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Chronic Social Stress and Ethanol Increase Expression of KLF11, a Cell Death Mediator, in Rat Brain

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Abstract

Major depressive disorder and alcoholism are significant health burdens that can affect executive functioning, cognitive ability, job responsibilities, and personal relationships. Studies in animal models related to depression or alcoholism reveal that the expression of Krüppel-like factor 11 (KLF11, also called TIEG2) is elevated in frontal cortex, which suggests that KLF11 may play a role in stress- or ethanol-induced psychiatric conditions. KLF11 is a transcriptional activator of monoamine oxidase (MAO) A and B, but also serves other functions in cell cycle regulation and apoptotic cell death. In the present study, immunohistochemistry was used to quantify intensity of nuclear KLF11, combined with an unbiased stereological approach to assess nuclei in fronto-limbic, limbic, and other brain regions of rats exposed chronically to social defeat or ethanol. KLF11 immunoreactivity was increased significantly in the medial prefrontal cortex, frontal cortex and hippocampus of both stressed rats and rats fed ethanol. However, expression of KLF11 protein was not significantly affected in the thalamus, hypothalamus or amygdala in either treatment group compared to respective control rats. Triple-label immunofluorescence revealed that KLF11 protein was localized in nuclei of neurons and astrocytes. KLF11 was also co-localized with the immunoreactivity of cleaved caspase 3. In addition, Western blot analysis revealed a significant reduction in anti-apoptotic protein, Bcl-xL, but an increase of caspase-3 expression in the frontal cortex of ethanol-treated rats compared to ethanol-preferring controls. Thus, KLF11 protein is up-regulated following chronic exposure to stress or ethanol in a region-specific manner and may contribute to pro-apoptotic signaling in ethanol-treated rats. Further investigation into the KLF11 signaling cascade as a mechanism for neurotoxicity and cell death in depression and alcoholism may provide novel pharmacological targets to lessen brain damage and maximize neuroprotection in these disorders.

Keywords

stress; chronic social defeat; chronic ethanol feeding; rats; ethanol-preferring rats; Kruppel-like factor 11 (Transforming growth factor-beta-inducible early gene 2); prefrontal cortex; hippocampus; apoptosis; cell death

Introduction

Major Depressive Disorder (MDD) and Alcohol Use Disorders (AUD) are debilitating disorders which can affect mood and cognition; moreover, these psychiatric illnesses are often comorbid (Grant and Harford, 1995). Chronic social stress and alcohol exposure are associated with molecular changes in the brain that are accompanied by up-regulation of monoamine oxidase (MAO) and transcriptional activator Kruppel-like factor 11 (KLF11; Ou et al., 2010; Grunewald et al., 2012, Duncan et al., 2012), as well as increased cell death (Carlsson et al., 1980; Ou et al., 2011; Yang et al., 2011).

Prolonged periods of psychological stress are associated with high levels of cortisol, dysregulated feedback in the hypothalamic-pituitary-adrenal axis, and in some cases, may precipitate MDD (Kendler et al., 1995). Glucocorticoid hypersecretion (Duval et al., 2006), and increases in MAO-A expression and catalytic activity (Harris et al., 2014; Johnson et al., 2011; Meyer et al., 2006; Meyer et al., 2009; Sacher et al., 2010) are associated with MDD. An abnormal increase of glucocorticoid levels over an extended period of time has been associated with atrophy of the hippocampus (Lee et al., 2002). In addition, dexamethasone, a synthetic glucocorticoid, increases MAO-A activity in human neuroblastoma and glioblastoma cells through its role as a cellular stressor (Ou et al., 2006). Furthermore, dexamethasone also induces MAO-B expression and activity in both neuronal cells (Tazik et al., 2009) and astrocytes (Carlo et al., 1996; Chen et al., 2011), and reduces the number of viable cells in primary hippocampal cultures (Yu et al., 2010). Previously, we reported that rats exposed to chronic social defeat (CSD) exhibited elevated blood glucocorticoids, MAO-A mRNA and catalytic activity, and KLF11 mRNA and protein (Grunewald et al., 2012). We further demonstrated that KLF11 is glucocorticoid-responsive and is necessary for glucocorticoid-induced MAO-A expression and catalytic activity (Grunewald et al., 2012). We have also documented an up-regulation of the KLF11-MAO-A pathway in the prefrontal cortex of mice exposed to chronic stress and post-mortem brain tissue from subjects with MDD (Harris et al., 2014). Activation of this pathway may contribute to cellular abnormalities because MAO can degrade a number of biogenic amines, including monoamine neurotransmitters, and subsequently produce reactive hydroxyl radicals which may cause cellular dysfunction and death (Youdim et al., 2004). Aside from the oxidation of monoamines, MAO-A is also directly involved in caspase-3-mediated apoptosis (Ou et al., 2006).

