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Ethanol withdrawal acts as an age-specific stressor to activate cerebellar P38 kinase

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Abstract

We investigated whether protein kinase P38 plays a role in the brain-aging changes associated with repeated ethanol withdrawal (EW). Ovariectomized young, middle-age and older rats, with or without 17 β -estradiol (E2) implantation, received a 90-day ethanol with repeated withdrawal. They were tested for active pP38 expression in cerebellar Purkinje neurons and whole-cerebellar lysates using immunohistochemistry and enzyme-linked immunosorbent assay, respectively. They were also tested for the Rotarod task to determine the behavioral manifestation of cerebellar neuronal stress and for reactive oxygen species (ROS) and mitochondrial protein carbonyls to determine oxidative mechanisms. Middle-age EW rats showed higher levels of pP38-positive Purkinje neurons/cerebellar lysates, which coincided with increased mitochondrial protein oxidation than other diet/age groups. Exacerbated motor deficit due to age-EW combination also began at the middle age. In comparison, ROS contents peaked in older EW rats. E2 treatment mitigated each of the EW effects to a different extent. Collectively, pP38 may mediate the brain-aging changes associated with pro-oxidant EW at vulnerable ages and in vulnerable neurons in a manner protected by estrogen.

Keywords

age; brain-aging-changes; cerebellum; 17 β -estradiol; ethanol withdrawal; female rats; mitochondrial protein carbonyls; phosphor-P38 (pP38); Purkinje neurons; reactive oxygen species

1. Introduction

P38 belongs to the family of mitogen-activated protein kinases that mediate signaling cascades and regulate cell fate in response to cellular stress (Arimoto et al., 2008). A

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Disclosure Statement

None of authors in this manuscript has any type of conflicts of interest including financial and personal matters.

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transient, moderate activation of P38 is associated with cell survival or differentiation, whereas the sustained or excess activation generally correlates with pathological conditions (Barca et al., 2008; Du et al., 2008; Giordano et al., 2008). P38 is activated upon phosphorylation (Moriguchi et al., 1996) and thus, pP38 is often measured as an indicator of P38 activation. P38 is also known as a stress-activated protein kinase because P38 is phosphorylated by stress signals such as inflammatory cytokines, heat shock or ischemia (Aydin et al., 2005). The known members of the P38 family include P38 α (Lee et al., 1994), P38 β (Jiang et al., 1996; Stein et al., 1997), P38 γ (Lechner et al., 1996; Li et al., 1996) and P38 δ (Jiang et al., 1997). Among these isozymes, P38 α and P38 β are highly expressed in brain areas that are vulnerable to ethanol/EW, such as cerebellum and cortex (Lee et al., 2000; Nonaka, et al., 2008; Xiong, et al., 2006).

The signaling role of P38 in the effects of ethanol has been demonstrated in a study in which a P38 inhibitor SB203580 attenuated ethanol-induced cell death in HT22 cells (Ku et al., 2007). Acute ethanol treatment led to P38 activation (Norkina et al., 2007) and augmented endotoxin-induced pP38 levels in a manner attenuated by a P38 inhibitor in human monocytes (Drechsler et al., 2006). P38 is also implicated in age-associated physiological or pathological conditions. For instance, while old men had a higher level of basal pP38 in skeletal muscles than young men, the opposite trend in age-associated pP38 levels occurred after strenuous exercise (Williamson et al., 2003). Pathological activation of P38 was shown in the brains of Alzheimer's disease patients (Hensley et al., 1999) and in the livers of aged rats after challenged with H₂O₂ (Vereker et al., 2000). These studies suggest that hyperactivation of P38 may reflect an aging process. Because aging is associated with estrogen depletion in females, we examined the protective role of estrogen in the homeostatic status of P38 at different ages. Previously, E2 attenuated the phosphorylation of P38 induced by angiotensin II in cells (Wu et al., 2009), cardiac hypertrophy in ovariectomized mice (van Eickels et al., 2001) and myocardial inflammation in ovariectomized rats (Wang et al., 2006). These studies suggest that E2 may attenuate P38 activation, especially when neurons are challenged by the stress of aging or EW.

In the current study, we used cerebellum, one of the most vulnerable brain areas to ethanol/EW insults (Ramadoss et al., 2007; Santucci et al., 2008). Although cerebellum contains multiple types of neurons, Purkinje neurons constitute the single output of all motor coordination in the cerebellar cortex. Therefore, damage to these neurons inevitably provokes impairment of motor behavior and coordination. In fact, a substantial loss of these neurons coincided with motor deficit in ethanol-withdrawn rats (Jung et al., 2002; Rewal et al., 2003). Using this brain area, we investigated whether EW provokes the age-specific activation of P38. First, we examined the effects of ethanol vs. EW on P38 activation by measuring protein levels of pP38 (I). Second, we assessed the effects of age-EW interaction on P38 activation by assessing pP38 in Purkinje neurons and in whole cerebellar lysates. The behavioral manifestation of cerebellar neuronal stress was assessed by a Rotarod test in which a shorter latency to fall from an accelerating rod indicated poorer cerebellar-related motor performance (II). Finally, the potential oxidative mechanisms underlying age-dependent activation of P38 and estrogen protection were investigated by measuring reactive oxygen species (ROS) and mitochondrial protein oxidation (III). In the initial part of this study (I), we used young male rats before superimposing age and the female hormone

estrogen. In the subsequent part of this study (II and III), we used female rats at the ages of 5–8 months (young), 12–15 months (middle-age) and 16–19 months (older) in a model of ovariectomy. Using this model, we provide empirical evidence that repeated EW provokes the hyperactivation of P38 in a manner that targets vulnerable ages and neurons and is protected by estrogen.

2. Materials and Methods

2.1. Subjects and Experimental groups

All rats were housed individually in a room with controlled temperature (22–25°C), humidity (55%) and lights (7 AM –7 PM). Body weights were recorded twice a week. All housing and procedures were in accordance with the guidelines of the Institutional Care and Use Committee of the National Research Council (NIH publication no. 85–23, revised 1996) and were approved by the University of North Texas Health Science Center Animal Care and Use Committee. All ethanol-withdrawn animals were sacrificed 14 days after the last ethanol dose (14th day of EW). Ethanol-consuming rats (named Ethanol-exposure group) continuously received an ethanol diet (6.5%) and were gradually withdrawn from an ethanol diet (5% for 3 days, 3% for 2 days and 2% for 2 days) to avoid EW stress. They were then sacrificed on the next morning. The ethanol diet (2%) bottle was available until they were sacrificed.

