Published in final edited form as:

J Neurosci Res. 2009 September ; 87(12): 2793–2802. doi:10.1002/jnr.22098.

Overexpression of Glycogen Synthase Kinase 3 β Sensitizes Neuronal Cells to Ethanol Toxicity

Ying Liu¹, Gang Chen², Cuiling Ma³, Kimberly A. Bower², Mei Xu², Zhiqin Fan¹, Xianglin Shi⁴, Zun-Ji Ke¹, and Jia Luo^{1,2,*}

¹Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

²Department of Internal Medicine, University of Kentucky College of Medicine, Lexington, Kentucky

³Department of Dermatology, Xijing Hospital, Xi'an, China

⁴Graduate Center for Toxicology, University of Kentucky College of Medicine, Lexington, Kentucky

Abstract

The developing central nervous system (CNS) is particularly susceptible to ethanol toxicity. The loss of neurons underlies many of the behavioral deficits observed in fetal alcohol spectrum disorders (FASD). The mechanisms of ethanol-induced neuronal loss, however, remain incompletely elucidated. We demonstrated that glycogen synthase kinase 3 β (GSK3 β), a multifunctional serine/threonine kinase, was involved in ethanol neurotoxicity. The activity of GSK3 β is negatively regulated by its phosphorylation at serine 9 (Ser9). Ethanol induced dephosphorylation of GSK3 β at Ser9 and the activation of Bax as well as caspase-3 in the developing mouse brain. These ethanol-induced alterations were ameliorated by the pretreatment of a GSK3 β inhibitor, lithium. To determine the role of GSK3 β in ethanol neurotoxicity, we overexpressed wild-type (WT), S9A mutant or dominant-negative (DN) mutant GSK3 β in a neuronal cell line (SK-N-MC). Ethanol only modestly reduced the viability of parental SK-N-MC cells but drastically induced caspase-3 activation and apoptosis in cells overexpressing WT or S9A GSK3 β , indicating that the high levels of GSK3 β or the active form of GSK3 β increased cellular sensitivity to ethanol. Contrarily, overexpression of DN GSK3 β conferred resistance to ethanol toxicity. Lithium and other specific GSK3 β inhibitors abolished the hypersensitivity to ethanol caused by WT or S9A overexpression. Bax, a proapoptotic protein, is a substrate of GSK3 β . Cells overexpressing WT or S9A GSK3 β were much more sensitive to ethanol-induced Bax activation than parental SK-N-MC cells. Our results indicate that GSK3 β may be a mediator of ethanol neurotoxicity, and its expression status in a cell may determine ethanol vulnerability.

Keywords

alcohol; apoptosis; development; fetal alcohol syndrome; neurodegeneration

Maternal alcohol consumption during pregnancy causes fetal alcohol spectrum disorders (FASD) that are characterized by a spectrum of structural anomalies and neurocognitive and

behavioral disabilities (Riley and McGee, 2005). Fetal alcohol syndrome (FAS), the most severe form of FASD, is the leading nonhereditary cause of mental retardation (Stratton et al., 1996; May and Gossage, 2001). Prenatal alcohol exposure can severely damage the developing brain, and neuronal death is a critical component of this injury. Our understanding of the mechanisms underlying alcohol-induced neuronal death, however, is fragmentary. Because neuronal loss plays a major role in the pathogenesis and outcome of FASD, identification of underlying cellular/molecular mechanisms is critically important.

Glycogen synthase kinase 3 (GSK3), a serine/threonine kinase originally identified as a regulator of glycogen metabolism, is a central component of the Wnt signaling pathway important for proper axis formation during embryonic development (Doble and Woodgett, 2003). There are two highly homologous forms of GSK3 in mammals, GSK3 α and GSK3 β . Both isoforms are ubiquitously expressed. The expression of GSK3 β in the central nervous system is developmentally regulated and localized primarily in neurons (Leroy and Brion, 1999; Coyle-Rink et al., 2002). GSK3 β is unusual in that it is largely regulated by inhibition. One mechanism through which GSK3 β can be inactivated is phosphorylation of serine 9 (Ser9; Grimes and Jope, 2001; Doble and Woodgett, 2003). Numerous protein kinases, such as Akt/protein kinase B (PKB), protein kinase C (PKC), p70 S6 kinase, p90Rsk, and protein kinase A (PKA), can phosphorylate GSK3 β at Ser9 and therefore inactivate GSK3 β (Grimes and Jope, 2001). GSK3 β is also capable of being regulated by an activation pathway. Activation of GSK3 β is accomplished by phosphorylation at tyrosine 216 (Tyr216). More than 40 proteins are substrates of GSK3 β , and these proteins have roles in a wide spectrum of cellular processes, including glycogen metabolism, transcription, translation, cytoskeletal regulation, cell differentiation, proliferation, transformation, and apoptosis (Grimes and Jope, 2001; Luo, 2009).

