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The Role of Presenilin-1 in the Excitotoxicity of Ethanol Withdrawal

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Abstract

Presenilin-1 (PS1) is a core component of γ-secretase that is involved in neurodegeneration. We have previously shown that PS1 interacts with a MAPK (JNK), and another MAPK (p38) is activated by ethanol withdrawal (EW), abrupt termination from chronic ethanol-exposure. EW is excitotoxic in nature, induces glutamate upregulation, and provokes neuronal damage. Here, we explored a potential mechanistic pathway involving glutamate, p38 (p38α isozyme), and PS1 that may mediate EW-induced excitotoxic stress. We used the prefrontal cortex of male rats withdrawn from a chronic ethanol diet. Additionally, we used ethanol withdrawn HT22 cells (mouse hippocampal) treated with the inhibitor of glutamate receptors (MK-801), p38α (SB203580), or γ-secretase (DAPT) during EW. Separately, ethanol-free HT22 cells were exposed to glutamate with or without SB203580 or DAPT. Protein levels, mRNA levels, and cell viability were assessed using immunoblotting, q-PCR, and Calcein assay, respectively. The prefrontal cortex of ethanol withdrawn rats or HT22 cells showed an increase in PS1 and p38α, which was attenuated by MK-801 and SB203580, but mimicked by glutamate treatment to ethanol-free HT22 cells. DAPT attenuated the toxic effect of EW or glutamate on HT22 cells. These results suggest that PS1 expression is triggered by glutamate through p38α, contributing to the excitotoxic stimulus of EW.

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Introduction

PS1 is a core component of enzyme complex γ-secretase that cleaves amyloid precursor protein (Parks and Curtis 2007), producing potentially neurotoxic Aβ peptide. γ-secretase also cleaves transmembrane proteins called Notch receptors, and thereby releases Notch intracellular domain (NICD) (De Strooper *et al.* 1999). The NICD then enters nucleus, activating transcriptional factors such as Hairy enhancer of split (HES1) (Tandon and Fraser 2002). Presenilin-2 (PS2) is a homologue of PS1 and displays a considerably less cellular activity than PS1-associated γ-secretase (Lai *et al.* 2003). While it is well known that the mutation of PS1 genes increases Aβ formation, the role of wild-type PS1 in brain is less explored and controversial. A decrease in PS1 occurred in the brain of aged mice (Thakur and Ghosh 2007; Kaja *et al.* 2014), suggesting the role of PS1 depletion in aging. On the other hand, an increase in PS1 occurred in the hippocampus of aged mice that exhibited premature memory loss and normal cDNA sequence of PS1 (Kumar *et al.* 2009). The authors have suggested that the increased PS1 might have contributed to the early loss of memory. The deleterious role of PS1 is supported by a study where neuronal cultures lacking wild-type PS1 show a low γ-secretase activity and neurotoxic amyloid peptide level (De Strooper *et al.* 1998). De Strooper's study suggests that PS1 is essential for γ-secretase activity, and excessive PS1 may be neurotoxic. Indeed, De Strooper (1998) stated that the inhibition of PS1 activity is a potential target for the treatment of neurodegenerative disorders. These studies lead to the speculation that changes in PS1 expression may depend on its interactive proteins.

As a potential interactive protein, PS1 may be associated with p38. p38 belongs to the superfamily of mitogen-activated protein kinases (MAPKs) that phosphorylate other proteins, thereby mediating a wide range of cellular reaction in responses to extracellular stimuli. p38 is also called a stress-activated protein kinase as it is activated by a variety of stresses. Examples are inflammatory cytokines, oxidative stress, hypoxia, ischemia, and tumor necrosis factor alpha (TNF-α) (reviewed by Cuadrado and Nebreda 2010),

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and abrupt withdrawal from chronic ethanol-exposure (Jung *et al.* 2011). A high level of p38 appears to be deleterious based on reports that excessive p38 mediates apoptosis (programmed cell death) (Cuenda and Rousseau 2007) and impedes cell survival (Jung *et al.* 2011). The over-activation of p38 has been shown in a mouse brain with mutant PS1 genes, suggesting PS1 and p38 association (Giraldo *et al.* 2014). We have previously demonstrated that an inhibitor of a MAPK (JNK) repressed PS1 transcription and γ-secretase-mediated-cleavage of amyloid precursor protein in SK-N-SH cells (Lee and Das 2008; Das *et al.* 2012). These studies indicate that certain MAPKs including p38 interact with PS1.

In the current study, we were interested in determining whether PS1 is involved in the excitotoxicity of abrupt ethanol withdrawal (EW) and if so, whether it depends on p38. EW stress is typically initiated upon the sudden cessation/reduction of long term heavy ethanol consumption. A clinical significance of EW is inferred from the fact that alcoholics must withdraw from ethanol for a detoxification process. The hallmark of EW stress is an excitatory property, meaning that an excitatory neurotransmitter such as glutamate is overly released in response to EW (Tsai *et al.* 1998; Prendergast *et al.* 2004). This is a physiologically serious problem as the high amount of glutamate triggers neuronal death, a phenomenon known as excitotoxicity (Leng and Chuang 2006). Indeed, EW-induced cell damage has been reported in multiple brain areas including hippocampus (Oliveira-da-Silva A *et al.* 2010), cortex (Nagy *et al.* 2002), and cerebellum (Jung *et al.* 2002). The neurotoxic effect of EW may involve p38 as EW-induced cell death is attenuated by a p38 inhibitor in our earlier study (Jung *et al.* 2011). Based on these studies, the current study investigated a potential mechanism by which PS1, p38 (p38α isozyme), and glutamate interact with each other to confer the excitotoxic effect of EW. We report that EW triggers an increase in PS1 through p38α, and this effect of EW is attenuated by a glutamate antagonist and mimicked by glutamate, thereby contributing to excitotoxic cell death.

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Methods

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 obtained 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).

Animal protocols

Male Sprague-Dawley rats were 3 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.

Ethanol diet and EW in rats

The overview of experimental designs involving EW rats is depicted in Figure 1. For an *in vivo* EW paradigm, we used the model of repeated EW (multiple episodes of EW) because it mimics the drinking pattern of human alcoholics (George *et al.* 1990; Becker and Hale 1993; NIAAA 1995; Krystal *et al.* 1997; Stephens *et al.* 2001). Many alcoholics repeat the vicious cycle of drinking and withdrawal, which increases the risk of brain damage (Ballenger and Post 1978; Brown *et al.* 1988). Rats (7 rats/group) were assigned to an ethanol or a control diet group. They 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. The control diet group was fed an identical liquid diet with the exception that dextrin (complex

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carbohydrate) isocalorically substituted for ethanol. Additionally, 7 rats were assigned to the ethanol-consuming group that received the aforementioned ethanol diet with the exception that these rats continuously received an ethanol diet. Notice that this study focuses on EW and the inclusion of the ethanol-consuming group is to demonstrate that the effect of EW on PS1 is not a merely the residual effect of ethanol-exposure. The concentration of ethanol was gradually increased to 7.5% during the first week of the ethanol diet, as described in our previous study (Jung *et al.* 2011). 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 the prefrontal cortex.

Blood ethanol concentrations

On the last day of an ethanol diet, blood ethanol concentrations were measured in rats that were assigned to the EW or the ethanol-consuming group. Three hours after placement of fresh diet bottles, 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 μl) was withdrawn and immediately mixed with 90 μl of ice-cold 0.55 M HClO4. Samples were centrifuged at 1,500 g for 10 min to sediment protein precipitate. Supernatants were adjusted to ~pH 5 with 200 μl 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 (Smolen et al., 1986) in which NAD+ reduction to NADH is coupled to ethanol oxidation by alcohol dehydrogenase (extinction coefficient $\varepsilon = 6.2\ mM^{-1} \cdot cm^{-1}$) in a Beckman DU 640 spectrophotometer.

In vitro EW and glutamate treatment

The overview of experimental designs involving *in vitro* experiments is depicted in Figure 1. HT22 cells were used for an *in vitro* model of EW and for all drug intervention studies using glutamate, a glutamate receptor antagonist (MK-801, 1 μM) (Santa Cruz, Dallas, TX), a p38α inhibitor (SB203580, 1 μM)

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(Thermo Scientific, Waltham, MA), or a γ-secretase inhibitor (DAPT, 10 μM) (Sigma Aldrich, St. Louis, MO). HT22 cell line has been originally developed by Dr. David Schubert in Salk Institute in 1994. The HT22 line was initially selected from HT4 cells based on glutamate sensitivity (Davis and Maher, 1994; Li et al. 1998). The glutamate sensitivity of HT22 cells was reproduced by our earlier study (Jung et al., 2005). The best known nature of EW is a hyperexcitatory effect on neurons through glutamate upregulation (Prendergast et al. 2004). Therefore, the HT22 cell line is an accepted *in vitro* model to assess excitatory neuronal damage associated with EW. Among four isoforms of p38 (p38α, β, γ, and δ), p38α is ubiquitously and significantly expressed in most cell types (reviewed by Cuadrado and Nebreda 2010) and in human brains (Wang *et al.* 1997). p38α is particularly responsive to stressful stimuli (Han *et al.* 1994) including stress associated with glutamate (Sun *et al.* 2003; Xing *et al.* 2015) and EW (Jung *et al.* 2011). We thus, focused on p38α among the four isoforms and used 1 μM of SB203580 as SB203580 is a selective p38α/β inhibitor at a dose of 1-5 μM (Mihara *et al.* 2008). HT22 cells were cultured in flasks until they reached 70% confluence, according to a method stablished by Perez *et al.* (2005). 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 μg/mL), at 37^{o}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 as was the case for EW in rats. The treatment with MK-801, SB203580, or DAPT 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 SB203580 or DAPT treatment to determine whether glutamate (Sigma Aldrich, St.Louis, MO) treatment alone resembles EW stress.