For the initial test of pP38, we used male rats (5 months old, Sprague-Dawley) and administered a short-term ethanol diet (6.5%, 5 weeks) to them. The usage of male rats and a short-term diet was to characterize the expression of pP38 in the absence of the influence of estrogen, age or aging associated with a lengthy diet. They were divided into the control dextrin, the ethanol-exposure or the EW group. For aging and estrogen studies, we used female rats (Fisher 344). They were approximately 8, 15 or 19 months old when tests began after a 90-day diet program. All female rats were ovariectomized, implanted with an E2 or a control oil pellet and allowed to recover for 2 weeks after the surgery. They were then divided into 5 groups, and we began a 25-day diet and a 5-day abrupt withdrawal cycle which was repeated 3 times. The 5 groups were 1) dextrin + oil, 2) dextrin + E2, 3) ethanol-exposure + oil, 4) EW + oil and 5) EW + E2 groups. Because we were interested in EW insults rather than ethanol insults, we did not include an ethanol-exposure + E2 group. Separately, ovariectomized rats implanted with oil pellets were subjected to an identical diet regimen and assigned exclusively for the analysis of blood ethanol concentrations (BEC). The dextrin diet was used as a sole control diet because we have repeatedly observed no significant difference between a dextrin diet vs. a regular chow diet or intermittent vs. continuous dextrin diets in vital signs or in experimental outcomes.

2.2. Ovariectomy and E2 implantation

Under isoflurane (2% v/v) anesthesia, 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. Immediately thereafter, Silastic pellets (s.c.) containing E2 or oil were implanted on the dorsal part of the rat, and incisions were closed with clips. The E2 pellet releases physiological concentrations of E2 (29 to 34 pg/ml) for 3 weeks and thus

was replaced every 3 weeks. According to this schedule, animals had two episodes of E2 implantation that took place on the 4th day of EW. At this point of EW, animals are typically recovered from the hyperexcitatory signs that are most severe during the first 24 hours of EW. Thus, the procedures of ovariectomy and E2 implantation unlikely influenced the hyperexcitatory stress of EW. Furthermore, rats implanted with a control pellet did not differ from non-surgery rats in vital signs, physical activities, or appearance. The method of preparing the hormone pellets was previously reported (Yang et al., 2001).

2.3. Chronic ethanol administration in a liquid diet and withdrawal

The amount of dextrin and ethanol was calculated in combination to adjust the concentration of ethanol to 6.5% v/v (Jung et al., 2002). Control animals were fed a liquid diet with dextrin isocalorically substituted for ethanol. The nutritionally balanced fresh diet (100 ml/rat) was prepared daily, poured into an L-shaped Plexiglass (Bio-Serv Frenchtown, NJ) and placed in each home cage every morning. On the last day of each diet cycle, a small aliquot (50 ml) of ethanol or dextrin diet was administered (7 AM) to ensure that all of the EW rats finished the same amount of ethanol by the time of the ethanol diet removal. Twelve hours later (7 PM), the diet tubes were removed, and regular chow pellets were placed in the home cage.

2.4. Collection of brain tissues

Rats were humanely sacrificed under anesthesia [xylazine (20 mg/kg, i.p.) and ketamine (100 mg/kg, i.p.)]. Cerebelli containing the vermis area were used for biochemical assays. For immunohistochemical assays, a separate set of rats was perfused intracardially with saline followed by 4% paraformaldehyde in a phosphate buffer. Brains were divided into halves by a midsagittal cut. A parasagittal cut was then made 3 mm to the left of the cerebellar midline, and this sample was used for the immunohistochemistry.

2.5. Blood ethanol concentrations (BEC)

On the last day of each diet cycle, whole blood (100 μ l) was collected from the tail vein 3 hours after placing fresh diet bottles. In brief, the rats were restrained in a Plexiglas rodent restraint device, and a syringe fitted with a 25 gauge needle was inserted toward the tail vein. Blood was collected and immediately mixed with 90 μ l of ice-cold, 0.55 M HClO₄. Samples were centrifuged at 1500 x g for 10 min 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 a colorimetric assay (Smolen et al., 1986) using a Beckman DU 640 spectrophotometer.

2.6. Accelerating Rotarod

This motor driven treadmill (Omnitech Electronics, Columbus, OH) measures running coordination and motor performance such that a shorter latency to fall from an accelerating rod indicates poorer motor performance. The rotor consists of 4 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 sec 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 min resting period between sessions (Rewal et al., 2004). The tests were not initiated until 7 days after the last ethanol diet to avoid the possibility that acutely occurring the hyperexcitatory signs of EW would obscure cerebellar-related motor deficit.

2.7. Immunoblotting

The hemi-cerebellum was weighed and added to lysis buffer containing ice-cold PBS, a protease inhibitor cocktail (EMD bioscience, CA), PMSF, and a phosphatase inhibitor cocktail. An aliquot was combined with an equal volume of lysis buffer [0.5M Tris-HCl (pH 6.8), 4.4% w/v SDS, 20% v/v glycerol, 2% v/v 2-mercaptoethanol]. After the samples were heated (95°C, 5 min) and sonicated (10 sec), Bradford assay was conducted to analyze protein concentrations according to manufacture's instructions (Biorad, Hercules, CA). A 30 µg of sample protein was electrophoresed on a 10% SDS-PAGE and then transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% fat free milk. The blot was washed in PBS containing 0.05% Tween-20 and probed overnight with a rabbit polyclonal antibody against P38 or a rabbit monoclonal antibody against pP38 (Thr180/Tyr182) (Cell Signaling, Danvers, MA). The blot was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for one hour at room temperature. Bands were detected using the UVP western blotting luminescence system and quantified by an image densitometer. Immunoblottings for β -actin were carried out as a positive and a loading control.

2.8. pP38 (Thr180/Tyr182) ELISA (enzyme-linked immunosorbent assay)

pP38 (Thr180/Tyr182) ELISA (Cell Signaling, Danvers, MA) was used to quantitate P38 activation in cerebellar extracts by measuring the protein level of an active form of P38 (pP38). Briefly, the coating antibody was diluted (1:100) in PBS, added to a microplate, and incubated overnight at 4°C. The plate contents were then discarded. The plate was washed with PBS (x3) and then blocked with blocking buffer for 2 hours. Cerebellar tissue lysates were then added to the well and incubated again for 2 hours. A detecting antibody (1:100) and a secondary antibody (1:1000) were sequentially added and incubated. Finally the plate was incubated at 37°C for 30 min and mixed with TMB substrates. STOP solution (100 µl) was added to each well to terminate the reaction. The plate was read using a microplate reader at an absorbance 450 nm. HeLa cells treated with vehicle or anisomycin were used to validate the ELISA analysis as positive controls. Lysis buffer was used as a blank condition.

2.9. Immunohistochemical analysis of pP38 or pSTAT1

In addition to pP38, STAT1 (Signal Transducer and Activator of Transcription), a direct substrate of pP38 was analyzed to confirm that pP38 was indeed functionally active. The fixed tissues were rinsed in 70% ethanol for overnight, dehydrated using different ethanol concentrations, xylene, and mixture of xylene/paraffin, and embedded in paraffin. The hemispheres were then cut into 8 µm-thick sagittal slices on a microtome. For consistency from animal to animal, multi-tissue blocks from the same groups were embedded together. The sections started approximately 1.5 mm parasagittal from the midline of the cerebellar vermis and ended after 250 sections at 2.5 mm. For each rat, the 9th section from the midline was used for the immunohistochemistry analysis (Jung et al., 2002). The slides were

deparaffinized in xylene, rehydrated through decreasing ethanol concentrations, and washed with PBS. The slices were then moisturized at 95°C for antigen retrieval. A primary antibody against pP38 (Thr180/Tyr182) or pSTAT1 (Ser727) (Cell signaling, Danvers, MA) was diluted (1:50) in blocking buffer (10% normal goat serum). The slices were rinsed in PBS and then incubated with broad spectrum poly HRP conjugate for 40 min. The antigen-antibody bindings were visualized with a diaminobenzidine (DAB) color reaction and examined with an inverted Carl Zeiss microscope and a digital camera. All photographs were taken of the cerebellar cortex containing Purkinje layers that showed a clear image across all treatment groups. Slides stained with non-immunoreactive serum used for a negative control had negligible DAB staining.