Alcoholism is a psychoactive substance use disorder resulting in neuropsychological difficulties in one-half of alcoholics (Dupont et al., 1996; Ducci et al., 2007). Pathologically, chronic alcohol use is accompanied by a reduction in the volume of gray matter in prefrontal cortex (Paul et al., 2008) and the densities (cells/volume of tissue) of neurons and glia observed in postmortem brain tissue from subjects with AUD (Miguel-Hidalgo et al., 2002;

Miguel-Hidalgo et al., 2006). In cell model systems, ethanol induces cell cycle delay and neuronal cell death (Luo and Miller, 1999; Luo et al., 1999; Chen et al., 2006; Johnson et al., 2007). Currently, the mechanisms associated with cellular dysfunction following chronic ethanol exposure are not fully understood. Though MAO-B has long been implicated in alcoholism (Carlsson et al., 1980), our group discovered that MAO-B is up-regulated *in vitro* as a result of exposure to physiologically-relevant concentrations of ethanol in a cell culture system (Ou et al., 2009) and *in vivo* in an animal model of chronic ethanol exposure (Ou et al., 2011). Furthermore, rats that were chronically fed ethanol demonstrated increased active caspase-3 and KLF11 protein, increased MAO-B mRNA and catalytic activity, and decreased anti-apoptotic Bcl-2 protein expression in the prefrontal cortex (PFC) (Ou et al., 2009). These findings suggest that stress- and ethanol-responsive signaling pathways involving KLF11 and MAO are responsive to CSD or chronic ethanol exposure in rats.

Krüppel-like factor 11 (KLF11), also known as TIEG2 (transforming growth factor-beta-inducible early gene 2), is an Sp1-like transcription factor belonging to the Sp/KLF zinc-finger family. KLFs are transcriptional regulators implicated in a broad range of cellular processes (Dang et al., 2000; Kaczynski et al., 2003), are distributed in various neural cells in the brain and have been implicated in a variety of human psychiatric and neurological disorders (Moore et al., 2011; Seo et al., 2012; Yin et al., 2013). KLF family members lack conservation outside the zinc-finger DNA-binding region which enables them to trans-activate or trans-repress target genes (Pearson et al., 2008). Specifically, KLF11 is capable of binding to four Sp/KLF binding sites in the MAO-A core promoter and further potentiates stress-induced MAO-A expression (Grunewald et al., 2012). Likewise, KLF11 transcriptionally activates MAO-B expression following chronic ethanol exposure (Ou et al., 2011) by binding to two clusters of overlapping Sp/KLF binding sites in the MAO-B core promoter (Ou et al., 2004). Additionally, KLF11 is reported to inhibit cell growth (Cook et al., 1998; Lomber et al., 2012) and induce apoptosis through direct transcriptional repression of Bcl-xL (Wang et al., 2007). KLF11 has also been shown to repress transcription of the reactive oxygen species (ROS) scavengers, superoxide dismutase 2 (SOD2) and catalase, and increase the rate of apoptosis in KLF11 transgenic mice (Fernandez-Zapico et al., 2003), thus providing further evidence *in vivo* of the pro-apoptotic role of KLF11. Either of these independent or converging KLF11-mediated events (Figure 6) may induce cell death and be actively involved in pathological processes associated with chronic stress or ethanol.

Our group has also documented increases in KLF11 and MAO in the prefrontal cortex of postmortem brain tissue from subjects diagnosed with MDD (Harris et al., 2014) and chronic alcohol dependence (Udemgba et al., 2013). However, whether the KLF11/MAO cascade is up-regulated broadly in whole brain or in a region-specific manner in these disorders is unknown. Therefore, we conducted a histological analysis of KLF11 protein expression in the frontal cortex; the medial prefrontal cortex (mPFC); two limbic regions: the hippocampus and the amygdala; the thalamus; and the hypothalamus in our rodent models related to depression and alcoholism (Figure 1). In addition, specific cell type localization of KLF11 was determined through triple immunolabeling studies and the differential effects of ethanol on the expression of neuroprotective protein, Bcl-xL and apoptotic procaspase-3

were analyzed by Western blot. These studies provide further functional insights into the regional differences of KLF11 protein expression following chronic social stress or ethanol exposure in rats.

Materials and Methods

Animal Care

All protocols for the animal experiments performed in this study were carried out according to the Ethical Guidelines on Animal Experimentation and were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. Upon arrival, rats were acclimatized for 3 days and allowed free access to Purina rat chow and tap water before undergoing experimentation.

Chronic Social Defeat

Twenty adult, male Wistar rats (weighing 180–220g; Charles Rivers Laboratories, Inc., Willmington, MA) were housed in individual cages in a temperature- and humidity-controlled room with a reversed 12:12-h light/dark cycle (Kieran et al., 2010). The resident-intruder paradigm (Miczek et al., 1982; Miczek et al., 2011) was used in the randomly assigned experimental group (N=10) to induce chronic social stress. The control group (N=10) was maintained and handled in the same manner as the experimental group except without exposure to chronic social stress. Each experimental “intruder” rat was transferred from its home cage to a cage holding one of ten male, Long Evans “resident” rats (weighing 580–620g; Charles Rivers Laboratories, Inc.). The intruder was described as being defeated by the resident if within three minutes the intruder displayed freezing behavior and submissive posturing. The intruder and resident were then immediately separated, and the intruder was kept in a small plastic wire mesh compartment within the resident’s cage for one hour. Afterwards, the intruder was removed from the small mesh cage and placed back into its home cage. This paradigm was repeated once daily for four days during week 1, two days for weeks 2 and 3, and four days during week 4 (Grunewald et al., 2012).