Immunoblotting

This method was used to assess the effects of EW on the expression of PS1, p38α, and PS1-activated molecules (NICD and HES1) in rat prefrontal cortex. This brain area and hippocampus are known to be

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sensitive to the damaging effect of excessive glutamatergic neurotransmission, chronic ethanol, and EW (Morgan *et al.* 1992; Fadda and Rossetti 1998). On the last day of the EW paradigm, the prefrontal cortex was collected from rats, and added to lysis buffer. The sample was then sonicated and an aliquot was used to determine protein concentration using the Bradford protein assay (Biorad, Hercules, CA). Another aliquot was combined with an equal volume of loading buffer, electrophoresed on a 10% SDS-PAGE, and then transferred onto a polyvinylidene fluoride membrane. Nonspecific binding sites were blocked with 5% fat free milk. The blot was washed in TBS-T and probed overnight with a mouse polyclonal antibody against PS1 (EMD Millipore, Billerica, MA) and NICD (Abcam, Cambridge, MA), or a rabbit monoclonal antibody against phosphor-p38α (an active form of p38α) (Abcam, Cambridge, MA) and HES1 (Abcam, Cambridge, MA). The blot was then washed and incubated with horseradish peroxidase - conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA) for 1 to 2 hours at room temperature. Bands were detected using the UVP (UVP, Upland, CA) western blotting luminescence system and quantified by an image densitometer. Immunoblotting for β-actin (Santa Cruz, Dallas, TX) was carried out as a positive and a loading control. For the immunoblotting of HT22 cells, the same aforementioned immunoblotting procedures were applied with the exception that cells were collected and combined with RIPA lysis buffer (Sigma Aldrich, St. Louis, MO) (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) and subsequently sonicated (30 seconds). Protein concentration was determined as mentioned above. Samples were then mixed with an equal volume of Laemmli sample buffer, heated (100ºC, 10 minutes) and loaded into BioRad precast gel.

Real-time polymerase chain reaction (PCR)

This assay was conducted to determine whether EW affects PS1 at the transcriptional level by measuring the mRNA level of PS1 in EW rats. Total RNA was isolated from the prefrontal cortex of rats using Trizol (Qiagen, Maryland), reagent following the manufacturer's instructions. RNA was converted to cDNA by adding random primers and Superscript III reverse transcriptase (Thermo Fisher, Waltham,

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MA). cDNA was then quantified using Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Real-time PCR was conducted to analyze mouse PS1 gene expression using an ABI PRISM 7000 (Thermo Fisher, Waltham, MA) sequence detection system with TaqMan primers (Thermo Fisher, Waltham, MA). Primer sequences were as follows: for PS1 forward, 5'-GCGGCGGGGAAGCGTATACC-3'; for PS1 reverse, 5'-GGCCAAGCTGTCTAAGGACCGC-3'. qPCR reactions were performed as follows: 50ºC for 2 minutes, 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. GAPDH was used as an internal control gene. Cycle threshold (Ct) values were calculated with SDS software v.2.3 (Thermo Fisher, Waltham, MA), using automatic baseline settings and a threshold of 0.2. The comparative Ct method was used to calculate the relative mRNA expression. The Ct value of GAPDH was also measured and subtracted from the corresponding Ct value for PS1 gene to calculate the ΔCt value.

Calcein-acetoxymethyl (Calcein-AM) ester viability assay

The physiological significance of PS1 expression was assessed by determining the effect of γ-secretase inhibitor DAPT on cell viability. This approach is based on the rationale that PS1 is a core component of γ-secretase, and a lack of PS1 dramatically drops the enzymatic activity (De Strooper *et al.* 1998). Therefore, using DAPT allows determining whether PS1 affects cell viability through its γ-secretase function. The membrane-permeant Calcein-AM ester dye (Invitrogen, Carlsbad, CA) was used to measure cell viability. Calcein-AM is a fluorogenic esterase substrate that permeates live cells that have esterase activity and membranes. Once hydrolysis of Calcein-AM by intracellular esterases begins, it produces Calcein, a strongly fluorescent compound that is retained in the cell cytoplasm. Briefly, HT22 cells received the aforementioned ethanol program. DAPT (10 μM) was applied to the cells during EW. The cells were then treated with Calcein-AM for 30 minutes. Separately, HT22 cells were treated with glutamate (3 mM) for 24 hours with or without DAPT 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

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containing 2.5 µM 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. Cell culture wells treated with methanol served as blanks. For the imaging of cell viability, separate HT22 cells were treated with vehicle or ethanol as described above. At 4 hours of EW, cell culture plates were placed on the fluorescent microscope platform. The pictures of live cells with fluorescent color were taken at the magnification of 20X. Since cell lines often do not accurately replicate the signaling environment in primary tissues; we separately used cortical primary neurons for Calcein assay to ensure that the toxic effect of EW on HT22 cells is replicated in primary neurons. Briefly, primary neurons from day 14 embryos of Sprague-Dawley rats were cultured on cell plates at a density of 20,000-60,000 cells per well. The plates had been coated with poly-D-lysine. The neurons grew for 8 days in a Neurobasal Medium, supplemented with B27, 2mM L-glutamine, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and 10% FBS before experiment. Neurons were maintained at 37℃ in a humidified incubator containing 5% CO2. Some neurons were exposed to 50 or 100 mM ethanol for 20 hours and then ethanol solution was removed for 4 hours per cycle for two cycles. The neurons were then treated with Calcein for 30 min and measured for viability.

Glutamate concentrations

The total levels of extracellular and intracellular glutamate were measured using the fluorometric glutamate ELISA method (Abcam, Cambridge, United Kingdom) (Wakabayashi et al., 2014; Amrani et al., 2014). Briefly, cortical tissues from ethanol withdrawn or control diet rats were homogenized in 100 µl of assay buffer. The supernatant was extracted by centrifugation (4◦C, 13,000 rpm, 5 min). Perchloric acid was added to remove any interfering proteins. Samples and reaction reagents were then pipetted into a 96-well plate, and incubated at 37◦C for 30 min. Fluorescence intensity was measured at 450 nm on Chromate absorbance plate reader. The glutamate concentrations were determined based on the protein content measured using the bicinchoninic acid assay (Thermo Scientific, Waltham, MA). Separately,

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HT22 cells at the end of the EW paradigm were washed with cold PBS and suspended in cold assay buffer. Cells were homogenized via a pipette and centrifuged (4°C, 13,000 rpm, 5 min) to remove insoluble material. Supernatant was collected, transferred to a tube in ice, and then plated in 96-well clear microplate for fluorescence intensity on a Chromate plate reader.

Data and statistical analysis

All numerical data are expressed as mean ± standard error of mean (SEM). For two group comparisons, we used Student t-test. For three group comparisons with one factor and four group comparisons with two factors, we used one-way and two-way ANOVA, respectively followed by Tukey post hoc analysis. For immunoblot data, each assay was repeated three or four times using multiple samples (N = 3 - 7). Data with the clearest band images were selected for statistical analysis and presented in figure sections. The results of cell viability, obtained in relative fluorescent units, were expressed as the percentage data relative to non-ethanol, control media values at 100%. P value was set less than 0.05 to indicate a statistically significant difference between groups.

Results

Ethanol consumption, blood ethanol concentrations, and body weight.

In general, rats drink the small amount of ethanol during the first week because they are learning the taste of an ethanol diet. Figure 2A shows the daily ethanol intakes that were normalized to body weights (Figure 2B) during each diet cycle. There were no significant differences in ethanol consumption or body weights between the EW and ethanol-consuming groups. Blood ethanol concentrations (Figure 2C) were 1.02 ± 0.06 mg/ml in the EW groups and 0.99 ± 0.04 mg/ml in the ethanol-consuming groups. Blood ethanol was not detected in the dextrin group.

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EW increases PS1 and p38α in the prefrontal cortex of rats.

Compared to control diet rats, rats in the EW ($p < 0.0001$) and ethanol-consuming ($p < 0.001$) groups showed an increase in PS1 protein [$F(2, 23) = 26.39$, $p < 0.0001$] (Figure 3A). PS1 protein level tended to be higher in the EW group than the ethanol-consuming group, but the difference was not statistically significant. Ethanol withdrawn rats also showed an increase in mRNA ($t = 10.08$, $df = 12$, $p < 0.0001$) level of PS1 compared to control diet rats (Figure 3B). The increase in PS1 in ethanol withdrawn rats concurred with an increase in p38α protein compared to control diet rats ($t = 6.761$, $df = 10$, $p = 0.0084$) (Figure 3C). The increasing effect of EW on p38α in prefrontal cortex is consistent with our previous observation that EW increases p38α in cerebellum (Jung *et al.* 2011; Ju *et al.* 2012). These results raise a possibility that EW-induced PS1 expression may depend on p38α.