2.10. Semi-quantitative analysis of pP38- or pSTAT1-positive Purkinje neurons

Brain slice samples were evaluated using the Carl Zeiss microscope, the image analysis program AxioVision 4 (Carl Zeiss, Thornwood, NY) and a previous method (Mausset et al., 2001) that was modified for our purpose. Three rats per treatment group were evaluated. Six microscopic fields per rat were selected such that two microscopic fields were randomly selected from each of anterior (lobes I to V), medius (VI to VII), and posterior (VIII to X) regions of the cerebellar cortex. We believe that this method of selecting the region of cerebellum was appropriate based on our previous demonstration that EW provoked a similar degree of Purkinje cell loss across anterior, medius, or posterior regions and that EW did not alter the length of Purkinje layer (Jung et al., 2002). All Purkinje cells with visible pP38- or pSTAT1-positive deposits were individually counted per microscopic field, and the length of the Purkinje layer per field was measured using the software to normalize the cell counts. Data were presented as the average number of pP38 or pSTAT1 positive Purkinje neurons/Purkinje layer (mm) from the 18 data points (3 rats/group, 6 fields/rat).

2.11. Total ROS

To determine the content of total ROS in cerebellar whole cell lysates, the fluorescence probe 2,7-dichlorofluorescein diacetate (DCFH) (Molecular Probes, Eugene, OR) was dissolved in absolute ethanol and added to sample proteins at 25 μ M DCFH followed by incubation at 37°C. The change in fluorescent intensity was measured using a fluorometer (Bio-TEK Instruments, Winooski, VT) at an excitation/emission wavelength of 485/535 nm. Data were presented as relative fluorescent intensity (Jakubowski and Bartosz, 2000).

2.12. Immunochemical detection of protein carbonyl

Cerebellar tissues were homogenized in 50 mM HEPES buffer (pH 7.2) containing 10 mM KCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and a proteinase inhibitor cocktail. To 1.0 ml of homogenate, 10 mM DNPH (dinitrophenylhydrazine, 0.2 ml) in 2 N HCl was added, and 2 N HCl (0.2 ml) was added to another 1 ml homogenate aliquot that was used as a blank control (Yan et al., 1997). The mixture was incubated and proteins were then precipitated with an equal volume of 20% trichloroacetic acid. Following washes (x3) with ethanol/ethyl acetate (1:1 v/v), the final precipitate was dissolved in 6 M guanidine HCl (2 ml, pH 2.3) and insoluble debris was removed by centrifugation. These samples were then analyzed by an immunoblot method as described previously (Yan et al., 1997).

2.13. Calcein-AM (calcein-acetoxymethylester) cell viability assay

HT22 cells (mouse hippocampal cell line) were treated with a vehicle medium (DMSO) or ethanol (50 mM or 100 mM) for 24 hours. Four hours later, the ethanol containing media was replaced with DMSO and then cells were collected (Jung et al., 2009). The P38 inhibitor, SB203580 (0–200 nM), was treated either during the 24-hour ethanol-exposure or during the 4-hour EW to test the effects of P38 activation induced by ethanol *per se* or EW on EW-induced cell death. Following the removal of the medium from the 96-well plates, the cells were rinsed with PBS and incubated with fluorogenic Calcein-AM (2.5 μ M, Molecular Probes, Eugene, OR) in PBS. Twenty min later, fluorescence intensity was determined using a Bio-Tek FL600 microplate reader (Winooski, VT) with an excitation/emission filter set at 485/530 nm. Cell culture wells treated with methanol served as a blank condition.

2.14. Statistical analysis

Data such as BEC (N=3 rats), Purkinje neuron count (N=18, 3 rats/group, 6 microscopic fields/rat), ELISA (N=4 or 5 rats) and total ROS (N=5–7 rats or 3 cell plates) were analyzed by two-way ANOVA (age x diet or SB203580 x dose of ethanol); Calcein data (N=4 wells) were analyzed by three-way ANOVA (SB203580 x window of treatment x dose of ethanol); and Rotarod data (N=4–7 rats) were analyzed by Multivariate-Repeated-Measure ANOVA. A post hoc Tukey's test was then conducted. Values were expressed as mean $\pm$ standard error of mean (SEM). The p value < 0.05 was used to indicate statistical significance. For qualitative data, pictures of a clear image were selected from 3 rats. Immunohistochemical images, neuronal counts and behavioral data were from the first set of rats. All other data from female rats were from the second set of rats except for the mitochondrial carbonyl data, which came from the third set of animals.

3. Results

I. Effects of Ethanol vs. EW on pP38

3.1. EW-induced P38 activation—We tested whether EW provokes P38 activation by measuring the active form of P38, pP38 (Figure 1). Compared to a dextrin diet, both ethanol-exposure ($p < 0.01$) and EW ($p < 0.001$) induced an increase in the levels of pP38 in young adult male rats [$F(2,6) = 13$, $p = 0.007$]. However, the increase was more profound during EW than during ethanol-exposure ($p < 0.01$). Neither ethanol nor EW significantly altered the protein levels of total P38. These data suggest that excessive activation of P38 occurs in ethanol-withdrawn cerebelli and that EW-induced P38 activation is not a residual effect of ethanol.

3.2. Cytotoxic P38 activation—We determined the cellular consequence of P38 activation (Figure 2). Three-way ANOVA indicated a significant interaction between the SB203580 treatment ($p < 0.05$), the dose of ethanol ($p < 0.01$) and the window of SB203580 treatment ($p < 0.01$) [$F(6,72) = 3$, $p = 0.023$]. EW suppressed cell viability in an ethanol-dose-dependent manner. The cell viability was protected when cells were treated with SB203580 during EW but not during ethanol-exposure. The protection by SB203580 appeared to be through a threshold effect rather than a dose-dependent effect because only the highest dose

(200 nM) of SB203580 was effective among the three doses. These data suggest that EW-induced rather than ethanol-induced P38 activation mediates the cell death associated with EW.

II. Effects of age on P38 activation

3.3. Ethanol consumption and BEC—Young, middle-age and older female rats consumed more ($p < 0.05$) of the ethanol diet in the last cycle ($\approx$ daily 16.5–17.3 g of ethanol/kg body weight) than in the first two cycles (daily 11.2–15 g/kg) of the diet program. The average diet consumption/day during a 90-day diet indicates that older rats drank slightly less ($p < 0.05$) than young or middle-age rats. Young (213 ± 5 g body weight), middle-age (241 ± 6 g), and older rats (249 ± 6 g) drank 15.6 ± 0.3 g, 15.2 ± 0.5 g, and 14.2 ± 0.4 g of ethanol/kg, respectively. Animals drank approximately 7 g/kg of ethanol by the time of blood collection. Average BECs from 3 diet cycles revealed that older rats had higher BEC (1.2 ± 0.03 mg/ml) than young (0.97 ± 0.02 mg/ml, $p = 0.017$) or middle-age (1 ± 0.04 mg/ml, $p = 0.03$) rats. When all quantitative data in the current study were normalized based on the BEC difference, it did not alter the statistical significance of the original data. There were no significant effects of E2 on ethanol consumption or BEC.