Chronic Ethanol Exposure

Twenty adult, ethanol-preferring male Wistar rats weighing 180–220 g were obtained from the Indiana University Alcohol Research Center (Li et al., 1979). The rats were randomly assigned to ethanol-fed or control groups (N=10/group). Following a three-day period of free access to liquid diets containing no ethanol (#710027, Dyets, Bethlehem, PA), rats in the ethanol-fed group were fed with the liquid ethanol diet (#710260, Dyets, Bethlehem, PA) which contained increasing concentrations of ethanol until a final diet containing 36% of calories from ethanol (6.4% EtOH) was achieved as follows: no EtOH for 3 days, 2.5% for 3 days, 5.0% for 5 days, and finally 6.4% for 17 days (Ou et al., 2011). In ethanol diets, glucose was isocalorically substituted with ethanol according to the Lieber-DeCarlie diet formula following the manufacturer’s instructions. Therefore, the liquid glucose control diet given to control rats contained the same number of calories as the ethanol liquid diet given to ethanol-treated rats. In addition, the blood ethanol concentrations achieved in this study were below 50 mM, a value that is within the physiological range observed in alcoholics (Henriksen et al., 1997; Yao et al., 2001; Ou et al., 2011).

Sacrifice

All rats from the chronic social defeat and ethanol studies were sacrificed by decapitation on day 29. Brains were immediately removed on ice. One hemisphere was fixed according to vendor instructions (Neuroscience Associates) with 4% paraformaldehyde at 4 C for 16h, and stored in phosphate buffered saline at 4°C until sectioning. The other hemisphere was freshly dissected on ice into different brain regions as described previously and flash frozen in liquid nitrogen and stored at –80°C for biochemical measures.

Immunofluorescence of KLF11 in Brain Regions of Rats Treated with Chronic Social Stress or Ethanol

All brains from rats that underwent CSD, chronic ethanol exposure, and their respective age/sex matched controls were embedded into two Multibrain® gelatin blocks and cut into 40 µm serial coronal free floating sections by Neuroscience Associates (Knoxville, TN). Sections were stored in antigen preserve (polyvinyl pyrrolidone, phosphate buffered saline, ethylene glycol) at –20°C until further use. Every 24th rat brain section (three total; Bregma 4.20mm, 3.20mm, & 2.20mm) spanning the mPFC and frontal cortex was selected for quantification of KLF11 immunofluorescence. Each adjacent section (Bregma –3.30mm) was used to quantify KLF11 expression in the hippocampus, thalamus, hypothalamus, and amygdala. After blocking with 5% goat serum, sections were incubated with rabbit anti-KLF11 polyclonal antibody (1:250; Cat# ARP38365, Lot# QC8151; A viva Systems Biology, San Diego, CA) overnight at 4 C. The KLF11 antibody used in this study is directed towards the C-terminal of human KLF11, corresponding to amino acids 423–472 of *Rattus norvegicus*. No staining was detected when the antibody was used on tissue from a KLF11 knockout mouse, which was generated at the University of Washington, Seattle, following standard homologous recombination techniques (Bonnetfond et al., 2011; Ou et al., 2013). Visualization of KLF11 immunoreactivity was made by secondary incubation with cy3-conjugated AffiniPure Goat anti-Rabbit IgG (1:1000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for two hours at room temperature. Brain sections of control and experimental groups sectioned from the same Multibrain® gelatin block were processed simultaneously to limit the intra- and inter-group variability due to experimental conditions. After incubations, sections were rinsed with phosphate-buffered saline (PBS), and mounted using Vectashield mounting medium with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to analyze under microscope.

Triple-labeling Immunofluorescence

Brain sections containing the frontal cortex or hippocampus from rats exposed to chronic ethanol were subjected to triple immunostaining for specific neuronal and astrocyte markers to reveal cell phenotype and subcellular distribution of KLF11 protein. Sections were blocked with 5% goat serum and incubated with rabbit anti-KLF11 antibody (1:250; Aviva Systems Biology, San Diego, CA) with mouse anti-Neu N monoclonal (1:500; Cat# ABN78, Lot# 2322047; Millipore, Billerica, MA) and chicken anti-GFAP polyclonal (1:1000; Cat# AB4647, Lot# 557236; Abcam, Cambridge, England) antibodies. Following primary antibody incubation, sections were visualized by incubation with FITC-conjugated goat anti-rabbit IgG (1:1000, Vector Laboratories, Inc., Burlingame, CA), Cy3 conjugated AffiniPure

goat anti-chicken (1:1000, Jackson ImmunoResearch, West Grove, PA), and Cy5 conjugated goat anti-mouse IgG. Lastly, all sections were rinsed with PBS and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA).

Double-labeling immunofluorescence

To determine KLF11 co-expression with active Caspase-3, additional sections were incubated with mouse anti-KLF11 monoclonal (1:250; Cat# SC136101, Lot# 60209; Santa Cruz Biotechnology, Dallas, TX) and rabbit anti-active caspase-3 polyclonal (1:250; Cat# 9661, Lot# 38; Cell Signaling Technology, Danvers, MA) antibodies. Visualization was made by secondary incubation with Cy2 conjugated goat anti-mouse (1:1000; Jackson) and Cy3 conjugated goat anti-rabbit (1:1000; Jackson). Lastly, all sections were rinsed with PBS and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories).