Glutamate mediates PS1 and p38α expression in ethanol withdrawn HT22 cells.

Glutamate is a major excitatory molecule that is known to be up-regulated upon EW stress. Given this, we hypothesized that glutamate mediates the increase in PS1 and p38α in EW rats. We tested this hypothesis using a glutamate receptor antagonist (MK-801) or glutamate itself. We selected MK-801, an antagonist of subtype [N-methyl-D-aspartate receptor (NMDA)] of glutamate receptors because EW-associated toxicity is often attributed to the excessive stimulation of NMDA receptors (Morgan *et al.* 1992; Hoffman and Tabakoff, 1994). When HT22 cells were treated with MK-801 during EW, they showed a lower level of PS1 ($p = 0.043$) [$F(1, 21) = 6.27$, $p = 0.01$ by a factor of EW; $F(1, 21) = 7.647$, $p = 0.0116$ by a factor of MK-801] and p38α ($p < 0.0001$) [$F(1, 24) = 134.1$, $p < 0.0001$ by a factor of EW; $F(1, 24) = 29.33$, $p < 0.0001$ by a factor of MK-801] compared to EW cells treated with vehicle (Figure 4A, 4B). MK-801 treatment to ethanol-free HT22 cells did not significantly alter the level of PS1 or p38α (Figure 4C, 4D). These data indicate that glutamate mediates the increased expression of PS1 and p38α during EW stress. If glutamate mediates EW effects on PS1 or p38α, glutamate treatment alone may resemble such effects of EW. We thus treated ethanol-free HT22 cells with glutamate for 24 hours.

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We observed a profound increase in PS1 ($t = 18.81$, $df = 15$, $p < 0.0001$) and p38α ($t = 6.345$, $df = 10$, $p < 0.0001$) protein expression in glutamate-treated HT22 cells compared to vehicle-treated control cells (Figure 4E, 4F). We next determined whether EW or glutamate increases glutamate production in cortical tissues and HT22 cells. We found that compared to control conditions, the total level of glutamate (intracellular and extracellular) concentrations were elevated in ethanol withdrawn cortical tissues ($t = 3.53$, $df = 14$, $p = 0.0033$) and HT22 cells ($t = 5.65$, $df = 6$, $p = 0.0013$) (Figure 4G, 4H). These results suggest that glutamate mediates the upregulation of PS1and p38α in response to EW stress.

EW or glutamate increases PS1 expression through p38α in HT22 cells.

The concurrence of PS1 and p38α in EW cells or glutamate-treated cells led us to explore whether PS1 is mechanistically linked to p38α in response to the excitotoxic effect of EW. To dissect the PS1- p38α link, we applied p38α inhibitor SB203580 (1 μM) to HT22 cells during each of the EW phases. Separately, we exposed ethanol-free cells to glutamate with or without cotreatment with p38α inhibitor SB203580. This cotreatment significantly lowered PS1 expression in both EW cells ($p = 0.015$) (Figure 5A, $F (1, 25) = 209$, $p < 0.0001$ by a factor of EW; $F (1, 25) = 12$, $p = 0.0016$ by a factor of SB203580, no interaction between two factors) and glutamate-treated cells ($p = 0.0043$) [Figure 5B, $F (1, 15) = 20.80$, $p = 0.0004$ by a factor of glutamate; $F (1, 15) = 8.178$, $p = 0.0119$ by a factor of SB203580, no interaction between two factors]. Unexpectedly, SB203580 treatment to control cells appeared to increase PS1 level compared to vehicle-treated control cells ($p < 0.05$). Nevertheless, the decreasing effect of SB203580 on PS1 during EW supports the idea that PS1 expression is a p38α-dependent molecular activity associated with the hyperactive stimulus nature of EW.

EW or glutamate increase NICD and HES1.

We further characterized a down-stream molecular activity that is triggered by PS1 during EW in rats. Notch is a transmembrane receptor family that mediates cell to cell communication and cleaved by the γ-

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secretase activity of PS1. Upon the cleavage, the intracellular domain of Notch receptors enters the nucleus and activates a transcription factor HES1, which is thought to regulate gene expression (reviewed by Tandon and Fraser 2002). Since PS1 expression is increased in EW rats (Figure 2A), it is reasonable to speculate that the expressions of NICD and HES1 are also increased in EW rats. We indeed observed that ethanol withdrawn rats showed an increase in both NICD ($t = 4.709$, $df = 10$, $p < 0.001$) and HES1 ($t = 3.932$, $df = 22$, $p = 0.007$) (Figure 6A, 6B). This effect of EW was mimicked by glutamate such that glutamate treatment to ethanol-free cells increased NICD ($p < 0.0001$) and HES1 ($p < 0.005$) expression (Figure 6C, 6D). The glutamate-induced increase in NICD ($p = 0.0056$) and HES1 ($p = 0.009$) was attenuated by a cotreatment with p38α inhibitor SB203580 (Figure 6C, 6D) [$F (2, 20) = 3.9$, $p = 0.06$ for NICD; $F (2, 20) = 27$, $p < 0.0001$ for HES1]. The glutamate-induced increase in NICD ($p = 0.0135$) and HES1 ($p < 0.0001$) was also attenuated by a cotreatment with γ-secretase inhibitor DAPT (Figure 6E, 6F) [$F (2, 21) = 6.107$, $p = 0.0081$ for NICD; $F (2, 21) = 13.28$, $p = 0.0002$ for HES1]. These results suggest that PS1-mediated Notch signaling molecules (NICD and HES1) may be involved in the excitotoxicity of EW in a manner that depends on p38α or γ-secretase.

γ-secretase inhibitor reduces the excitotoxic effects of EW or glutamate.

PS1 is a main component of γ-secretase, and thus, we determined the physiological significance of PS1 over-expression by measuring the effect of γ-secretase inhibitor DAPT on cell viability. Compared to control HT22 cells, EW cells showed a lower viability ($p < 0.0001$) in a manner that was ameliorated by DAPT ($p = 0.0003$) treatment (Figure 7A) [$F (1, 58) = 20.00$, $p < 0.0001$ as a factor of EW; $F (1, 58) = 12.41$, $p = 0.0008$ as a factor of DAPT]. In the microscopic examination of cell viability (Figure 7D), the EW cell group showed much less green fluorescent color compared to the groups of control, EW with DAPT, and control with DAPT. Only intact cells can hydrolyze Calcein-AM by intracellular esterases, producing Calcein a strongly fluorescent compound, indicating that EW-induced cytotoxicity is attenuated by DAPT treatment. Similarly to the case for EW, glutamate decreased cell viability ($p = 0.0006$) in a

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manner that was ameliorated by DAPT ($p = 0.045$) cotreatment (Figure 7B) [$F (1, 58) = 19.15$, $p < 0.0001$ as a factor of glutamate; $F (1, 58) = 11.7$, $p < 0.0001$ as a factor of DAPT]. We finally assessed whether EW-induced HT22 cell death is reproduced in cortical primary neurons. We observed that EW inhibited the viability of the primary neurons [$F (2, 12) = 146.1$, $p < 0.0001$]. Thus, 77 ± 7% and 57.6 ± 1.7% of cells survived from EW after exposure to 50 mM ($p < 0.001$) and 100 mM ($p < 0.0001$) of ethanol, respectively (Figure 7C). These results indicate that the overexpression of PS1 may contribute to excitotoxic cell damage through its γ-secretase function.

Discussion

The key finding of the current study is that EW provokes PS1 upregulation through p38α activation in a manner mediated by glutamate. Furthermore, a γ-secretase inhibitor ameliorates the toxic effect of EW or glutamate. These observations provide first empirical evidence that PS1 contributes to the excitotoxic stress associated with EW.

PS1 is at the catalytic core of γ-secretase that catalyzes neurotoxic Aβ peptide (Aβ42) production. PS1 exerts multifunctional effects on brain through interacting with signaling and apoptotic proteins (Ni *et al.* 2001; De Strooper and Annaert 2001). The homeostatic level of the interacting proteins is essential for cellular survival. Therefore, the aberrant or over-expression of PS1 and its interactive proteins may endanger cell survival. JNK (MAPK) inhibition repressed PS1 transcription (Das *et al.* 2012), suggesting that JNK-MAPK interacts with PS1. p38 is another MAPK activated by stress such as inflammatory cytokines and heat stress. Our earlier study where p38α expression was increased by EW (Jung *et al.* 2011) prompted us to test whether EW increases PS1 as a mediator of excitotoxicity. We observed that EW increased PS1 protein and mRNA level, accompanied by p38α increase. The concurrence of p38α and PS1 suggests that these two proteins are connected to the excitotoxic pathway.