3.4. Age-dependent motor deficit in ethanol-withdrawn rats—Behavioral manifestation of cerebellar neuronal stress was assessed using a Rotarod test in 4 diet groups (dextrin, dextrin + E2, EW, and EW + E2) at the ages of 8 (young), 15 (middle-age) or 19 (older) months. Ethanol-exposure groups were not tested because they were intoxicated due to gradual EW. EW (+oil pellet) rats had the shortest latencies (poorest) among the diet groups on most of the 5-day-test days, especially in the middle-age and older groups [$F(3,56) = 3$, $p = 0.038$ by diet; $F(2,56) = 5$, $p = 0.011$ by age]. Only young, dextrin rats performed better as they repeated the task for 5 days (day-effect, $p < 0.01$). Because there was no significant effect of repeating tasks, except for the young, dextrin rats, we computed an average latency across the 5 days [$F(3,57) = 3.5$, $p = 0.021$ by diet; $F(2,57) = 5.4$, $p = 0.007$ by age] (Figure 3). The average latency for 5 days was also shorter in EW rats than in other young, diet groups ($p < 0.01$). The shorter latency in young, EW rats was further shortened ($p < 0.01$) in middle-age EW rats. However, the latency in middle-age EW rats did not significantly differ from that in older EW rats, suggesting that the onset of age-EW interaction occurs at a middle-age (15 months old). A latency decrease was more profound in EW middle-age rats than in dextrin middle-age rats ($p < 0.05$), indicating an adverse effect of age-EW combination. While E2 *per se* did not alter the latency, it increased the latency of EW rats at all three ages.

3.5. P38 activation in whole cerebellar extracts—When the level of pP38 was determined in the whole cellular extraction of cerebellum, the level was increased only in the middle-age EW rats among all of the diet and age groups [$F(3, 72) = 6$, $p < 0.01$ by diet; $F(2, 72) = 10$, $p < 0.01$ by age] (Figure 4). Neither age *per se* nor E2 *per se* significantly altered the total cerebellar level of pP38.

3.6. P38 activation in Purkinje neurons—Although pP38 immunoreactivity was found in other types of neurons, such as granular neurons, we focused on Purkinje neurons

because Purkinje neurons are the sole output of the cerebellar cortex and play a major role in cerebellar motor function (Watanabe, 2008). The immunohistochemical photographs revealed that the pP38 immunoreactivity (dark deposits) was more distinctively visualized in the ethanol-withdrawn Purkinje neurons along the Purkinje layer than that in the other diet groups across all three ages (Figure 5A–D). The number of pP38-positive Purkinje neurons did indeed differ by diet [$F(3,204) = 57, p < 0.001$] and by age [$F(2,204) = 22, p < 0.001$]; middle-age EW rats had a higher number of pP38-positive Purkinje neurons than young EW or older EW rats ($p < 0.01$) (Figure 5E). E2 treatment protected against the EW effects across all three ages ($p < 0.01$). Among dextrin groups, the middle-age rats also had a higher number of pP38-positive neurons than young rats ($p < 0.05$), but the magnitude of the difference was smaller than the difference between young and middle-age EW rats ($p < 0.05$). These data indicate that P38 activation targets Purkinje neurons upon EW insults in a manner that is exacerbated at a vulnerable age and is protected by estrogen. In order to confirm that the pP38 identified is indeed functionally active (phosphorylates a substrate), we measured the level of phospho-STAT1 (pSTAT1). STAT1 is a direct substrate of pP38 (Kovarik et al., 1999). Please note that the sole purpose for this STAT1 test is to confirm functionally active pP38, not to test the mechanistic involvement of STAT1. This test employed only middle-age groups as a representative case. Similar to the pP38 results, EW rats had a higher number of pSTAT1-positive Purkinje neurons than dextrin ($p = 0.005$) or EW+E2 ($p = 0.001$) rats [$F(2,51) = 29, p = 0.001$] (Figure 6), suggesting functional activation of pP38 during EW.

III. Oxidative Mechanisms

3.7. Total ROS in rats and HT22 cells—We determined whether an oxidative pathway is involved in EW-induced P38 activation. In all three age groups, EW rats had a higher ROS content than dextrin ($p < 0.001$), ethanol-exposure ($p < 0.01$) or EW+E2 ($p < 0.01$) rats [$F(3, 72) = 456, p < 0.001$] (Figure 7A). Between ages [$F(2, 72) = 9, p < 0.01$], older rats had moderately higher ROS contents than young or middle-age rats ($p < 0.05$), except for dextrin rats which showed no difference between age groups. Neither age *per se* nor E2 *per se* (data not shown) significantly altered the ROS content. Although EW predominantly provoked the generation of ROS, both the exacerbating effect of age-EW combination and the protective effects of E2 appeared to be moderate. To determine P38 and redox interaction during EW, we inhibited EW-induced P38 activation and measured ROS in HT22 cells. As in the *in vivo* case, EW significantly increased the ROS content [$F(2, 18) = 996, p < 0.001$ by ethanol-dose] (Figure 7B). SB203580 treatment restricted to the EW phase, but not the ethanol-exposure phase, protected against the EW-induced ROS generation [$F(2, 18) = 212, p < 0.001$ by the SB203580 treatment window; $F(4, 18) = 78, p < 0.001$ by the interaction between the ethanol-dose and the SB203580 window]. These results suggest that EW-induced P38 activation perturbs a redox balance during EW.

3.8. Mitochondrial protein oxidation—Since mitochondria are believed to be vulnerable to oxidative stress and aging, we tested whether mitochondrial protein oxidation age-dependently coincides with P38 activation during EW (Figure 8). EW rats showed protein carbonylation more severely than dextrin rats at all 3 ages. Between age groups, the middle-age EW rats exhibited stronger carbonyl signals than young or older EW rats at

certain molecular weights of proteins. In all 3 age groups, EW did not alter the levels of proteins stained with Coomassie blue staining *per se* (Jung et al., 2008). Given these findings, an age-EW combination appears to exacerbate mitochondrial protein oxidation at a vulnerable age.

4. Discussion

We have demonstrated that EW provokes the hyperactivation of protein kinase P38. The activation of P38 was more severe in middle-age (15 months) EW rats, who also displayed more severe oxidation of mitochondrial proteins than other diet (control dextrin, ethanol-exposure, and/or EW+E2) and age groups (8 and 19 months). Furthermore, it was the middle-age at which the adverse age-EW interaction began to affect cerebellar neuron-related motor deficit. These results suggest that the activation of P38 targets a vulnerable age and neurons challenged with EW. Such effects of EW do not appear to be due to a difference in body weights or ethanol kinetics because there was no correlation between body weights, BEC, and the degree of damage associated with EW. P38 activation has both beneficial (Peart et al., 2007) and deleterious (Campos et al., 2006) effects and thus, we tested a cellular consequence of P38 activation. As expected, excessive activation of this kinase was toxic to cells (Figure 2), and EW-induced rather than ethanol-induced activation of P38 contributed to the EW cytotoxicity.