Quantification of KLF11 Intensity

Fluorescent images were captured and visualized using a Zeiss Observer Z1 imaging microscope system (Oberkochen, Germany). For each section, a montage picture was obtained using a 10× magnification field (Figure 1A, mPFC and frontal cortex; Figure 1B, hippocampus, thalamus, hypothalamus, and amygdala). Each region of interest (ROI) was defined and contours were drawn as described in the rat brain atlas (Paxinos and Watson, 1986). Then PFC, frontal cortex, hippocampus (granule cell layer of DG; pyramidal cell layer of CA2/3 and CA1), thalamus, hypothalamus, and amygdala were then masked to allow random assignment of sampling frames onto the masked area by SlideBook 5.0 software (Innovative Intelligence, Inc., Denver, CO). Each masked ROI contained between 6–9 sampling frames. The dimensions used for sampling frame volume were set to 100µm (x-axis) × 100µm (y-axis) × 10µm (z-axis), yielding a total sampling volume of 100,000 µm³ per sampling site. Within each optical dissector frame, a series of z-stage images were taken at one µm intervals using a 40× objective with numerical aperture of 0.55. All photomicrographs from the individual dissector frame were then compiled and stacked for analysis. Next, a mask was generated for each sampling site which allowed unbiased, quantitative assessment of fluorescence intensity in individual cell nuclei. Only well-focused and fully visible nuclei within each sampling site were then contoured and masked, which allowed intensities of individual KLF11 nuclei to be represented in sampling site datasets. In all, fluorescence intensity was quantified for ~38,000 cell nuclei in this study.

Western Blot Analysis

Frontal cortical tissue from each ethanol-treated (N=9) and ethanol-preferring control (N=9) rat was homogenized in RIPA buffer (Sigma-Aldrich, St. Louis, MO) with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL) and lysates were centrifuged at 4 °C (11,500 rpm) for 10 min to pellet and eliminate cell debris. Supernatants were then stored at –80°C until use. Protein concentrations were calculated using the BCA Protein Assay Kit (Pierce, Rockford, IL). Twenty-five micrograms of total protein from each sample were separated in 10.5% SDS-PAGE gel in a BioRad mini tank apparatus and transferred to PVDF membranes by a BioRad Trans-blot turbo transfer system. Membranes were then blocked with 5% nonfat dry milk for 1h at room temperature, followed by primary incubation with mouse anti-Bcl-xL (1:250; Santa Cruz) or rabbit Caspase-3 (1:250; Santa

Cruz) antibodies for 1h at room temperature. After secondary antibody incubation (goat anti-mouse IgG or goat anti-rabbit IgG; 1:1000; Santa Cruz) for 2h at room temperature, bands were visualized by horse-radish peroxidase reaction using SuperSignal Chemiluminescent Substrate (Pierce). As a control for sample loading, the same blot, after having been immunostained for Bcl-xL or Caspase-3, was stripped for 20 min at room temperature in Restore Western Blot Stripping Buffer (Fisher Scientific, Pittsburgh, PA) and then re-probed with mouse anti-actin (1:10,000; Millipore) primary antibody incubation for 1h at room temperature, followed by goat anti-mouse (1:1000; Santa Cruz) secondary antibody incubation for 2h at room temperature (Ou et al., 2009). All band intensities were calculated and normalized to those of actin using Quantity One Plus analysis software (Johnson et al., 2011).

Data Analysis

The intensity of KLF11 immunoreactivity of each nucleus within an individual sampling site was automatically processed into an excel file by imaging analysis software (Slidebook 5.0). Next, mean intensity of KLF11 from all nuclei within an individual sampling site was calculated. After gathering mean KLF11 nuclear intensity for each sampling site, all sampling site intensities within an individual region of interest (ROI) were averaged. Lastly, intensities from each mPFC, frontal cortex, hippocampus, thalamus, hypothalamus, or amygdala ROI were averaged across all rat brain sections containing these regions. Mean background values were subtracted from individual intensity values (Waters, 2009). Statistical analysis was performed using a two-tailed, unpaired student t-test (GraphPad Prism) with probability 0.05 deemed to be statistically significant between each stress- or ethanol-treated group and their respective control groups. All data are presented as means $\pm$ SEM.

Results

Chronic Social Stress Exposure increases KLF11 Immunoreactivity in the Rat Medial Prefrontal Cortex, Frontal Cortex and Hippocampus

For male Wistar rats exposed to chronic social stress over 28 days, KLF11 immunoreactivity, measured by immunofluorescence, was significantly increased by: 24% (239.98 ± 13.16 vs. 193.63 ± 13.72 ; Mean $\pm$ SEM) in the medial prefrontal cortex ($t(58) = 2.44$, $p = 0.017$; Figure 2B(mPFC), bars 2 vs. 1), 20% (232.61 ± 13.67 vs. 193.2 ± 13.69) in the frontal cortex ($t(58) = 2.04$, $p = 0.046$; Figure 2B(FC), bars 4 vs. 3), and 36% (227.46 ± 13 vs. 167.06 ± 24.83) in pyramidal cells of the CA1 subregion of hippocampus ($t(18) = 2.16$, $p = 0.044$; Figure 2B(CA1), bars 12 vs. 11) compared to unexposed control rats. However, no significant changes in KLF11 nuclear intensity were noted in the amygdala, thalamus, hypothalamus, or CA2/3 and dentate gyrus subregions of hippocampus of stressed rats compared to control rats.