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We found a consistent tendency that an increase in PS1 is more prominent under EW than ethanol-exposure. EW is hyperexcitatory in nature, and thus, the PS1 increase may reflect the role of PS1 in hyperexcitatory stress. Notice that the brains of ethanol withdrawn rats were collected three weeks after ethanol removal. Therefore, the PS1 upregulation may be involved in EW mechanisms rather than the residual effect of ethanol-exposure. The upregulation of excitatory molecule glutamate is a well-established mechanism mediating the hyperexcitatory nature of EW (Prendergast *et al.* 2004). Glutamate binds to metabotropic or ionotropic glutamate receptors that are coupled with intracellular G proteins or form an ionic channel, respectively. In many cases, EW-associated toxicity is associated with the excessive stimulation of the N-methyl-d-aspartate (NMDA) receptor, an ionotropic glutamate receptor (Morgan *et al.* 1992; Hoffman and Tabakoff 1994; Thomas et al., 1998). When cells were treated with a NMDA antagonist (MK-801) during EW, both PS1 and p38α expressions were decreased. These results substantiate that glutamate triggers EW-induced PS1and p38α expression. Should glutamate mediate the PS1 increase in ethanol withdrawn rats, glutamate treatment to ethanol-free cells would produce a similar effect to EW. As expected, there was a substantial increase in PS1 and p38α in ethanol-free cells treated with glutamate. One can hence argue that the over-expression of PS1 accompanied by p38α during EW is an excitatory molecular activity triggered by glutamate. In fact, the role of p38 in glutamate toxicity has been reported by Kawasaki et al (1997). They observed that glutamate-treated cerebellar granule cells underwent apoptosis in a manner that was attenuated by a p38α inhibitor.

The increase in PS1-mRNA in ethanol withdrawn rats suggests that EW affects PS1 at the level of transcription. It is also possible that EW affects PS1 through other proteins. The stability of presenilin proteins depends on its post-translational modification by phosphorylation (Walter *et al.* 1999; Fluhrer *et al.* 2004; Massey *et al.* 2004). Being a kinase, p38α may phosphorylate, stabilize, and increase the level of PS1 available for mediating down-stream molecular activities. Such functional interaction between p38α and PS1 appears to be evoked by the hyperactivity of neurons as seen in our results that p38α

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inhibitor attenuates the increasing effect of EW or glutamate on PS1. A puzzling result of our study is that p38α inhibitor treatment to control cells appeared to increase PS1 level. This is opposite to the decreasing effect of a p38α inhibitor on PS1 in EW/glutamate-treated cells. A potential explanation is that a p38α inhibitor treatment to control cells lowers a p38α below a normal level. Cells may sense that this level of p38α is too low to maintain the physiological level of PS1, resulting in a rebound increase in PS1. PS1-p38 link is supported by a different model of CNS pathology. Excessive Aβ peptide imposed stress to actin fibers through p38α activation (Song *et al.* 2002). p38α was significantly activated in the cortex of demented mice aged 7 or 12 months (Savage *et al.* 2002), accompanied by Aβ peptide deposition (Savage *et al.* 2002). Since PS1 mediates Aβ production, these studies provide an indirect evidence of p38α-PS1 link. Mice carrying mutated PS1 genes exhibit a high level of phosphorylated-p38α in the hippocampus, further supporting PS1-p38 link (Giraldo *et al.*, 2014). A presenilin homologue induces apoptosis in a manner blocked by p38α inhibition (Sun *et al.* 2001), suggesting that p38α and presenilin interaction mediates the reduced cell viability.

PS1 cleaves many transmembrane proteins, and later these cleaved fragments are transported to the nucleus where they interact with transcription factors. This signaling pathway regulates the transcription of crucial genes for neuronal survival and function. Notch receptors (Notch 1-4) are transmembrane proteins that enable the Notch pathway to transduce signals between neighboring cells. Notch signaling mediates different intercellular communications that are essential for determining neuronal fates during development and in the adult. PS1 as a component of γ-secretase cleaves the intracellular domain of the Notch receptors, releasing NICD into the cytoplasm. Thereafter, NICD translocates to the nucleus where it forms an active transcriptional complex with the DNA binding protein, activating transcription factors (Weinmaster 2000). Among the NICD-inducible transcription factors, the best defined are HES1 families of transcriptional regulators that mediate the downstream responses of Notch signaling. Given this scenario, one can expect that PS1-evoking stress in turn increases NICD and then HES1. Although this

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was expected, it was amazing to see that ethanol withdrawn rats show a robust increase in NICD and HES1. This effect of EW was reproduced by glutamate treatment to ethanol-free cells but not in the presence of a p38α inhibitor or a γ-secretase inhibitor. These results suggest that the increase in NICD and HES1 is associated with excitotoxicity in a manner that depends on p38α or γ-secretase.

Our previous study has shown that a p38α inhibitor treatment attenuates the toxic effect of EW on HT22 cells (Jung *et al.* 2011), demonstrating that the excessive level of p38α is a part of a cell death pathway. PS1 has also been implicated in cell death: the deletion of PS1 in mouse embryonic cells mitigated the cytotoxicity of a DNA damaging reagent (Song *et al.* 2013). This phenotype was reversed by re-expression of PS1 (Song *et al.* 2013), which leads to the assumption that PS1 contributes to the dell death. Being a main component of γ-secretase, the reduced cell viability associated with PS1 may be attributed to γ-secretase. Li *et al.* (2011) have demonstrated that the over-expression of normal or mutant PS1 is sufficient to increase the amount and activity of γ-secretase. A high level of intracellular Ca^{2+} triggered hippocampal cell deaths in a manner that was attenuated by a γ-secretase inhibitor (Choi *et al.* 2010), suggesting that γ-secretase mediates the cell death. A single treatment with a γ-secretase inhibitor reduced brain damage associated with ischemia/reperfusion in mice (Arumugam *et al.* 2006). Arumugam *et al.* (2006) proposed that a high level of γ-secretase increases cellular vulnerability to apoptosis, thereby resulting in cell death. Our results are in accordance with these studies in that γ-secretase inhibitor treatment allowed more cells to survive from the toxic effect of EW or glutamate. Notice that DAPT was applied to cells during EW after ethanol was removed. The cellular protection achieved by DAPT treatment during EW raises an important mechanistic point that PS1 through its γ-secretase function may mediate excitotoxicity when the level of PS1 is increased beyond a physiological level. As mentioned earlier, the γ-secretase activity of PS1 releases NICD (De Strooper *et al.* 1999) which in turn activates HES1. It is hence conceivable that cell death seen in our study is also attributed to NICD and HES1 activation.

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We finally assessed glutamate concentrations under EW conditions. Compared to control conditions, glutamate concentrations were increased in the brain tissues and HT22 cells collected three weeks and four hours after ethanol removal, respectively. This timing difference can be a limitation of this study. We selected this time based on our preliminary observation that PS1 level was significantly increased in brain tissues and HT22 cells under these timing conditions. In addition, we measured a total level of glutamate including extracellular and intracellular glutamate, and thus, our data on glutamate level can provide only indirect evidence of glutamate elevation. Further, we were unable to assess the physiological relevance of the glutamate concentrations used with exogenous glutamate application. Nevertheless, the increase in glutamate concentrations under our *in vivo* and *in vitro* EW condition raises a possibility that repeated EW results in the prolonged accumulation of glutamate, which may permanently alter signaling pathways.

In conclusion, our studies highlight that the excitotoxic effect of EW involves the sequential or concerted molecular activities of glutamate, p38α, PS1, and PS1's down-stream molecules (Figure 8). These observations may provide a new mechanistic insight into understanding neurochemistry underlying excitotoxic brain disorders like EW.

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Authorship Contribution:

Participated in research design: Jung and Das

Conducted experiments: Metzger and Das

Performed data analysis: Jung

Wrote or contributed to the writing of the manuscript: Jung, Das, and Metzger

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Figure legends

Figure 1. A schematic diagram of EW or glutamate treatment. The EW paradigm was applied to rats and HT22 cells. Male rats received a control or an ethanol diet for four weeks followed by withdrawal for three weeks per cycle for two cycles. Next morning, the prefrontal cortex was collected for the measurements of PS1, p38α, NICD, and HES1. For the *in vitro* EW paradigm, HT22 cells were exposed to ethanol (0 or 100 mM) for 20 hours and withdrawn for four hours per cycle for two cycles. Vehicle solution and glutamate antagonist MK-801, p38α inhibitor SB203580, or γ-secretase inhibitor DAPT was applied to cells during each of 4-hour-EW phases. The cells were then subjected to the measurements of PS1, p38α, NICD, HES1, or cell viability. For glutamate paradigm, HT22 cells were exposed to glutamate for 24 hours with or without SB203580 or DAPT cotreatment. The cells were then subjected to the measurements of PS1, p38α, NICD, HES1, or cell viability.

Figure 2. Ethanol consumption, blood ethanol concentrations, and body weight. Male rats aged 3 months received an ethanol diet (7.5% v/v ethanol) for four weeks followed by withdrawal for three weeks per cycle for two cycles. Dextrin replaced ethanol for a control diet. Ethanol consumption (N = 7 rats/group) was measured once a day, and body weight (N = 7 rats/group) was measured twice a week. Data are the average of weekly ethanol consumption or body weights. Blood ethanol (N = 3 rats/group) was measured using colorimetric assay at the time of euthanasia. There were no significant differences in ethanol consumption, body weights, or blood ethanol concentrations between the EW and the ethanol-consuming groups.