A growing body of evidence suggests that GSK3 β is an important modulator of neuronal survival (Beurel and Jope, 2006). It appears that GSK3 β activity correlates inversely with neuronal viability (Crowder and Freeman, 2000; Hetman et al., 2000; Lucas et al., 2001; Song et al., 2002). For example, overexpression of GSK3 β to specific regions of the brain in mice results in neuronal death in these regions (Lucas et al., 2001). GSK3 β has been linked to several key neuropathological mechanisms of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD; Bhat and Budd, 2002; Kaytor and Orr, 2002) and mediates neuronal damage caused by various insults (Chen et al., 2004; Beurel and Jope, 2006). It is unclear whether GSK3 β is involved in ethanol neurotoxicity. In this study, we sought to address several important questions regarding the role of GSK3 β in ethanol neurotoxicity. Is GSK3 β involved in ethanol neurotoxicity? Will the status of GSK3 β expression determine cellular sensitivity to ethanol? What is the downstream component of GSK3 β responsible for ethanol-induced neurodegeneration? With the combination of in vivo and in vitro model systems, we demonstrate that GSK3 β mediates ethanol neurotoxicity and ethanol activates Bax, a proapoptotic protein in a GSK3 β -dependent manner. Furthermore, we show that the expression status of GSK3 β in a cell determines sensitivity to ethanol.

MATERIALS AND METHODS

Materials

All antibodies except the anti-tubulin and anti-Bax antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-tubulin antibody was purchased from Sigma (St. Louis, MO). Anti-Bax antibody was obtained from Alexis Biochemicals (San Diego, CA). GSK3 β inhibitors (TDZD-8 and LiCl) were purchased from Calbiochem (La Jolla, CA). An Annexin V FITC Apoptosis Detection Kit was obtained from Biovision Inc. (Mountain View, CA). Other chemicals were obtained from Sigma.

Animals and Treatment

C57BL/6 mice were obtained from Shanghai Laboratory Animals Co. Ltd. (Shanghai, China) and maintained at the Animal Facility of the Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS). All procedures were performed in accordance with the guidelines set by the Animal Care and Use Committee of SIBS. Each 7-day-old mouse pup in a litter was assigned to one of the following groups: control (saline), ethanol, lithium, or ethanol plus lithium. An ethanol exposure paradigm, which had been shown to induce robust neurodegeneration in mice (Olney et al., 2002), was employed. The mice were injected subcutaneously with saline or ethanol (2.5 g/kg, 20% solution in saline) twice at 0 hr and 2 hr. A previously described method of lithium chloride (LiCl) injection was adapted, with some modifications (Zhong et al., 2006). Briefly, mice received an injection of LiCl (40 mg/kg body weight; 4 mg/ml) intraperitoneally 30 min prior to the first ethanol injection. At specified times after the first ethanol injection, the brains were removed and processed for immunoblotting or immunohistochemical study.

Sample Preparation and Immunoblotting

After treatment, mice were anesthetized by intraperitoneal injection of chloral hydrate (350 mg/kg), and the cerebral cortex was immediately dissected. The tissues were frozen in liquid nitrogen and stored at -80°C . Proteins were extracted as previously described (Wang et al., 2007). Briefly, tissues were homogenized in an ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 0.5% NP-40, 0.25% SDS, 5 $\mu\text{g}/\text{ml}$ leupeptin, and 5 $\mu\text{g}/\text{ml}$ aprotinin. Homogenates were centrifuged at 20,000g for 30 min at 4°C , and the supernatant fraction was collected.

The immunoblotting procedure has been previously described (Chen et al., 2004). Briefly, aliquots of the protein samples (30 μg) were separated on a SDS-polyacrylamide gel by electrophoresis. The separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5% BSA or 5% nonfat milk in 0.01 M PBS (pH 7.4) and 0.05% Tween-20 (TPBS) at room temperature for 1 hr. Subsequently, the membranes were probed with primary antibodies directed against target proteins overnight at 4°C . After three quick washes in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL). The immune complexes were detected by the enhanced chemiluminescence method (Amersham). In some cases, the blots were stripped and reprobed with either an anti-tubulin or an antiactin antibody. The density of immunoblotting was quantified with the software Quantity One (Bio-Rad, Hercules, CA).

