampus, collectively overriding E2's antioxidant protection for hippocampal behaviors.

More severe mitochondrial membrane swelling in the hippocampus of ethanol-withdrawn female than male rats may be the indication that some sex difference exists in molecular activities associated with mitochondrial membranes. We approached CcO located in mitochondrial membranes and essential for mitochondrial respiration. In the mitochondria, electrons are transferred across electron transport chains, finally to the terminal enzyme complex CcO that helps generate ATP. The deficiency of this enzyme is one of the most common defects found in human mitochondrial diseases [69]. Our assessment of CcO activity revealed that EW decreased CcO activity more severely in female hippocampus than male hippocampus, and E2 treatment only marginally improved the activity of CcO in hippocampus. This was different from cerebellum in that EW decreased CcO activity less severely in female cerebellum than male cerebellum, and E2 treatment substantially increased the CcO activity of cerebellum. A sex difference in the effect of EW on CcO hence appears to depend upon a diet (dextrin or EW) and brain regions. A higher basal activity of cerebellar CcO in male rats than female rats is in fact consistent with our previous study where male mice showed a higher basal expression of cerebellar CcO than female mice [70]. This may be a compensatory mechanism by which high CcO activity increases mitochondrial respiration to supply sufficient ATP for cerebellar functions in males [70]. The severe inhibition of CcO risks cell survival as it can suppress mitochondrial respiration. As we have previously reported, EW suppressed mitochondrial respiration, and further so in the presence of CcO inhibitor NaN_3 (Figure 9B). NaN_3 suppresses mitochondrial respiration more severely in ethanol-withdrawn hippocampal cells (HT22 cells) than control cells (Figure 9B). This observation leads to the speculation that CcO inhibition is particularly deleterious to hippocampal neurons in the hyperexcitatory milieu like EW. We tested this idea by treating HT22 cells with NaN_3 and glutamate. This approach is to mimic the property of EW that suppresses CcO and upregulates glutamatergic neurotransmission. NaN_3 treatment resulted in cell death of which magnitude was 4-fold greater ($34.8 \pm 3.4\%$ cell death) in the presence of glutamate compared to non-glutamate (normal) condition ($8.5 \pm 2.1\%$ cell death) (Figure 10). Further, E2 was unable to protect hippocampal cells from EW in the presence of NaN_3 . The profound inhibition of CcO by EW in the hippocampus of female rats may hence blunt E2's protection to the extent unable to improve hippocampal behavior. Relevant to the vulnerability of hippocampal CcO to a hyperexcitatory milieu is a study done by Llewellyn et al. [55]. In that study, compared to cerebellar CcO, hippocampal CcO was more suppressed by a genetic (UBE3A gene) defect that creates the hyperexcitatory syndromes such as recurrent seizures. The article did not specify a sex of animals they used. If they used female mice, their study is consistent with our findings that hippocampal CcO in female rats is more suppressed by EW than cerebellar CcO in female rats. At the very least, our results suggest that EW suppresses CcO in female hippocampus to a degree that diminishes E2's antioxidant protection for hippocampal behavior.

In conclusion, our studies highlight that EW provokes a sex specific oxidative stress, and E2's antioxidant protection depends on a target brain area under the condition of hyperexcitatory EW. This sex difference is unlikely attributed to blood ethanol concentrations that showed no significant difference between sexes. These observations may warrant further studies to substantiate a sex and region specific oxidative brain damage in ethanol-withdrawn subjects for a better management of EW in men and women.

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Abbreviations

BEC	Blood ethanol concentrations
E2	17 β -estradiol
EW	Ethanol withdrawal
Vt.E+Co-Q10	Vitamin E + Co-enzyme Q10
CcO	Cytochrome c oxidase
MDA	Malondialdehyde
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances

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Highlights

- Ethanol withdrawal increases oxidative stress less severely in the cerebellum and hippocampus of female rats than male rats.
- Ethanol-withdrawn female rats show better and worse cerebellar- and hippocampus-related behaviors than male rats, respectively.
- Antioxidant 17 β -estradiol protects cerebellar behaviors but not hippocampal behaviors from ethanol withdrawal in female rats.