Figure 3. Effects of EW on PS1 and p38α in rats. Male rats aged 3 months received an ethanol diet (7.5% v/v ethanol) for four weeks followed by withdrawal for three weeks per cycle for two cycles. Dextrin (control) replaced ethanol for a control diet. Next morning, rats in the EW group were humanely

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sacrificed and the prefrontal cortex was collected to measure PS1 protein (3A), PS1 mRNA level (3B), and p38α protein (3C). Rats in the ethanol-consuming group received an ethanol diet continuously and were sacrificed at the end of ethanol-exposure while they were intoxicated (3A). β-actin was measured as a loading control for all proteins. Compared to control diet rats, all of PS1 protein (**$p < 0.0001$), PS1 mRNA (*$p < 0.0001$), and p38α protein (*$p = 0.0084$) levels were elevated in EW rats. The level of PS1 protein was also elevated in ethanol-consuming rats (*$p < 0.001$). *, ** vs. control. N = 3 - 7 rats/group.

Figure 4. Effects of glutamate antagonist MK-801 or glutamate on PS1 and p38α.

HT22 cells were exposed to ethanol (0 or 100 mM) for 20 hours and withdrawn for four hours per cycle for two cycles. MK-801 (1 μM) was applied to cells during each of 4-hour-EW phases. The cells were then collected and proteins were isolated to measure PS1 and p38α protein using the immunoblot method. β-actin was used for a loading control. MK-801 treatment significantly lowered PS1 (*$p = 0.043$) and p38α (*$p < 0.0001$) protein in EW cells (4A, 4B) but not in control cells (4C, 4D). Separately, HT22 cells were exposed to glutamate (3 mM) for 24 hours. The cells were then collected and proteins were isolated to measure PS1 and p38α protein using the immunoblot method. Compared to control cells, glutamate-treated cells show a substantial increase in the level of PS1 protein (*$p < 0.0001$) and p38α protein (*$p < 0.0001$) (4E, 4F). *vs. control or EW + MK-801. N = 4 cell culture plates/group. Separately, glutamate concentrations were measured in the aforementioned ethanol withdrawn cortical tissues and HT22 cells using the fluorometric ELISA. The total level of glutamate (intracellular and extracellular) concentrations were elevated in ethanol withdrawn cortical tissues (*$p = 0.0033$) and HT22 cells (*$p = 0.0013$) (Figure 4G, 4H). N= 4 rats or 7 wells/group.

Figure 5. Effects of p38α inhibitor SB203580 on PS1. HT22 cells were exposed to ethanol (0 or 100 mM) for 20 hours and withdrawn for four hours per cycle for two cycles. SB203580 (1 μM) was applied to cells during each of 4-hour-EW phases. Separately, HT22 cells were exposed to glutamate (3 mM) for

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24 hours with or without cotreatment with SB203580 (1 μM). Cells were then collected and proteins were isolated to measure PS1 protein using the immunoblot method. β-actin was used for a loading control. EW (*p = 0.0001, 5A) or glutamate (*p = 0.0018, 5B) treatment increased PS1 expression in a manner that was attenuated by SB203580 cotreatment. †p = 0.015 vs. EW, †p = 0.0043 vs. Glutamate. N = 4 cell culture plates/group. Some statistical symbols are omitted for figure clarity.

Figure 6. Effects of EW or glutamate on NICD and HES1. Male rats aged 3 months received an ethanol diet (7.5% v/v ethanol) for four weeks followed by withdrawal for three weeks per cycle for two cycles. Dextrin replaced ethanol for a control diet. At the end of the EW paradigm, rats were humanely sacrificed and the prefrontal cortex was collected to measure NICD and HES1 protein (6A, 6B) using the immunoblot method. β-actin was measured as a loading control. Compared to control diet rats, both NICD (*p < 0.001) and HES1 (*p = 0.007) (6A, 6B) levels were elevated in EW rats. *vs. control. N = 3 or 4 rats/group for this figure. Separately, HT22 cells were exposed to glutamate (3 mM) for 24 hours with or without cotreatment with p38α inhibitor SB203580 (1 μM) or γ-secretase inhibitor DAPT (10 μM). The cells were then collected and proteins were isolated to detect NICD and HES1 protein using the immunoblot method. β-actin was used for a loading control. Glutamate treatment resulted in an increase in NICD (*p < 0.0001, 6C) and HES1 (*p < 0.005, 6D). SB203580 treatment significantly lowered both NICD (†p = 0.0056) and HES1 (†p = 0.009) protein levels in glutamate-treated cells (6C, 6D). DAPT cotreatment with glutamate also lowered NICD (†p = 0.0135) and HES1 (†p < 0.0001) expression (6E, 6F). N = 4 cell culture plates/group. Some statistical symbols are omitted for figure clarity.

Figure 7. Effects of γ-secretase inhibitor DAPT on cell viability. HT22 cells were exposed to ethanol (0 or 100 mM) for 20 hours and withdrawn for four hours per cycle for two cycles. γ-secretase inhibitor DAPT (10 μM) was applied to HT22 cells during each of 4-hour-EW phases. At the end of the EW paradigm, cell viability was assessed using Calcein-AM assay. Separately, HT22 cells were exposed to

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glutamate (3 mM) for 24 hours with or without DAPT cotreatment, and then tested for cell viability. Compared to control cells, the cell viability was reduced under the condition of EW ($p < 0.0001$, 7A) or glutamate ($p = 0.0006$, 7B) in a manner that was mitigated by DAPT treatment. The microscopic examination of cell viability (Figure 7D) showed a strong green fluorescent color in control cells with or without DAPT treatment. By comparison, the fluorescent color is much less visible in the EW cell group, a phenotype reversed by DAPT treatment to EW cells. *vs. control. †vs. EW ($p = 0.0003$) or glutamate ($p = 0.045$). Some statistical symbols are omitted for figure clarity. Figure C shows the effect of EW on the viability of cortical primary neurons that received the aforementioned EW paradigm. Like HT22 cells, EW reduces the viability of cortical primary neurons. *$p < 0.001$ and **$p < 0.0001$ vs. control (0 mM ethanol).

Figure 8. The hypothetical pathway of the excitotoxic effects of EW. In this study, EW as well as glutamate treatment to ethanol-free cells increased p38α, PS1, NICD, and HES1 expression. MK-801 (a glutamate antagonist) treatment inhibited EW-induced increase in p38α and PS1, suggesting that glutamate mediates the p38α and PS1 expression. SB203580 (a p38α inhibitor) treatment inhibited EW/glutamate-induced PS1 expression, suggesting that p38α mediates the PS1 expression. EW provoked an increase in NICD and HES1 expression. This effect of EW was reproduced by glutamate treatment to ethanol-free cells, a phenomenon that was attenuated by SB203580 or DAPT (γ-secretase inhibitor). DAPT treatment ameliorated EW/glutamate-induced reduced cell viability, suggesting that the γ-secretase function of PS1 mediates the cell death. These data suggest that the sequential or concerted activities of p38α, PS1, NICD, and HES1 may mediate excitotoxic stress involving glutamate in response to EW.

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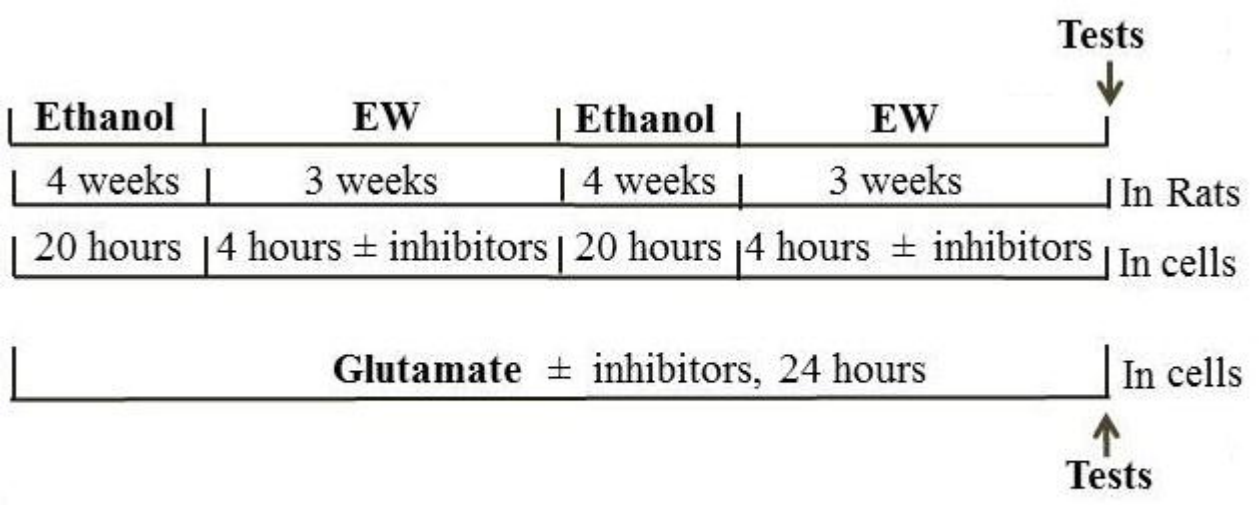
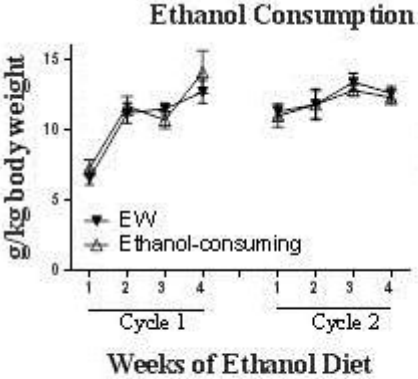