The role of P38 activation in brain-aging changes has been reported in a model of neurodegeneration (Kelleher, et al., 2007). In that study, transgenic mice that formed the pathological aggregation of tau proteins had excess levels of pP38, and this level increased with age. In the current study, we asked whether P38 activation is involved in cerebellar aging that suffers from EW and E2 depletion. Cerebellum governs motor behavior through Purkinje neurons, which play a principal role in motor coordination and motor learning (Watanabe, 2008). For instance, cerebellar ataxic mice had a loss of Purkinje neurons but had no changes in the cerebellar granular neurons (Becker et al., 2009). Purkinje neuron knock-out mice showed more severe motor deficit in a Rotarod test than granular neuron knock-out mice (Levin et al., 2006). More relevant to the current study, EW rats exhibited poor motor performance that was accompanied by a substantial loss of Purkinje neurons and persistent cerebellar dysfunction (Jung et al., 2002; Rewal et al., 2003). These studies prompted us to test whether EW-induced P38 activation targets the Purkinje neurons. Indeed, an increase in the number of pP38-positive Purkinje neurons was found in all EW rats in a manner that peaked in middle-age EW rats and was protected by E2 treatment. The localization of pP38 in ethanol-withdrawn Purkinje neurons may imply that P38 activation intimately interferes with the neuronal integrity. In order to confirm that the pP38 identified in the Purkinje neurons is indeed functionally active (phosphorylates its substrate), we assessed phosphorylated STAT1 in the neurons. STAT1 is the best characterized direct substrate of pP38 (Kovarik et al., 1999). As was the case for pP38, EW rats showed a higher number of pSTAT1-positive Purkinje neurons than dextrin or EW+E2 rats. Given these findings, it is evident that the increase in the expression of pP38 in ethanol-withdrawn Purkinje neurons reflects an increase in the function of pP38.

The susceptible neuronal response of middle-age rats to the P38 activating EW was coherent with behavioral manifestation in that EW-induced motor deficit was exacerbated by age, and the exacerbation began at a middle age. These observations suggest that this age window (≈ 15 months) is a critical transition period. The vulnerability of middle-age rats to P38 activation was not limited to neurons; it was also seen at the whole-cellular level. However, compared to pP38-positive Purkinje neurons, of which numbers were increased in all EW rats (highest at a middle age), cerebellar whole-cell lysates showed that among all age and diet groups, only the middle-age EW group had increased pP38. Such a difference informs two potentially important aspects: neurons may be more susceptible in general to P38 activation than other types of cells and older-age rats may develop an adaptation to a stressful neuronal and cellular milieu. The former notion is supported by our pilot study in which pP38 was not co-localized with astrocytes even under a vulnerable condition (Supplementary Figure). Svensson et al. (2003) also reported no co-localization between pP38 and astrocytes in the lumbar spinal cord after treatment with a hyperalgesic substance. Unlike the case with P38 in older EW rats, in which the number of pP38-positive Purkinje neurons returned to the level of young EW rats, an exacerbated motor-deficit due to age-EW combination began at a middle age and was sustained at an older age. However, this behavioral result may still reflect an adaptive response in older rats because the exacerbated motor deficit seen in middle-age EW rats was not further exacerbated by aging, perhaps due to a tolerance that older rats have developed. In addition, the not-exactly-paralleled response between neurons and behavior at an older age implies that P38 is not the sole contributor to the functional consequence of the neurons.

A previous study reported the vulnerability of middle-age groups to aging symptoms such that the onset of memory impairment occurred at the age of 12 months in female rats (Markowska et al., 1999). The susceptibility of middle-age rats may be the reflection of cellular and neuronal alterations associated with transition stress from young to old endogenous systems. The variety of alterations may collectively perturb the neuronal integrity of the cerebellum, providing favorable conditions for P38 activation upon EW insults. In this scenario, older animals may develop adaptations and tolerances to the altered cellular and neuronal milieu and thus become more resistant to the EW stress. Alternatively, age-associated alteration may depend on specific organelles or molecules. For instance, when $\bullet\text{O}_2$ - and H_2O_2 were measured in mitochondrial fractions, the content peaked in middle-age rats (Guarnieri et al., 1992). The susceptibility of the middle-age rats to oxidative insults in Guarnieri's study is in line with our results, which indicate that the oxidation of mitochondrial proteins is more severe in middle-age EW rats than in young or older EW rats (Figure 8). In comparison, when total ROS content was measured in the whole-cerebellar lysates (not specific organelles), it peaked at an older age (19 months) (Figure 7A). The ROS results suggest that a middle age is not always the most vulnerable period and that the vulnerable age at which a maximum damage occurs may vary depending upon target molecules or organelles or the nature of stress.

Based on the idea that oxidative stress is attributed to P38 activation (Galluzzi et al., 2009; Valles' et al., 2008), we hypothesized that EW permits a link between the oxidative pathway and P38 activation. This hypothesis was at least partly confirmed by our P38 inhibition study in which SB203580 (P38 inhibitor) treatment restricted to the EW phase attenuated ROS

generation in HT22 cells. This cell line is an effective model of oxidative stress. While HT22 cells contain a minimum amount of glutamate receptors (Zaulyanov et al., 1999), they contain the glutamate/cystine antiporter for the delivery of cystine into neuronal cells that mediates the synthesis of an antioxidant glutathione. Therefore, HT22 cellular injury is often associated with oxidative stress (Tan et al., 1998) including EW-induced cellular oxidation (Jung et al., 2009). Such crosstalk between oxidative stress and P38 activation might have contributed to the more severe mitochondrial protein oxidation occurring with the more severe P38 activation, in middle-age EW rats than in young or older EW rats (Figure 4 and 8). Therefore, it is tempting to speculate that EW-induced ROS (Figure 7A) trigger the phosphorylation of P38 (Huot et al., 1997, Figure 4) as an initial step. Later, a reciprocal interaction (Figure 7B) between the active pP38 and oxidative pathways is permitted at a vulnerable age. Adverse down-stream effectors are in turn triggered, inflicting further oxidative damage to mitochondrial proteins (Figure 8). While neurons are repeatedly exposed to the chaotic milieu, perhaps irreversible neuronal damages occur with behavioral consequences (Figure 3). Because it has an antioxidant property during EW insults (Figure 7A, Rewal et al., 2004), E2 may interfere with any of these steps, protecting neurons from EW.