Figure 1. Sex difference in the effects of EW on oxidative stress in the cerebellum of rats
 Gonad intact female and male rats received an ethanol diet (0 or 7.5% v/v) for four weeks followed by EW for three weeks per cycle for two cycles. Some female rats were ovariectomized with a vehicle (corn oil) or an E2 pellet replacement. At the end of the diet program, cerebelli were collected to measure $O_2^{\bullet-}$, MDA, and protein carbonyls. EW increased the amount of $O_2^{\bullet-}$, MDA, and protein carbonyls less severely in female rats than male rats, and in E2-treated ovariectomized rats than vehicle-treated ovariectomized rats. Compared to dextrin-diet male rats, a moderately smaller amount of $O_2^{\bullet-}$ ($^{\dagger}p = 0.017$) and protein carbonyls ($^{\dagger}p < 0.001$) were found in dextrin-diet female rats. Depicted are mean $\pm$ SEM for 6–10 rats/group. * $p < 0.001$ vs. respective dextrin-diet rats. $^{\dagger\dagger}p < 0.002$ vs. ethanol-withdrawn female rats. $^{\ddagger}p < 0.001$ vs. EW rats.

Figure 2. Sex difference in the effects of EW on mitochondrial membrane swelling in cerebellum Female and male rats received an ethanol diet (0 or 7.5% v/v) for four weeks followed by EW for three weeks per cycle for two cycles. Upon euthanasia, cerebelli were collected to measure mitochondrial membrane swelling by recording an absorbance decline at 540 nm. The magnitude of EW-induced mitochondrial membrane swelling relative to a dextrin condition is illustrated as a gray area. A smaller gray area indicates smaller mitochondrial membrane swelling induced by EW. Female cerebellum showed less severe mitochondrial membrane swelling induced by EW than male cerebellum. Depicted are mean $\pm$ SEM for 7 rats/group.

Figure 3. Effects of E2, antioxidants, or cyclosporin A on mitochondrial membrane swelling in the cerebellum of ethanol-withdrawn rats

Ovariectomized rats received an ethanol diet (0 or 7.5% v/v) for four weeks followed by EW for three weeks per cycle for two cycles with or without E2 or Vt.E+Co-Q10 treatment.

Cyclosporin A was added to sample solution at the time of mitochondrial membrane swelling assay. The magnitude of the protective effect of E2, Vt.E+Co-Q10, and cyclosporin A on EW-induced mitochondrial membrane swelling is illustrated as a gray area. All of E2, Vt.E+Co-Q10, and cyclosporin A ameliorated EW-induced mitochondrial membrane swelling. Depicted are mean $\pm$ SEM for 7 rats/group.

Figure 4. Sex difference in the effects of EW on Rotarod performance in rats

Gonad intact female and male rats received an ethanol diet (0 or 7.5% v/v) for four weeks followed by EW for three weeks per cycle for two cycles. Some female rats were ovariectomized with E2 or Vt.E+Co-Q10 treatment. At the end of the diet program, rats were tested for the Rotarod task. Figure 4A and 4B show an average latency from the 5-days data. Ethanol-withdrawn rats showed a shorter latency to fall from Rotarod than dextrin rats, and this effect of EW was less severe in female than male rats. For dextrin-diet rats, the Rotarod performance of female rats was better than male rats ($^{\dagger}p < 0.0079$). Ethanol-withdrawn ovariectomized rats treated with E2 or Vt.E+Co-Q10 showed a longer latency than ovariectomized rats treated with vehicle. Figure 4C and 4D show an average latency from each day data for 5 days. Latency to fall from Rotarod increased as rats repeated the task for 5 days. However, the magnitude of the motoric learning did not significantly differ between treatment or sex groups. $^*p = 0.02$ or $^{**}p < 0.01$ vs. respective dextrin. $^{\dagger\dagger}p = 0.0004$ vs. ethanol-withdrawn male rats. $^{\dagger}p < 0.01$ vs. ovariectomized ethanol-withdrawn rats. Depicted are mean $\pm$ SEM for 6–10 rats/group.