Tests
Ethanol
EW
Ethanol
EW
4 weeks
3 weeks
4 weeks
3 weeks
In Rats
20 hours
4 hours ± inhibitors
20 hours
4 hours ± inhibitors
In cells
Glutamate ± inhibitors, 24 hours
In cells
Tests

Figure 1



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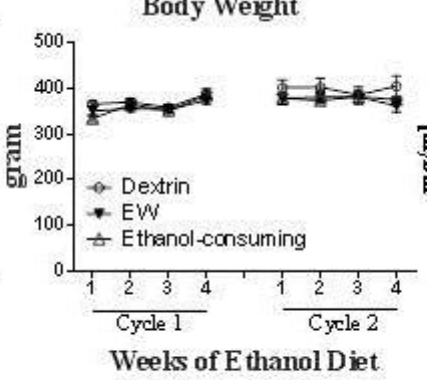

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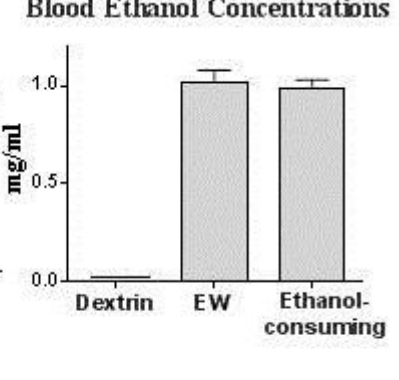
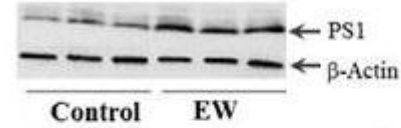
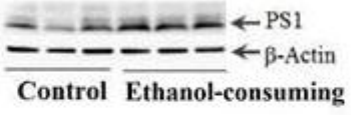
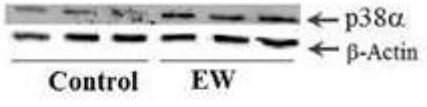
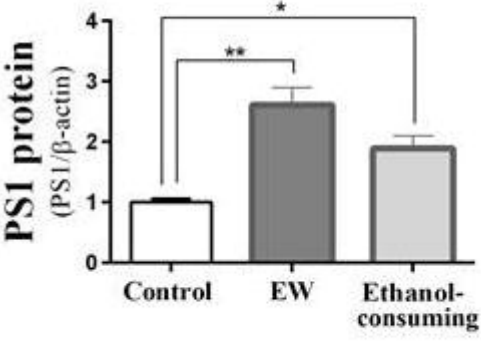


Figure 2C









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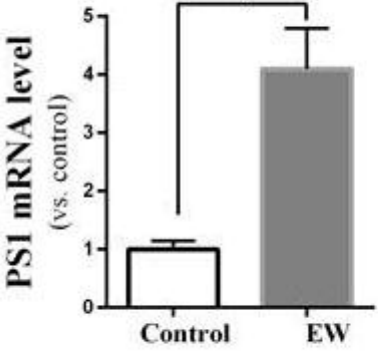

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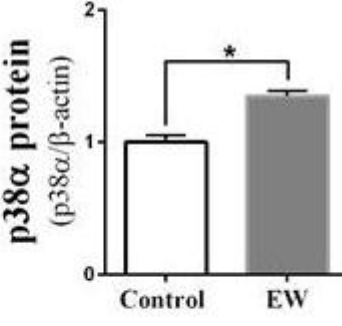

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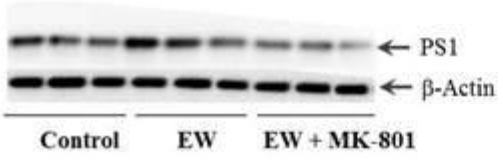

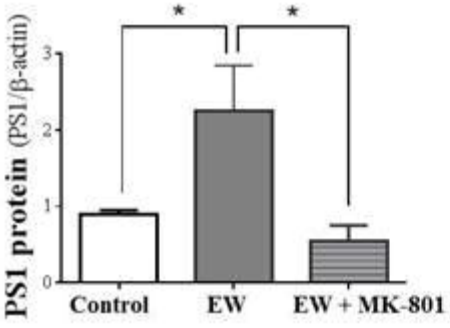

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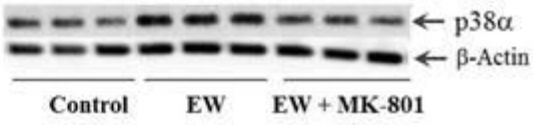

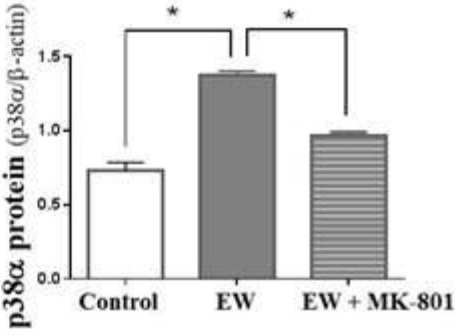

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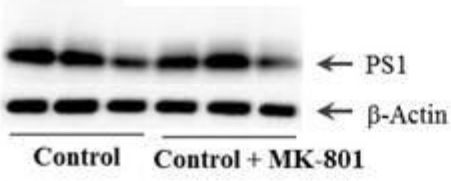

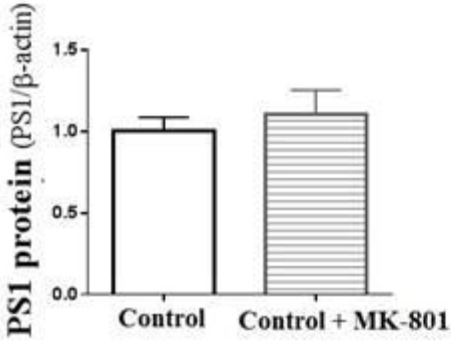

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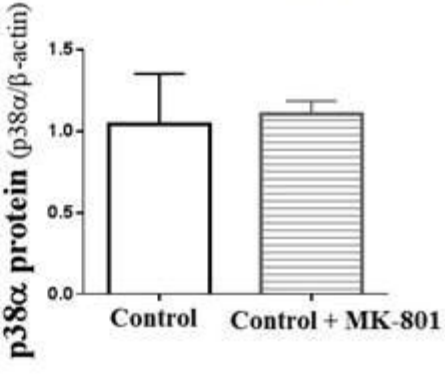

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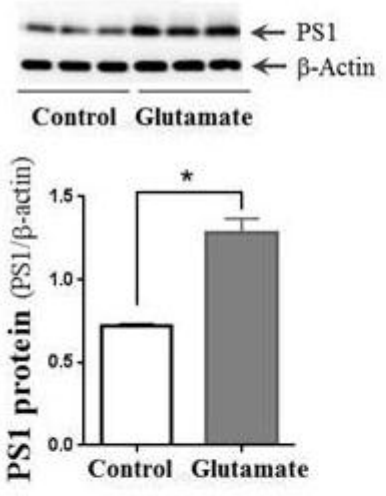

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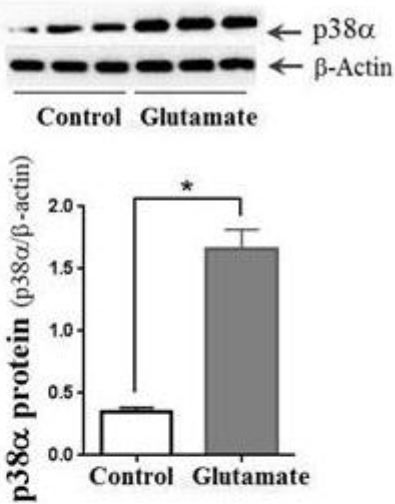

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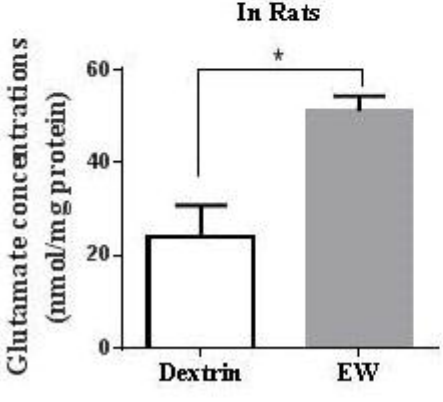