We previously observed that E2 protected against EW-induced motor deficit that was accompanied by the loss of cerebellar Purkinje neurons (Jung et al., 2002). At the level of P38, Valles et al. (2008) suggested that E2 prevented oxidative stress, which in turn inhibited P38 activation and protected neurons from Amyloid β -toxicity. In contrast, trauma-hemorrhage decreased the protein level of pP38, and E2 treatment increased it toward a control level (Hsu et al., 2007). In our study, EW increased the levels of pP38 in middle-age EW rats, and E2 treatment decreased it toward a control level. It is not clear what underlies the mechanisms of the inhibiting and the activating effects of E2 on P38. Presumably, E2 may modulate the pathological conditions of P38 in a direction toward the homeostatic status of P38. It should be also noted that the magnitude of E2 protection is not uniform across ages and is modest, especially against total ROS. This may be because E2 is neither a pure antioxidant nor a pure P38 inhibitor. Instead, multiple mechanisms triggered by E2 may crosstalk with each other, collectively executing E2 protection.

In conclusion, our study provides empirical evidence that EW acts as a stressor to activate P38 at a vulnerable age and in vulnerable neurons. The adverse age-EW interaction may in part result from cross-talk between pP38 and oxidative pathways. It is our hope that this work contributes to a new insight into the mechanistic involvement of P38 in aging females simultaneously suffering from EW and E2 depletion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Arimoto K, Fukuda H, Imajoh-Ohmi S, Saito H, Takekawa M, 2008 Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways. *Nat. Cell. Biol.* 10, 1324–1332. [PubMed: 18836437]
- Aydin MV, Sen O, Kayaselcuk F, Bolat F, Tufan K, Caner H, Altinors N, 2005 Analysis and prevalence of inflammatory cells in subtypes of lumbar disc herniations under cyclooxygenase-2 inhibitor therapy. *Neurol. Res.* 27, 609–612. [PubMed: 16157010]
- Barca O, Costoya JA, Senaris RM, Arce VM, 2008 Interferon-beta protects astrocytes against tumour necrosis factor-induced apoptosis via activation of p38 mitogen-activated protein kinase. *Exp. Cell. Res.* 314, 2231–2237. [PubMed: 18501892]
- Becker EB, Oliver PL, Glitsch MD, Banks GT, Achilli F, Hardy A, Nolan PM, Fisher EM, Davies KE, 2009 A point mutation in TRPC3 causes abnormal Purkinje cell development and cerebellar ataxia in moonwalker mice. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6706–6711. [PubMed: 19351902]
- Campos CB, Bedard PA, Linden R, 2006 Requirement of p38 stress-activated MAP kinase for cell death in the developing retina depends on the stage of cell differentiation. *Neurochem. Int.* 49, 494–499. [PubMed: 16782232]
- Drechsler Y, Dolganiuc A, Norkina O, Romics L, Li W, Kodys K, Bach FH, Mandrekar P, Szabo G, 2006 Heme oxygenase-1 mediates the anti-inflammatory effects of acute alcohol on IL-10 induction involving p38 MAPK activation in monocytes. *J. Immunol.* 177, 2592–2600. [PubMed: 16888021]
- Du J, Yang S, Wang Z, Zhai C, Yuan W, Lei R, Zhang J, Zhu T, 2008 Bone morphogenetic protein 6 inhibit stress-induced breast cancer cells apoptosis via both Smad and p38 pathways. *J. Cell. Biochem.* 103, 1584–1597. [PubMed: 17879955]
- Galluzzi L, Blomgren K, Kroemer G, 2009 Mitochondrial membrane permeabilization in neuronal injury. *Nat. Rev. Neurosci.* 10, 481–494. [PubMed: 19543220]
- Giordano G, Klintworth HM, Kavanagh TJ, Costa LG, 2008 Apoptosis induced by domoic acid in mouse cerebellar granule neurons involves activation of p38 and JNK MAP kinases. *Neurochem. Int.* 52, 1100–1105. [PubMed: 18164102]
- Guarnieri C, Muscari C, Caldarera CM, 1992 Mitochondrial production of oxygen free radicals in the heart muscle during the life span of the rat: peak at middle age. *EXS* 62, 73–77. [PubMed: 1333312]
- Hensley K, Floyd RA, Zheng NY, Nael R, Robinson KA, Nguyen X, Pye QN, Stewart CA, Geddes J, Markesbery WR, 1999 p38 kinase is activated in the Alzheimer's disease brain. *J. Neurochem.* 72, 2053–2058. [PubMed: 10217284]
- Hsu JT, Hsieh YC, Kan WH, Chen JG, Choudhry MA, Schwacha MG, Bland KI, Chaudry IH, 2007 Role of p38 mitogen-activated protein kinase pathway in estrogen-mediated cardioprotection following trauma-hemorrhage. *Am. J. Physiol. Heart Circ. Physiol.* 292, H2982–2987. [PubMed: 17293487]
- Huot J, Houle F, Marceau F, Landry J, 1997 Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. *Circ. Res.* 80, 383–392. [PubMed: 9048659]
- Jakubowski W, Bartosz G, 2000 2,7-dichlorofluorescein oxidation and reactive oxygen species: what does it measure? *Cell. Biol. Int.* 24, 757–760. [PubMed: 11023655]
- Jiang Y, Chen C, Li Z, Guo W, Gegner JA, Lin S, Han J, 1996 Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). *J. Biol. Chem.* 271, 17920–17926. [PubMed: 8663524]
- Jiang Y, Gram H, Zhao M, New L, Gu J, Feng L, Di Padova F, Ulevitch RJ, Han J, 1997 Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38delta. *J. Biol. Chem.* 272, 30122–30128. [PubMed: 9374491]
- Jung ME, Yan LJ, Forster MJ, Simpkins JW, 2008 Ethanol withdrawal provokes mitochondrial injury in an estrogen preventable manner. *J. Bioenerg. Biomembr.* 40, 35–44. [PubMed: 18210193]
- Jung ME, Wilson AM, Ju X, Wen Y, Metzger DB, Simpkins JW, 2009 Ethanol withdrawal provokes opening of the mitochondrial membrane permeability transition pore in an estrogen-preventable manner. *J. Pharmacol. Exp. Ther.* 328, 692–698. [PubMed: 19050172]