Figure 5. Sex difference in the effects of EW on oxidative stress in the hippocampus

Gonad intact female and male rats received an ethanol diet (0 or 7.5% v/v) for four weeks followed by EW for three weeks per cycle for two cycles. Some female rats were ovariectomized with a vehicle (corn oil) or an E2 pellet replacement. At the end of the diet program, a higher amount of $O_2^{\bullet-}$, MDA, and protein carbonyls were found in ethanol-withdrawn rats vs. the respective dextrin groups, female rats vs. male rats, and E2-treated, ethanol-withdrawn ovariectomized rats vs. vehicle-treated rats. Dextrin-diet rats did not show a significant sex difference in oxidative markers. Depicted are mean $\pm$ SEM for 6–10 rats/group. * $p < 0.0001$ vs. respective dextrin rats. † $p < 0.001$ vs. ethanol-withdrawn male rats. ‡ $p < 0.01$ vs. ovariectomized ethanol-withdrawn rats.

Figure 6. Sex difference in the effects of EW on mitochondrial membrane swelling in hippocampus

Gonad intact female and male rats received an ethanol diet (0 or 7.5% v/v) for four weeks followed by EW for three weeks per cycle for two cycles. Some female rats were ovariectomized with a vehicle, E2 pellet, or Vt.E+Co-Q10 treatment. At the end of the diet program, hippocampi were collected to measure mitochondrial membrane swelling by recording an absorbance decline at 540 nm. The magnitude of EW-induced mitochondrial membrane swelling relative to a dextrin-diet is illustrated as a gray area. Female hippocampus showed more severe mitochondrial membrane swelling induced by EW than male hippocampus. Neither E2 nor Vt.E+Co-Q10 treatment significantly altered mitochondrial membrane swelling. Depicted are mean $\pm$ SEM for 7 rats/group.

Figure 7. Sex difference in the effects of EW on water-maze performance in rats

Gonad intact female and male rats received an ethanol diet (0 or 7.5% v/v) for four weeks followed by EW for three weeks per cycle for two cycles. Some female rats were ovariectomized with a vehicle (corn oil), E2 pellet, or Vt.E+Co-Q10 treatment. At the end of the diet program, rats were trained to find a visible platform for 5 days (data not shown). From the next day, rats were subjected to the acquisition test for 4 days and the retention memory test for two days by recording the latency to find a hidden platform. Compared to dextrin-diet rats, ethanol-withdrawn female rats but not male rats showed a longer time (poor performance) to find a platform for both acquisition and retention tests (7A). Among dextrin-diet rats, female rats took a longer time to find a platform during the acquisition ($p = 0.02$) and the retention phase ($p < 0.001$) than male rats. After ovariectomy, the time to find a plate form did not differ among the groups of dextrin, EW, EW+E2, and EW+Vt.E+Co-Q10 rats. * $p = 0.02$ or † $p < 0.01$ vs. dextrin-diet female rats. †† $p < 0.01$ vs. ethanol-withdrawn male rats. Depicted are mean $\pm$ SEM for 6–10 rats/group.

Figure 8. A sex difference in the effect of EW on CcO activity

Gonad intact female and male rats received an ethanol diet (0 or 7.5% v/v) for four weeks followed by EW for three weeks per cycle for two cycles. Some female rats were ovariectomized with a vehicle or E2 pellet treatment. Upon euthanasia, cerebellum and hippocampus were collected to measure the activity of CcO using a spectrophotometry method. EW suppressed CcO activity less severely in female cerebellum than male cerebellum, but more severely in female hippocampus than male hippocampus. Unlike EW, the cerebellum of dextrin-diet female rats showed a lower ($p < 0.0001$) activity of CcO than that of dextrin-diet male rats. CcO activity in the hippocampus did not significantly differ between dextrin-diet female and male rats. E2 treatment to ovariectomized rats increased CcO activity of ethanol-withdrawn rats, and this effect of E2 was smaller in hippocampus than cerebellum. Depicted are mean $\pm$ SEM for 7 rats/group. * $p < 0.0001$ vs. respective dextrin rats. ^{††} $p < 0.01$ vs. ethanol-withdrawn male rats. [†] $p < 0.0005$ vs. dextrin-diet male rats. [‡] $p < 0.0001$ vs. EW rats. ^{‡‡} $p = 0.0255$ vs. cerebellar EW+E2 rats. Depicted are mean $\pm$ SEM for 6–10 rats/group.