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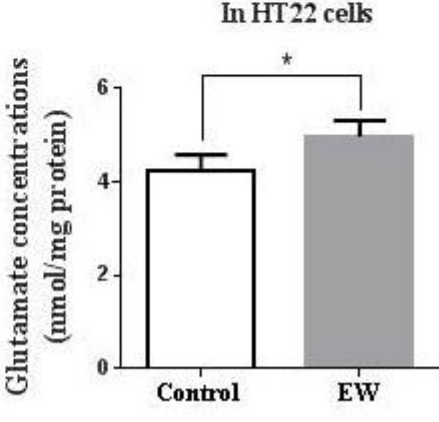

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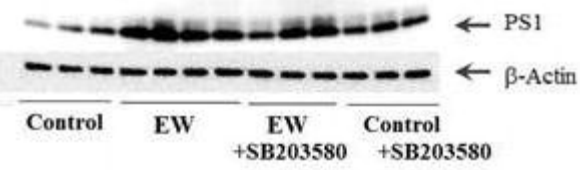

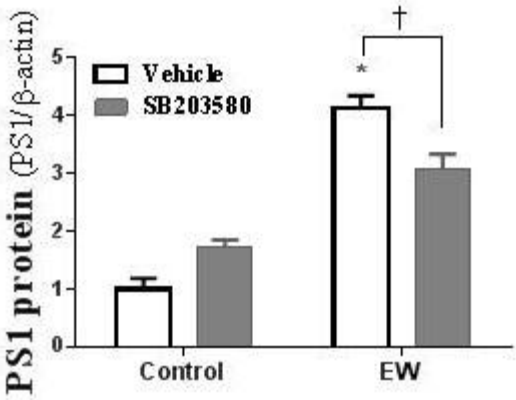

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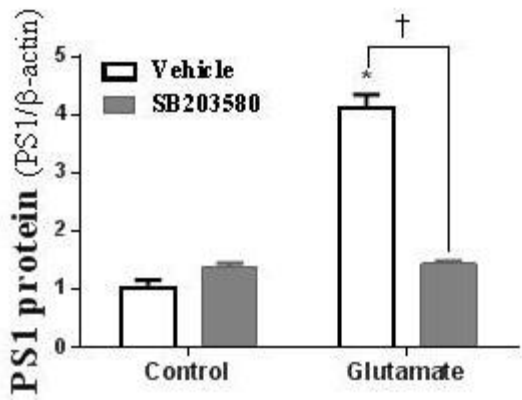

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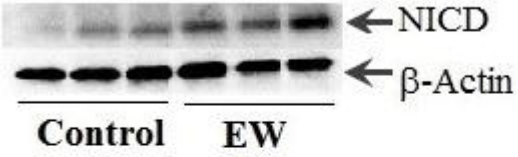

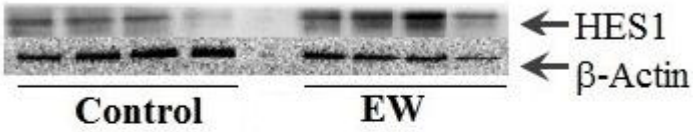

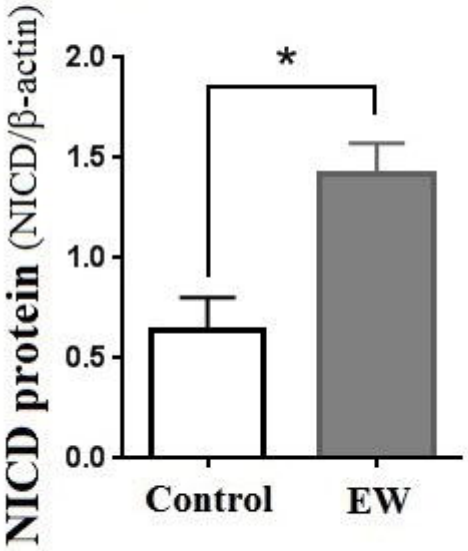

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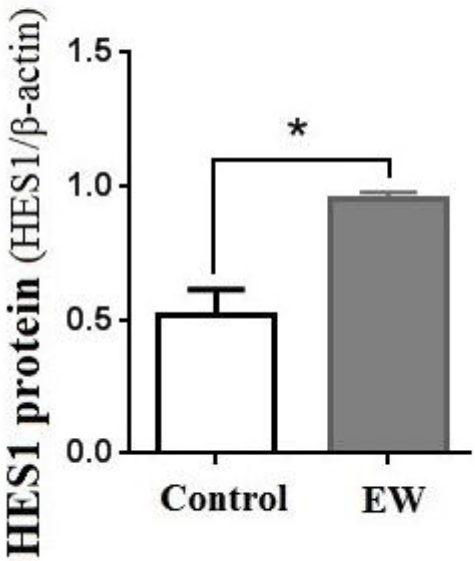

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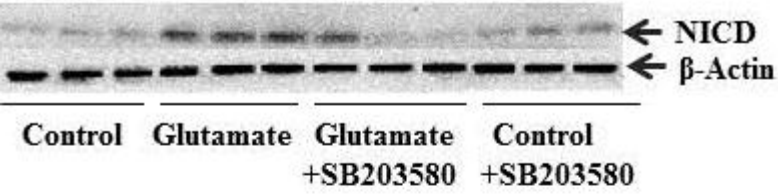

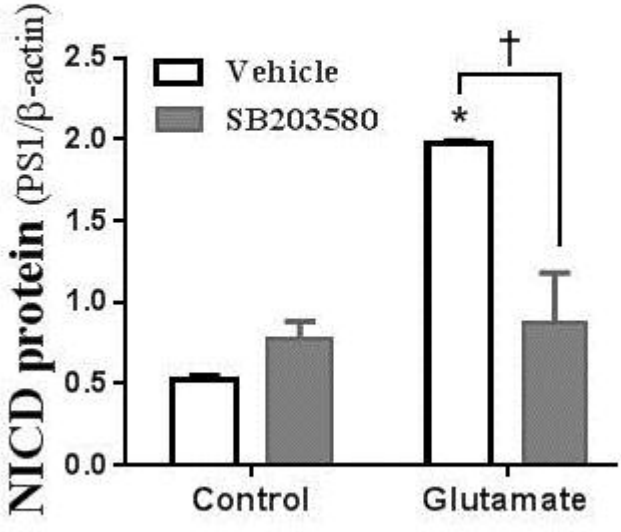

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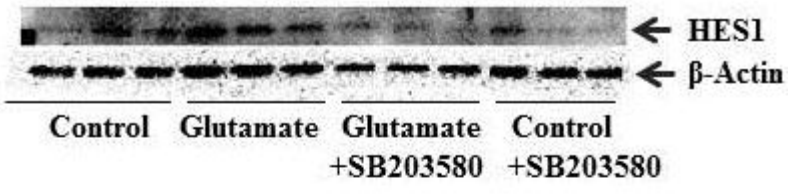

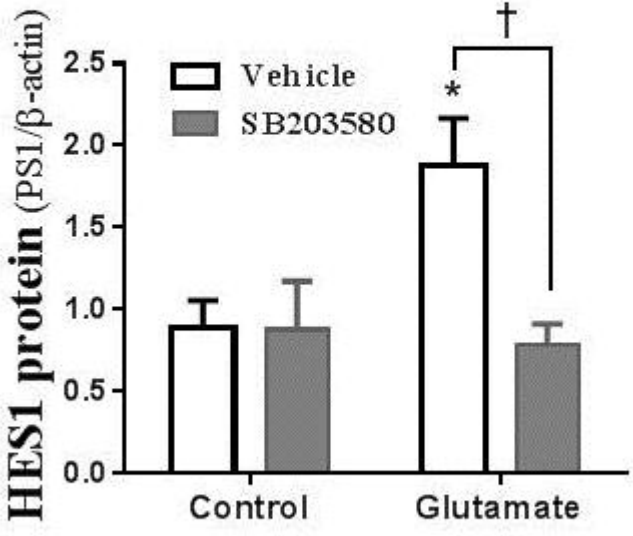

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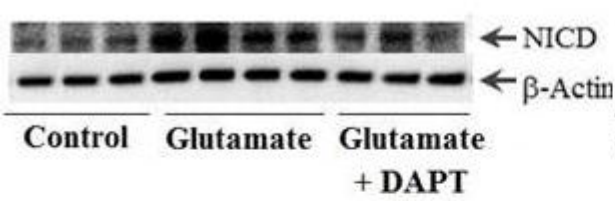
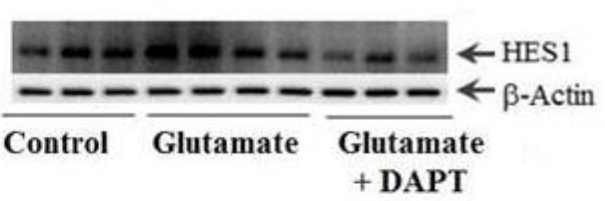
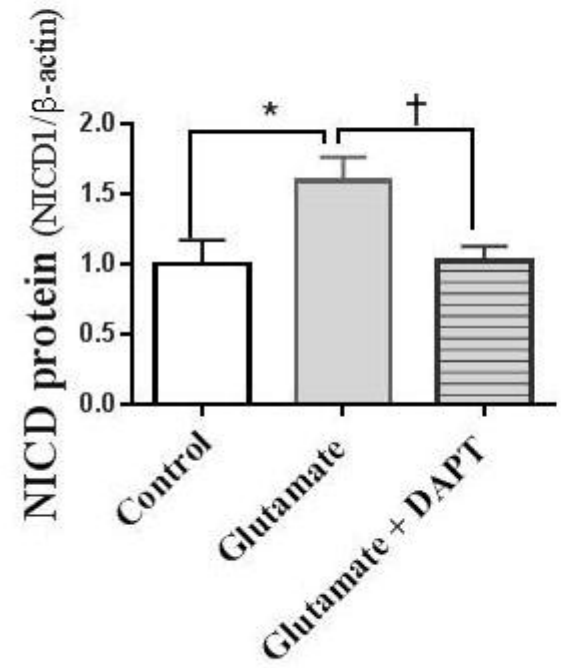