Chronic Ethanol Exposure increases KLF11 Protein Expression in the Rat Medial Prefrontal Cortex, Frontal Cortex, and Hippocampus

Similarly, male Wistar rats that were chronically exposed to ethanol over 28 days revealed a significant increase in KLF11 nuclear immunoreactivity by: 16% (226.57 ± 12.09 vs.

194.93±10.12) in the medial prefrontal cortex ($t(58) = 2.01$, $p = 0.049$; Figure 3B(mPFC), bars 2 vs. 1), 17% (237.78±10.74 vs. 203.37±10.95) in the frontal cortex ($t(58) = 2.24$, $p = 0.028$; Figure 3B(FC), lanes 4 vs. 3), 29% (214.54±14.28 vs. 167.31±16.24) in pyramidal cells of the CA2/3 sub-region of hippocampus ($t(18) = 2.18$, $p = 0.042$; Figure 3B(CA2/3), bars 10 vs. 9), and 36% (225.27±11.24 vs. 166.43±18.18) in pyramidal cells of the CA1 sub-region ($t(18) = 2.75$, $p = 0.013$; Figure 3B(CA1), bars 12 vs. 11) compared to ethanol-preferring control rats. There was a trend for an increase in KLF11 expression in granule cells of the hippocampal dentate gyrus (21%, $p = 0.075$; Figure 3B, bars 8 vs. 7), and in the amygdala (27%, $p = 0.078$; Figure 3B, bars 6 vs. 5) of ethanol-fed rats compared to control rats. As with rats exposed to CSD, exposure to chronic ethanol had no significant effect on the intensity of KLF11 immunoreactivity in the thalamus or hypothalamus.

KLF11 Protein is Expressed in Neurons and Astrocytes and is Co-localized with Cleaved Caspase-3

To determine which cells express KLF11, triple immunofluorescence staining was conducted with specific cell markers to identify KLF11 expression in neurons or astrocytes in the brain of rats. KLF11 immunoreactivity was present in nuclei of neurons and astrocytes (Figure 4A & B) in hippocampus and medial prefrontal cortex of ethanol treated rats. In addition, KLF11 immunoreactivity was present in cells which expressed cleaved caspase-3 (C-CASP3; Figure 5A). It is worth noting that KLF11-positive nuclei only express a mild degree of C-CASP3 and KLF11-immunoreactive (IR) signal is not observable in cells which showed abundant C-CASP3, suggesting that KLF11 may be more heavily involved in pro-apoptotic signaling before the cleavage of Caspase-3. Together, these data further suggest that ethanol or stress increases KLF11 protein in neurons and astrocytes in key executive and limbic brain regions which could contribute to the pathophysiology of depression and alcoholism.

Chronic Ethanol Exposure Increases Caspase-3, but Decreases Anti-apoptotic Bcl-xL Expression

Indeed, chronic ethanol feeding increased KLF11 immunoreactivity and also co-localized with C-CASP3 (Figure 5A). This observation suggests that KLF11 may initiate the apoptotic process in cells (co-localizing with C-CASP3 during the onset of apoptosis). To test the hypothesis that KLF11 is involved in pro-apoptotic signaling, we evaluated protein expression of an anti-apoptotic protein, Bcl-xL, and the apoptotic protein, procaspase-3 (Caspase-3). Western blot analysis demonstrated a significant increase of Caspase-3 by 49% in the frontal cortex compared to ethanol-preferring controls ($t(16) = 2.24$, $p = 0.04$; 1.61±.2 vs 1.08±.13, Figure 5B). In addition, rats that were chronically fed ethanol showed a significant reduction of Bcl-xL by 36% in the frontal cortex of rats exposed to ethanol compared to ethanol-preferring controls ($t(16) = 2.44$, $p = 0.027$; .69±.13 vs. 1.08±.09, Figure 5C). These results support enhanced pro-apoptotic signaling following chronic exposure to ethanol which are accompanied by an increase in KLF11 expression, suggesting KLF11 may mediate apoptotic cell death signaling in the frontal cortex of rats exposed to ethanol.

Discussion

For either chronically stressed rats or ethanol-fed rats, the expression of KLF11 protein, a MAO-transcriptional activator, was significantly increased in the medial prefrontal cortex, frontal cortex, and hippocampus but not in the thalamus, hypothalamus or amygdala. This is the first study to measure expression of KLF11 protein by immunohistochemical methods in several brain regions of male Wistar rats in rodent models related to depression and alcoholism. In rodent models and human neuronal cell line (SH-SY5Y), our group has demonstrated that expression of KLF11 protein and mRNA is elevated in response to stress/dexamethasone (Grunewald et al., 2012) or ethanol (Lu et al., 2008; Ou et al., 2011). We have also observed that KLF11 is up-regulated in postmortem brain tissue of subjects meeting criteria for MDD or alcohol dependence (Harris et al., 2014; Udemgba et al., 2013). The current study provides further *in vivo* support of KLF11 as a stress- and ethanol-responsive gene, which is functionally capable of initiating signaling cascades that lead to cellular stress (including up-regulation of MAO) and apoptosis.