- Jung ME, Yang SH, Brun-Zinkernagel AM, Simpkins JW, 2002 Estradiol protects against cerebellar damage and motor deficit in ethanol-withdrawn rats. *Alcohol* 26, 83–93. [PubMed: 12007583]
- Kelleher I, Garwood C, Hanger DP, Anderton BH, Noble W, 2007 Kinase activities increase during the development of tauopathy in htau mice. *J. Neurochem.* 103, 2256–2267. [PubMed: 17908241]
- Kovarik P, Stoiber D, Eyers PA, Menghini R, Neiningner A, Gaestel M, Cohen P, Decker T, 1999 Stress-induced phosphorylation of STAT1 at Ser727 requires p38 mitogen-activated protein kinase whereas IFN-gamma uses a different signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13956–13961. [PubMed: 10570180]
- Ku BM, Lee YK, Jeong JY, Mun J, Han JY, Roh GS, Kim HJ, Cho GJ, Choi WS, Yi GS, Kang SS, 2007 Ethanol-induced oxidative stress is mediated by p38 MAPK pathway in mouse hippocampal cells. *Neurosci. Lett.* 419, 64–67. [PubMed: 17420100]
- Lechner C, Zahalka MA, Giot JF, Moller NP, Ullrich A, 1996 ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 93, 4355–4359. [PubMed: 8633070]
- Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, 1994 A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 372, 739–746. [PubMed: 7997261]
- Lee SH, Park J, Che Y, Han PL, Lee JK, 2000 Constitutive activity and differential localization of p38alpha and p38beta MAPKs in adult mouse brain. *J. Neurosci. Res.* 60, 623–631. [PubMed: 10820433]
- Levin SI, Khaliq ZM, Aman TK, Grieco TM, Kearney JA, Raman IM, Meisler MH, 2006 Impaired motor function in mice with cell-specific knockout of sodium channel Scn8a (NaV1.6) in cerebellar purkinje neurons and granule cells. *J. Neurophysiol.* 96, 785–793. [PubMed: 16687615]
- Li HL, Wu S, Rottenberg H, 1996 Alcohol inhibits the depolarization-induced stimulation of oxidative phosphorylation in synaptosomes. *J. Neurochem.* 66, 1691–1697. [PubMed: 8627327]
- Markowska AL, 1999 Sex dimorphisms in the rate of age-related decline in spatial memory: relevance to alterations in the estrous cycle. *J. Neurosci.* 19, 8122–8133. [PubMed: 10479712]
- Mausset AL, de Seze R, Montpeyroux F, Privat A, 2001 Effects of radiofrequency exposure on the GABAergic system in the rat cerebellum: clues from semi-quantitative immunohistochemistry. *Brain Res.* 912, 33–46. [PubMed: 11520491]
- Moriguchi T, Toyoshima F, Gotoh Y, Iwamatsu A, Irie K, Mori E, Kuroyanagi N, Hagiwara M, Matsumoto K, Nishida E, 1996 Purification and identification of a major activator for p38 from osmotically shocked cells. Activation of mitogen-activated protein kinase 6 by osmotic shock, tumor necrosis factor-alpha, and H2O2. *J. Biol. Chem.* 271, 26981–26988. [PubMed: 8900184]
- Nonaka Y, Miyajima M, Ogino I, Nakajima M, Arai H, 2008 Analysis of neuronal cell death in the cerebral cortex of H-Tx rats with compensated hydrocephalus. *J. Neurosurg. Pediatr.* 1, 68–74. [PubMed: 18352806]
- Norkina O, Dolganiuc A, Shapiro T, Kodys K, Mandrekar P, Szabo G, 2007 Acute alcohol activates STAT3, AP-1, and Sp-1 transcription factors via the family of Src kinases to promote IL-10 production in human monocytes. *J. Leukoc. Biol.* 82, 752–762. [PubMed: 17575268]
- Peart JN, Gross ER, Headrick JP, Gross GJ, 2007 Impaired p38 MAPK/HSP27 signaling underlies aging-related failure in opioid-mediated cardioprotection. *J. Mol. Cell. Cardiol.* 42, 972–980. [PubMed: 17407780]
- Ramados J, Lunde ER, Chen WJ, West JR, Cudd TA, 2007 Temporal vulnerability of fetal cerebellar Purkinje cells to chronic binge alcohol exposure: ovine model. *Alcohol Clin. Exp. Res.* 31, 1738–1745. [PubMed: 17681031]
- Rewal M, Jung ME, Simpkins JW, 2004 Role of the GABA-A system in estrogen-induced protection against brain lipid peroxidation in ethanol-withdrawn rats. *Alcohol Clin. Exp. Res.* 28, 1907–1915. [PubMed: 15608608]
- Rewal M, Jung ME, Wen Y, Brun-Zinkernagel AM, Simpkins JW, 2003 Role of the GABAA system in behavioral, motoric, and cerebellar protection by estrogen during ethanol withdrawal. *Alcohol* 31, 49–61. [PubMed: 14615011]

- Santucci AC, Cortes C, Bettica A, Cortes F, 2008 Chronic ethanol consumption in rats produces residual increases in anxiety 4 months after withdrawal. *Behav. Brain Res.* 188, 24–31. [PubMed: 18061285]
- Smolen A, Marks MJ, Smolen TN, Collins AC, 1986 Dose and route of administration alter the relative elimination of ethanol by long-sleep and short-sleep mice. *Alcohol. Clin. Exp. Res.* 10, 198–204. [PubMed: 3521378]
- Stein B, Yang MX, Young DB, Janknecht R, Hunter T, Murray BW, Barbosa MS, 1997 p38–2, a novel mitogen-activated protein kinase with distinct properties. *J. Biol. Chem.* 272, 19509–19517. [PubMed: 9235954]
- Svensson CI, Marsala M, Westerlund A, Calcutt NA, Campana WM, Freshwater JD, Catalano R, Feng Y, Protter AA, Scott B, Yaksh TL, 2003 Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing. *J. Neurochem.* 86, 1534–1544. [PubMed: 12950462]
- Tan S, Wood M, Maher P, 1998 Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. *J. Neurochem.* 71, 95–105. [PubMed: 9648855]
- Valles SL, Borrás C, Gambini J, Furriol J, Ortega A, Sastre J, Pallardo FV, Vina J, 2008 Oestradiol or genistein rescues neurons from amyloid beta-induced cell death by inhibiting activation of p38. *Aging Cell.* 7, 112–118. [PubMed: 18031570]
- van Eickels M, Grohe C, Cleutjens JP, Janssen BJ, Wellens HJ, Doevendans PA, 2001 17beta-estradiol attenuates the development of pressure-overload hypertrophy. *Circulation* 104, 1419–1423. [PubMed: 11560859]
- Vereker E, O'Donnell E, Lynch MA, 2000 The inhibitory effect of interleukin-1beta on long-term potentiation is coupled with increased activity of stress-activated protein kinases. *J. Neurosci.* 20, 6811–6819. [PubMed: 10995825]
- Wang M, Tsai BM, Reiger KM, Brown JW, Meldrum DR, 2006 17-beta-Estradiol decreases p38 MAPK-mediated myocardial inflammation and dysfunction following acute ischemia. *J. Mol. Cell. Cardiol.* 40, 205–212. [PubMed: 16427650]
- Watanabe M, 2008 Molecular mechanisms governing competitive synaptic wiring in cerebellar Purkinje cells. *Tohoku J. Exp. Med.* 214, 175–190. [PubMed: 18323688]
- Williamson D, Gallagher P, Harber M, Hollon C, Trappe S, 2003 Mitogen-activated protein kinase (MAPK) pathway activation: effects of age and acute exercise on human skeletal muscle. *J. Physiol.* 547, 977–987. [PubMed: 12562918]
- Wu M, Han M, Li J, Xu X, Li T, Que L, Ha T, Li C, Chen Q, Li Y, 2009 17beta-estradiol inhibits angiotensin II-induced cardiac myofibroblast differentiation. *Eur. J. Pharmacol.* 616, 155–159. [PubMed: 19470381]
- Xiong W, Kojic LZ, Zhang L, Prasad SS, Douglas R, Wang Y, Cynader MS, 2006 Anisomycin activates p38 MAP kinase to induce LTD in mouse primary visual cortex. *Brain Res.* 1085, 68–76. [PubMed: 16581040]
- Yan LJ, Levine RL, Sohal RS, 1997 Oxidative damage during aging targets mitochondrial aconitase. *Proc. Natl. Acad. Sci. U.S.A.* 94, 11168–11172. [PubMed: 9326580]
- Yang SH, He Z, Wu SS, He YJ, Cutright J, Millard WJ, Day AL, Simpkins JW, 2001 17-beta estradiol can reduce secondary ischemic damage and mortality of subarachnoid hemorrhage. *J. Cereb. Blood Flow Metab.* 21, 174–181. [PubMed: 11176283]
- Zaulyanov LL, Green PS, Simpkins JW, 1999 Glutamate receptor requirement for neuronal death from anoxia-reoxygenation: an in Vitro model for assessment of the neuroprotective effects of estrogens. *Cell Mol. Neurobiol.* 19, 705–718. [PubMed: 10456232]

Figure 1. EW-induced P38 activation.