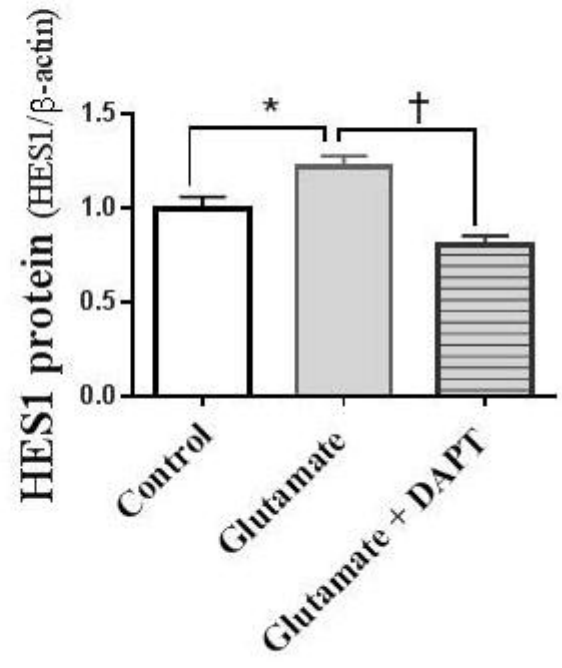
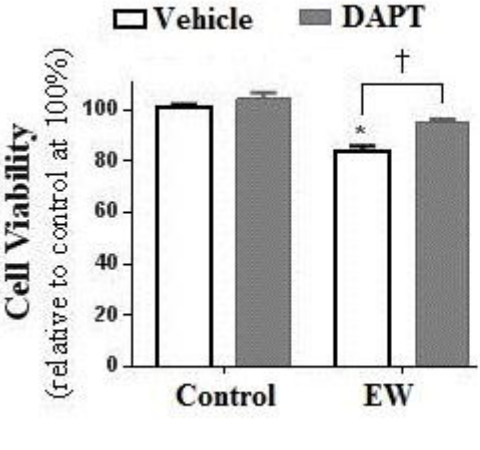


Figure 6E

Figure 6F



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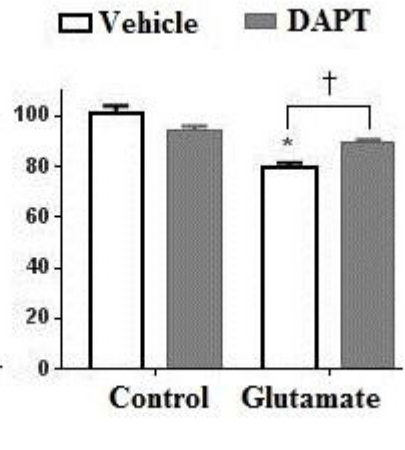

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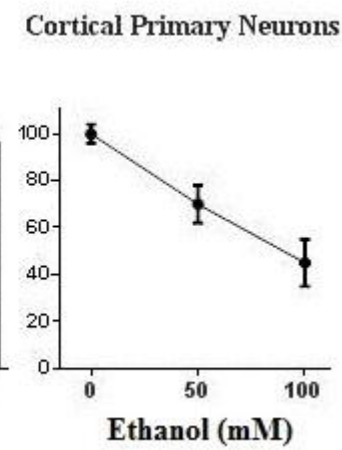

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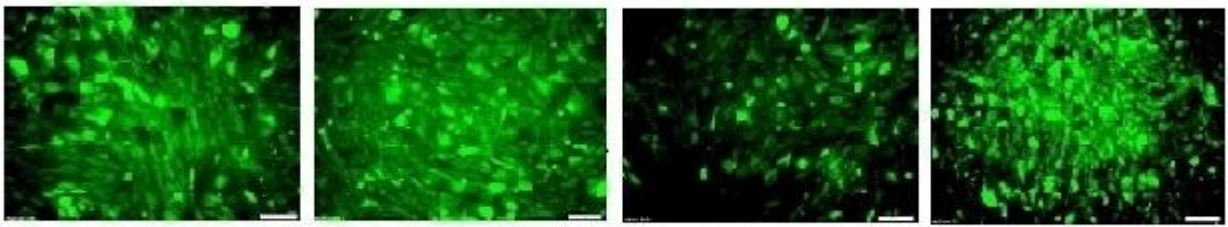

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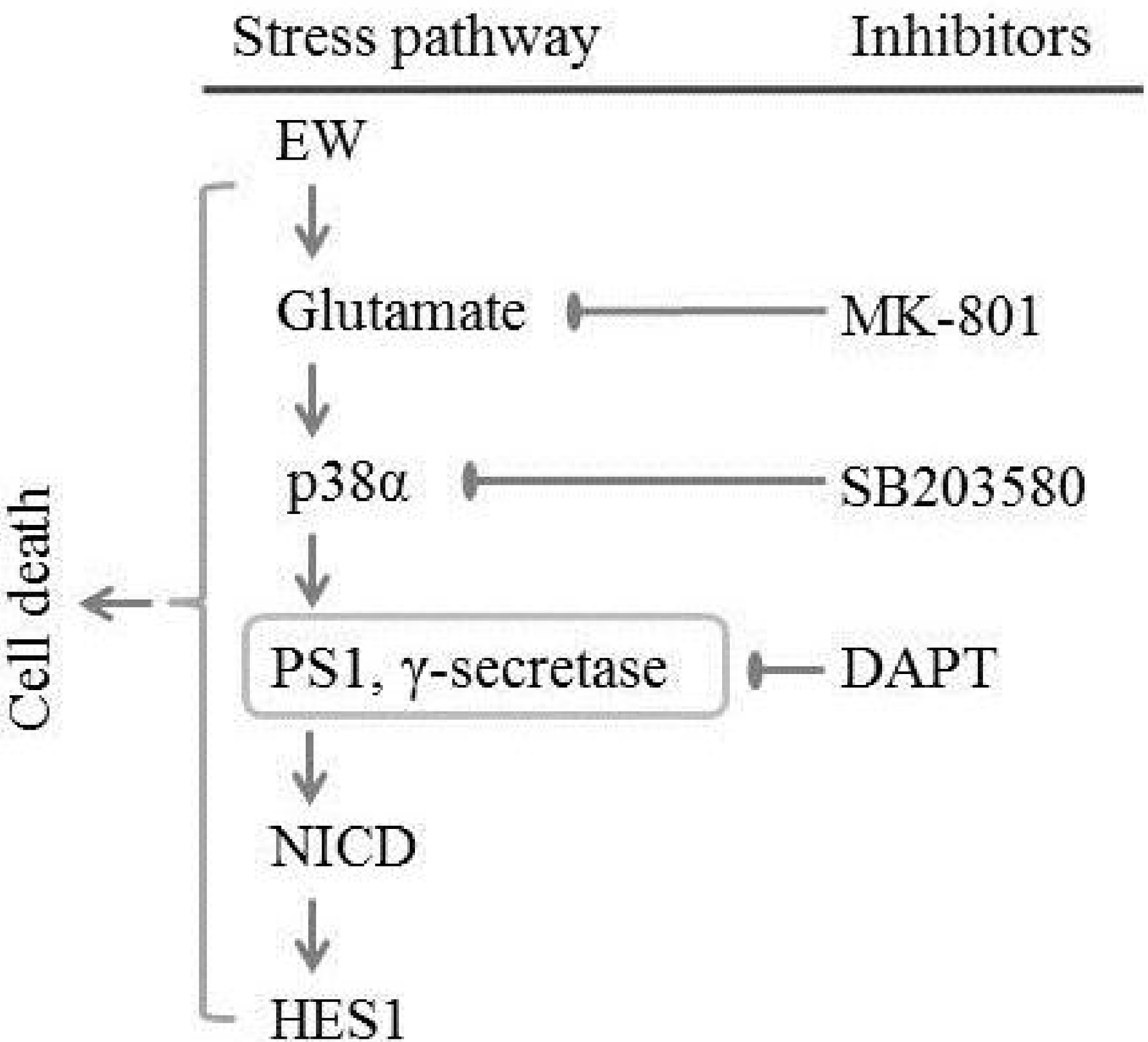
Stress pathway
Inhibitors
EW
Glutamate
MK-801
p38α
SB203580
Cell death
PS1, γ-secretase
DAPT
NICD
HES1

Figure 8