Figure 9. Effects of EW on Ψ_m and mitochondrial respiration in hippocampal cells

HT22 cells were exposed to control media or 100 mM ethanol for 20 hours and were withdrawn for 4 hours per cycle for two cycles. Cells were treated with JC-1 dye, and then observed with a fluorescence microscope. Control cells show largely red fluorescence (high Ψ_m), but ethanol-withdrawn cells show more green (low Ψ_m) and less red fluorescence. Yellow colors are from the merge of red and green fluorescence. Blue colors are from DAPI (4',6-diamidino-2-phenylindole) that stains nuclei. Magnification was at $100\times$ (Figure 9A). For mitochondrial respiration (Figure 9B), HT22 cells were plated in a 24-well microplate and subjected to the aforementioned EW paradigm with or without E2 treatment. Mitochondrial respiration was measured using the XF respirometer. Immediately after basal O_2 consumption rate was read, NaN_3 (CcO inhibitor, $1\ \mu M$) was added to cells. Compared to control cells, ethanol-withdrawn cells showed lower mitochondrial respiration. This effect of EW was smaller after E2 treatment. When NaN_3 was injected to EW+E2 cells, the mitochondrial respiration did not differ from that of vehicle-treated ethanol-withdrawn cells. * $p < 0.002$ or ** $p < 0.001$ vs. control cells. † $p < 0.05$ vs. NaN_3 treatment. Depicted are mean $\pm$ SEM for 6 wells/group

Figure 10. The effect of a CcO inhibitor on cell viability

HT22 cells were exposed to glutamate (3 mM) for 24 hours with or without NaN₃ (1 μM) treatment. Separately, HT22 cells were subjected to ethanol exposure (0 or 100 mM) for 20 hours and withdrawal for four hours per cycle for two cycles. Cells were treated with E2 and/or NaN₃ during each of EW phases. Cell viability was assessed using Calcein-AM assay. Compared to control cells at 100%, NaN₃ lowered cell viability (**p* < 0.02) and further so in the presence of glutamate (****p* < 0.0001). E2 treatment failed to increase the viability of ethanol-withdrawn cells in the presence of NaN₃. **p* < 0.02, ***p* < 0.001, ****p* < 0.0001 vs. control cells. †*p* < 0.005 vs. glutamate. ‡*p* < 0.005 vs. EW+E2. *N* = 6 wells/each condition. Depicted are mean ± SEM for 6 wells/group.

Table 1
Sex specific effects of EW on oxidative, mitochondrial, and behavioral markers

Our current findings on a sex specific effect of EW on oxidative, mitochondrial, and behavioral markers are listed in Table 1.

Brain area	Cerebellum				Hippocampus					
Sex	Female rats			Male rats		Female rats			Male rats	
Diet/Treatment	Dextrin	EW	E2/antioxidant	Dextrin	EW	Dextrin	EW	E2/antioxidant	Dextrin	EW
O2-	*	**	Protective	**	***	*	**	Protective	*	***
CO	*	***		**	*****	*	**		*	***
MDA	*	**		*	***	*	**		*	***
Mitochondrial swelling	*	**		**	***	*	***		No effect	*
	*	**	**	***	**	***	*	*		
Behaviors	*	*	Protective	*	***	*	***	Less effect	*	***
CcO	***	***		*	*****	*	***		*	***
Conclusion	Female cerebellum and hippocampus are less vulnerable to EW-induced oxidative stress in part due to antioxidant effects of E2.				Antioxidant effects of E2 are not sufficient to protect hippocampal mitochondria and behaviors from EW in part due to CcO vulnerability.					
	Antioxidant effects of E2 protect cerebellar mitochondria and behaviors from EW.				Antioxidant effects of E2 are not sufficient to protect hippocampal mitochondria and behaviors from EW in part due to CcO vulnerability.					

A greater number of arbitrary symbol “*” was used to indicate a greater adverse effect of EW on each parameter. A bigger size of “*” was used to indicate the worst effect of EW.