CSD is a validated rodent model for the induction of depression-related behaviors in rats and mice (Kieran et al., 2010; Vialou et al., 2010; Grunewald et al., 2012). The ethanol-preferring line of rats is a validated choice for investigating chronic ethanol feeding as a model for alcoholism (Li et al., 1979; Ou et al., 2011). In humans, the prefrontal cortex and hippocampus are strongly affected in depression. In MDD, these regions display altered cerebral blood flow (Drevets, 2000) and reductions in volume (Bremner, 2000; Bremner et al., 2002; Cobb et al., 2013; Sheline et al., 1996; Koolschijn et al., 2009). In a rat model of repeated stress, there is a significant decrease in apical dendritic spine density and length in the mPFC (Radley et al., 2006). Moreover, a stress paradigm of chronic immobilization in male Wistar rats showed dendritic atrophy and decreased expression of brain-derived neurotrophic factor (BDNF) in the hippocampus (Lakshminarasimhan and Chattarji, 2012). Similarly, the prefrontal cortex and hippocampus are affected by chronic ethanol exposure in humans. In subjects with AUD, imaging studies have shown volumetric reductions in the amygdala (Wrase et al., 2008), hippocampus (Agartz et al., 1999; Sullivan et al., 1995), frontal lobe (Pfefferbaum et al., 1997), and prefrontal and orbitofrontal cortices (Durazzo et al., 2011; Beck et al., 2012; Kril et al., 1997). Furthermore, ethanol reportedly opposes the effects of some neurotrophic factors on survival of primary rat hippocampal neurons (Mitchell et al., 1998). Whether or not KLF11 is actively involved in processes leading to volumetric reductions in MDD and AUD is still unknown. Much of the literature has cited the pro-apoptotic role of KLF11 in cell culture models, and indeed these findings may not be relevant in the adult mammalian brain. Additional experiments are necessary, including rodent studies such as brain-specific KLF11 knock out mice), to afford a deeper understanding of KLF11 and pro-apoptotic signaling in mammals.

Diverse functions are served by the brain regions in which KLF11 demonstrates responsiveness to chronic social stress or ethanol. The mPFC [including the medial (frontal) agranular, anterior cingulate, prelimbic and in fralimbic subdivisions]of rats appears to be involved in higher order cognitive functioning, and through interconnections among these four subdivisions, is capable of exerting control over many aspects, including affective components of goal-directed behavior(Hoover and Vertes, 2007). The rat frontal cortical

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areas (Fr1, Fr2, Fr3) are somatotopically organized (Neafsey et al., 1986) and contain the primary motor cortex (Fr1; (Zilles et al., 1993)), but also incorporate primate premotor characteristics (Uylings and van Eden, 1990). The hippocampus (dentate gyrus, CA1, CA2/3) is known for its role in learning and memory. Surprisingly, the expression of KLF11 protein was increased similarly in both chronic social stress and ethanol consumption, demonstrating that KLF11 may play a comparable role in both conditions. In fact, MDD and alcoholism both are associated with similar neuropathologies, including changes to glial cells and apoptotic markers in the PFC, suggesting some genetic components are shared between the disorders (Miguel-Hidalgo et al., 2002; Miguel-Hidalgo & Rajkowska, 2003).

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KLF11 immunoreactivity was significantly increased in the mPFC, frontal cortex and hippocampus following chronic social stress or ethanol, suggesting that KLF11 is more responsive in these brain areas rather than those in the thalamus, hypothalamus or amygdala. Interestingly, regions that displayed a significant increase in KLF11 expression are consistent with human imaging studies showing decreased volume in the same comparative regions of patients diagnosed with depression or alcoholism, supporting the hypothesis that KLF11 contributes to the pathophysiology of these disorders. Furthermore, KLF11 immunoreactivity was more pronounced in the hippocampus of ethanol-treated rats compared to socially-stressed rats. This finding may account for some of the pathological consequences of chronic ethanol exposure in this ethanol-vulnerable region by regulating expression of other genes involved in cellular function and homeostasis.

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In addition, our triple-labeling immunofluorescence revealed for the first time that KLF11 was localized in both neurons and astrocytes. KLF11 also displayed a sub-cellular localization to the cell nucleus, which is consistent with its role as a transcription factor. KLF11 was co-localized with the apoptotic protein, cleaved caspase-3 (Figure 5A), which suggests that KLF11-mediated cell death signaling involves the Caspase-3 pathway. To further evaluate ethanol-induced pro-apoptotic signaling in our rodent model, we analyzed protein expression of Caspase-3 and Bcl-xL. Ethanol-fed rats showed higher expression of Caspase-3, but reduced expression of Bcl-xL compared to control rats (Figures 5B & C), providing further evidence of the association between cell death and KLF11, which is also a candidate for Caspase-3 promoter regulation through Sp1-binding sites (Sudhakar et al., 2008). Together, these results suggest that ethanol induces KLF11-mediated cell death signaling. Further studies to elucidate the role of KLF11 in the complex intrinsic apoptosis signaling pathway are necessary to understand how genes responsible for cell viability or death could be regulated by KLF11.