The protein levels of cerebellar pP38 were assessed using an immunoblot method 2 weeks after a 5-week ethanol diet (6.5% v/v) in young adult male rats. The levels of pP38 were much higher during EW than during ethanol-exposure, but the levels of total P38 did not significantly differ between diet groups. * $p < 0.01$, ** $p < 0.001$ vs. a control dextrin (=100%). Depicted are mean $\pm$ SEM for 3 rats/group.

Figure 2. Cytotoxic P38 activation.

The effect of EW on HT22 cell viability was measured 4 hours after 24-hour ethanol exposure. EW decreased cell viability relative to control cell viability at 100% (statistical symbols were omitted to simplify figures). SB203580 (200 nM) treatment during the EW phase (right), but not the ethanol-exposure phase (left), mitigated EW-induced cell death. * $p = 0.039$, ** $p = 0.009$ vs. vehicle (non SB203580). Depicted are mean $\pm$ SEM for 4/treatment.

Figure 3. Age-dependent motor deficit in ethanol-withdrawn rats.

Ovariectomized rats were implanted with oil or E2 pellets, received a 90-day diet regimen, a 25-day ethanol- (6.5% v/v) or a control dextrin diet followed by 5-day abrupt withdrawal, and the cycle was repeated 3 times. Beginning at the 7th day of EW, the rats were tested for the Rotarod task (3 sessions/day, 5 days). Data were presented as an average latency from the 5-day data. Motor deficit (shorter latency) was found in young EW rats ($p < 0.01$) compared to other diet groups, became more severe ($p < 0.01$) in middle-age EW rats, and then remained at a similar level in older EW rats. * $p < 0.01$ vs. dextrin, dextrin + E2 or EW + E2 at each age. † $p < 0.05$ vs. 8-month-old dextrin. ‡ $p < 0.01$ vs. 8-month-old EW. Depicted are mean $\pm$ SEM for 4–7 rats/group.

Figure 4. P38 activation in whole cerebellar extracts.

Ovariectomized rats underwent repeated EW as described in the Figure 3 legend. At the 14th day of EW, the protein level of pP38 was measured in the cerebellar whole-cell lysates using ELISA assay. The middle-age EW rats had a higher level of pP38 than other diet or age groups (left). E2 *per se* did not significantly alter the level of pP38 in dextrin diet rats (right).

* $p < 0.01$ vs. all other groups. Depicted are mean $\pm$ SEM for 4 or 5 rats/group.

Figure 5. pP38-positive Purkinje neurons.

Ovariectomized rats underwent repeated EW as described in the Figure 3 legend. At the 14th day of EW, the left hemisphere containing the cerebellar vermis was processed for immunohistochemical analysis. All photographs were taken of the cerebellar cortex area containing Purkinje layers that showed a clear image across all treatment groups. Dark deposits marked with arrows indicate pP38 immunoreactivity in Purkinje neurons along the Purkinje layer, and they distinctively appeared in the EW groups across all 3 ages (Figure 5A-D). EW rats had a higher number of pP38-positive Purkinje neurons/Purkinje layer (mm)

than dextrin, ethanol or EW+E2 rats, particularly at 15 months old (Figure 5E). * $p < 0.01$ vs. dextrin or EW+E2 at 8 or 19 months old; ** $p < 0.01$ vs. dextrin, Ethanol or E2 at 15 months old; † $p < 0.01$ vs. 8- or 19-month-old EW; ‡ $p < 0.05$ vs. 8-month-old-dextrin. A 5-fold (Figure 5A) or 20-fold (Figure 5B-D) magnification was used to take pictures of representative groups (15 months old) or all age/diet groups, respectively. Figure 5A shows cerebellar lobule III and IV and Figure 5B-D show lobule II. The scale bar indicates an actual length of 200 μm (Figure 5A) or 50 μm (Figure 5B-D). Depicted are mean $\pm$ SEM for 6 microscopic fields/rat for 3 rats/group (Figure 5E).

Figure 6. pSTAT-positive Purkinje neurons.

Ovariectomized rats underwent repeated EW as described in the Figure 3 legend. At the 14th day of EW, the left hemisphere containing the cerebellar vermis was processed for immunohistochemical analysis to identify pSTAT1-positive Purkinje neurons. Photographs were taken of the cerebellar cortex of lobule II containing Purkinje layers of middle-age rats (15 months). Dark deposits marked with arrows indicate pSTAT1 immunoreactivity in Purkinje neurons, and they distinctively appeared in the EW group. EW rats had a higher number of pSTAT1-positive Purkinje neurons/Purkinje layer (mm) than dextrin (* $p = 0.005$) or EW+E2 († $p = 0.001$) rats. A 20-fold magnification was used to take pictures. The scale bar indicates an actual length of 50 μm . Depicted are mean $\pm$ SEM for 6 microscopic fields/rat for 3 rats/group.

Figure 7. Total ROS in rats and HT22 cells.

Ovariectomized rats underwent repeated EW as described in the Figure 3 legend (left).

Within the same age groups, ROS content in whole-cerebellar lysates was higher in the EW group than in other diet groups. Between age groups, a higher ROS content was found in the older EW group than in the young ($**p < 0.01$) or the middle-age ($*p < 0.05$) EW groups. $^{\dagger}p < 0.05$ vs. young or middle-age ethanol; $^{\ddagger}p < 0.05$ vs. young or middle-age EW+E2. In HT22 cells (right), total ROS were measured at 4 hours of EW from 24-hour ethanol exposure (0–100 mM). SB203580 (200 nM) treatment during the EW phase but not the ethanol-exposure phase decreased EW-induced ROS generation. $*p < 0.01$, $**p < 0.001$ vs. non-ethanol control. $^{\dagger}p < 0.01$ vs. cells treated with SB203580 (200 nM) during ethanol-exposure at 50 mM or 100 mM of ethanol. Depicted are mean $\pm$ SEM for 5–7 animals/group or 4 cell plates/group.

Figure 8. Mitochondrial protein oxidation.

Ovariectomized rats implanted with oil pellets underwent repeated EW as described in the Figure 3 legend. The carbonylation of mitochondrial proteins with molecular weights equal to or less than 130 KDa is shown in this picture. The EW group exhibited much stronger carbonyl signals (dark bands) than the dextrin groups especially in the middle-age rats at certain molecular weights, including those indicated with arrows. Cerebellar mitochondria were pooled from 5 rats/group.