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In the present study, we determined that nuclear KLF11 protein expression is up-regulated in a brain region-specific manner in rats exposed to chronic stress or ethanol. We previously documented that glucocorticoids and ethanol up-regulate KLF11 in rats (Grunewald et al., 2012). Mechanistically, ethanol and subsequent oxidative stress (Chen et al., 2006), as well as glucocorticoid-induced leucine zipper (Bereshchenko et al., 2014), up-regulates transforming growth factor- β (TGF- β) which in turn can signal the transcription of KLF11 through the Smad pathway (Johnsen et al., 2002; Yan et al., 2009; Lui et al., 2010). Sp1 is also affected by ethanol (Jin et al., 2013; Do et al., 2013) and is capable of regulating KLF11 through Sp1 binding sites (Kuroda et al., 2009).

In parallel to KLF11, glucocorticoids also up-regulate KLF15 (Asada et al., 2011) and KLF9 (Bonett et al., 2009; Bagamasbad et al., 2012). As immediate early genes, KLF family members, which sometimes perform as competing or complimentary cues in transcriptional regulation of their targets, serve diverse functions in response to a number of different stimuli (Cook et al., 1998; Asano et al., 1999; Lomberg & Urrutia, 2005; Wang et al., 2007; Pearson et al., 2008; Lomberg et al., 2012). For example, KLF9 has been shown to be critical for neurogenesis (Scobie et al., 2009) and KLF15 is associated with neural stem cell maintenance and reduction in apoptosis (Ohtsuka et al. 2011; Masuno et al., 2011).

Therefore, apoptosis mediated by KLF11 is, in part, opposed by other KLFs, and thus expected to involve a conserved and tightly regulated mechanism for apoptotic induction. Consistent with our results, KLF11 promotes the activation of Caspase-3 (Lomberg et al., 2012) and mimics TGF β effects by inducing apoptotic cell death and activation of Caspase-3 in OLI-neu cells (Gohla et al., 2008). Caspase-3 promoter activation requires binding at Sp1-binding sites (Sudhakar et al., 2008) and KLF11 increases the expression of Caspase-8 through repression of anti-apoptotic, Bcl-xL (Wang et al., 2007). KLF11 is also a candidate for Bcl-2, Bim, Caspase-8 (Hirose and Horvitz, 2013; Liedtke et al., 2003; Liedtke et al., 2005), as well as Caspase-9 transcriptional regulation, as they contain Sp1-binding sites in the promoter region (Seto et al., 1988; Grillot et al., 1997; Gogada et al., 2013). Further, KLF11 appears to mediate the preliminary steps of the apoptotic process, as evidenced by our KLF11-C-CASP3 double-immunohistochemistry, where KLF11 is inversely co-localized with the degree of C-CASP3 immunoreactivity. Moreover, executioner events at the final phases of cell death may involve 1) high activated Caspase-3 levels which will induce DNA fragmentation and abolish the functional gene transcription for synthesis of new KLF11; and 2) cleavage and proteolysis of existed KLF11/Sp1 and other nuclear proteins by high amounts of activated caspase 3 as reported by Rickers et al. in anti-IgM-induced B-cell apoptosis (Rickers et al., 1999; Brockstedt et al., 1998), which could explain why KLF11 is functionally important in the early, but not late, stages of apoptosis. Therefore, it is our hypothesis that KLF11, which is induced by glucocorticoids or ethanol, may promote cell death by regulating anti-apoptotic (Bcl-xL) and apoptotic factors (Caspases), oxidative stress scavengers (SOD2, Catalase), and MAO (with subsequent ROS) expression which collectively results in neural cell death (Figure 6).

In summary, our preliminary immunohistochemical analysis of the expression of KLF11 (TIEG2) protein, a cell death mediator and transcriptional activator of MAO, reveals that KLF11 immunoreactivity is significantly increased in the mPFC, frontal cortex and hippocampus in male Wistar rats following exposure to chronic social stress or ethanol. However, no significant changes in KLF11 expression were detected in the thalamus, hypothalamus or amygdala in either of these paradigms, suggesting that KLF11 protein expression is selectively affected by chronic social stress or ethanol in different brain areas. In these models, changes in KLF11 expression are targeted to brain regions important for executive functioning and learning/memory. The changes in KLF11 expression in these rat brain regions behaviorally match human brain regions relevant to neuropsychological deficiencies seen in chronic alcoholism or major depressive disorder (Clark et al., 2009; Tarter and Alterman, 1984; Nicolas et al., 1993), supporting the hypothesis that KLF11 mechanistically contributes to the pathophysiology in these regions. Our data provides

further insight into the regional distribution of KLF11 in psychiatric conditions, such as major depressive disorder or alcohol use disorders, which could contribute to novel pharmacological strategies to mitigate stress- or ethanol- induced neurotoxicity and brain tissue injury.

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Figure 1. Selected areas of the rat brain used for KLF11 quantification

(A) Representative montage (visualized with DAPI, blue) obtained with a 10x objective depicting the medial frontal cortex (mPFC) and frontal cortex regions (Bregma 3.20mm).

(B) Representative montage (visualized with DAPI, blue) obtained with a 10x objective depicting the DG, CA2/3, CA1, thalamus, hypothalamus, and amygdala (Bregma -3.30mm).

The areas of the rat brain are numbered from 1 to 8 as follows: 1. Medial Prefrontal Cortex; 2. Frontal Cortex; 3. Hippocampal CA1 (pyramidal layer); 4. Hippocampal CA2/3 (pyramidal layer); 5. Hippocampal Dentate Gyrus (granular layer); 6. Thalamus; 7. Hypothalamus; and 8. Amygdala.

Figure 2. Chronic social defeat stress increases KLF11 immunoreactivity in the rat brain Rats were exposed to chronic social defeat stress for 28 days (an animal model for depression) and nuclear KLF11 immunoreactivity was quantified. **Upper Panel (A).** Representative photomicrographs of nuclei (DAPI, blue) and KLF11 protein (cy3, red) obtained with a 20× objective in the mPFC of control or stressed rats. **Lower Panel (B).** Quantitative analysis of KLF11 immunoreactivity revealed a significant increase in mPFC, frontal cortex, and CA1 sub-region of hippocampus, but was unchanged in the DG and CA2/3 sub-regions of hippocampus, thalamus, hypothalamus or amygdala of rats exposed to chronic social defeat compared to controls. Data represents the group (N=10 in either control or stressed group) mean $\pm$ SEM (* $p < 0.05$).

Figure 3. Chronic ethanol exposure increases KLF11 immunoreactivity in the rat brain

Rats were fed ethanol or control diets for 28 days (an animal model for alcohol dependence) and nuclear KLF11 immunoreactivity was quantified. **Upper Panel (A).** Representative photomicrographs of nuclei (DAPI, blue) and KLF11 protein (cy3, red) obtained with a 20× objective in the mPFC of control or ethanol-fed rats. **Lower Panel (B).** Quantitative analysis of KLF11 immunoreactivity revealed a significant increase in mPFC, frontal cortex, CA1 and CA2/3 subfields of hippocampus, but was not significantly affected in the thalamus, hypothalamus or amygdala of ethanol-treated rats compared to ethanol-preferring controls. Data represents the group (N=10 in either control or ethanol-treated group) mean ± SEM (* $p < 0.05$).

Figure 4. Nuclear Localization of KLF11 in neurons and astrocytes

KLF11 expression is present in the nuclei of neurons and astrocytes. Rat brain sections were subjected to triple-immunolabeling to discern the specific phenotypic expression of KLF11 immunoreactivity. **Upper Panel (A)** KLF11 (FITC, green) expression is present in nuclei of both neurons (NeuN, cy5, magenta) and astrocytes (GFAP, cy3, red, indicated by arrows) in hippocampal dentate gyrus. GCL = granular cell layer. **Lower Panel (B)** KLF11 (FITC, green) expression is present in both of neurons (NeuN, cy5, magenta) and astrocytes (GFAP, cy3, red, indicated by arrows) in mPFC.

Figure 5. Ethanol-fed rats display increased markers of apoptosis

KLF11 is co-localized with cleaved caspase-3 (C-CASP3) and ethanol-fed rats show an increase in caspase-3, but decrease in expression of potent anti-apoptotic protein, Bcl-xL. Rats were fed with an ethanol diet or isocaloric control diet for 28 days; the co-localization of KLF11 and C-CASP3 was examined by double immunofluorescence and protein levels of Bcl-xL and caspase-3 were examined by Western blot analysis. (A). Double immunofluorescence labeling of KLF11 (green) with C-CASP3 (red) in mPFC of rats fed with an ethanol diet. Cells which express KLF11 and C-CASP3 positive nuclei are indicated by arrows. (B). Upper Panel: Representative Western blots showing the immunolabeling of caspase-3 in the prefrontal cortex of 3 untreated controls and 3 ethanol-treated rats. Anti-actin antibody was used as protein loading controls. Lower panel: Quantitative graph depicting the average optical density of caspase-3/actin ratios in the ethanol-preferring control group (N=9) and the ethanol-fed group (N=9). Expression of caspase-3 was significantly increased in ethanol-treated rats compared to ethanol-preferring controls (mean $\pm$ SEM; * p < .05). (C). Upper Panel: Representative Western blots showing the immunolabeling of Bcl-xL in the prefrontal cortex of 3 untreated controls and 3 ethanol-treated rats. Anti-actin antibody was used as protein loading controls. Lower Panel: Quantitative graph depicting the average optical density of Bcl-xL/actin ratios in the ethanol-preferring control group (N=9) and the ethanol-fed group (N=9). Expression of Bcl-xL was significantly decreased in ethanol-treated rats compared to ethanol-preferring controls (mean $\pm$ SEM; * p < .03).

Figure 6. Proposed pathways of stress- and ethanol-induced KLF11 pro-apoptotic signaling

Ethanol is capable of inducing TGFβ and SP1, which can in turn activate KLF11.

Glucocorticoids induce KLF11 expression. KLF11 upregulates MAO, which produces hydrogen peroxide as a byproduct of monoamine catabolism, and downregulates oxidative stress scavengers, SOD2 and Catalase. SP1-binding sites in the promoters of Bcl-xL, caspase-3, -8, and -9 make them viable targets of KLF11 regulation. Oxidative stress and changes in expression to anti-apoptotic and apoptotic proteins can signal the cleavage of caspase 3 which initiate executioner events leading to cleavage of SP1 and SP1-like transcription factors, transcription inhibition, and apoptosis. Arrows and bars represent activation and repression of the following targets, respectively. Solid line indicates potential direct effect; broken line indicates indirect effect.