Immunohistochemistry

After treatments, the mice were deeply anesthetized with chloral hydrate (350 mg/kg), then perfused with saline followed by 4% paraformaldehyde in 0.1 M potassium phosphate buffer (pH 7.2). The brains were removed and postfixed in 4% paraformaldehyde for an additional 24 hr, then transferred to 30% sucrose. The brain was sectioned at 40 μm with a sliding microtome (Leica Microsystems, Wetzlar, Germany).

The procedure for immunohistochemistry staining has been described elsewhere (Ke et al., 2005). Briefly, free-floating sections were incubated in 0.3% H_2O_2 in methanol for 30 min at room temperature and then treated with 0.1% Triton X-100 for 10 min in PBS. The sections were washed with PBS three times, then blocked with 1% BSA and 0.01% Triton X-100 for 1 hr at room temperature. The sections were incubated with an anti-active caspase-3 antibody (at dilution of 1:1,000) overnight at 4°C . Negative controls were performed by omitting the primary antibody. After rinsing in PBS, sections were incubated

with a biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA; 1:200) for 1 hr at room temperature. The sections were washed three times with PBS, then incubated in avidin-biotin-peroxidase complex (Vector; 1:100 in PBS) for 1 hr and developed in 0.05% 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) containing 0.003% H₂O₂ in PBS. The images were recorded with an Olympus microscope (BX61) equipped with a DP70 digital camera.

Cell Culture and Ethanol Exposure Protocol

Human SK-N-MC cells obtained from ATCC were grown in Eagle's MEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 µg/ml gentamicin, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO₂. A method utilizing sealed containers was used to maintain ethanol concentrations in the culture medium. With this method, ethanol concentrations in the culture medium can be accurately maintained (Luo et al., 2001). A pharmacologically relevant concentration of 400 mg/dl was used in this study. In general, the concentration for in vitro studies is higher than that required to produce a similar effect in vivo (Luo et al., 2001).

Cell Transfection and Establishing Stable Transfectants

SK-N-MC cells stably expressing various GSK3β constructs were established as previously described (Ma et al., 2008). V5-tagged GSK3β constructs (wild-type, S9A, and K85R) carried by vector pcDNA3 were generous gifts from Dr. Thilo Hagen (University Hospital Nottingham, Nottingham, United Kingdom). Cell transfection was carried out with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Stable cell clones expressing exogenous GSK3β were selected by the treatment of G418 (600 µg/ml) for 3–4 weeks. Positive clones were verified by the expression of a V5 tag protein as well as the evidence of GSK3β overexpression. The clones expressing the highest levels of exogenous GSK3β were selected for subsequent experiments.

Determination of Cell Viability and Apoptosis

Cell viability was determined by MTT assay as previously described (Chen et al., 2004). Apoptosis was measured by using an Annexin V FITC Apoptosis Detection Kit as previously described (Wang et al., 2007). Briefly, cells (1×10^6) were suspended in 500 µl binding buffer (10 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂) and treated with 10 µl of FITC-conjugated antiannexin V antibody and 10 µl of propidium iodide (PI) for 10 min in the dark at room temperature. The samples were analyzed by flow cytometry (Ex = 488 nm; Em = 530 nm).

STATISTICAL ANALYSIS

Differences among treatment groups were tested by ANOVA. Differences with $P < 0.05$ were considered statistically significant. In cases in which significant differences were detected, specific post hoc comparisons between treatment groups were examined with Student-Newman-Keuls tests.

RESULTS

Lithium Ameliorates Ethanol-Induced Caspase-3 Activation in the Developing Brain

Results from immunohistochemistry and immunoblotting analysis indicated that ethanol caused a duration-dependent activation of caspase-3 in mouse cerebral cortex (Fig. 1). The induction of active caspase-3 was evident at 4 hr after ethanol injection, and maximal activation occurred between 8 and 12 hr after ethanol exposure. It must be noted that caspase-3 activity in homogenized brain tissue reflected both neuronal and nonneuronal cell death. More importantly, pretreatment with lithium, a GSK3β inhibitor, diminished ethanol-

mediated up-regulation of active caspase-3 (Fig. 2). To determine whether ethanol activated GSK3 β , we examined the effect of ethanol on GSK3 β phosphorylation. The activity of GSK3 β is negatively regulated by its phosphorylation at Ser9 but positively by its phosphorylation at Tyr216 (Doble and Woodgett, 2003). As shown in Figure 3, ethanol caused a dephosphorylation of GSK3 β at Ser9 but had little effect on the phosphorylation at Tyr216 or the expression of total GSK3 β . Because Bax is a substrate of GSK3 β and a proapoptotic protein, we sought to determine whether ethanol activated Bax. An antibody directed against an active form of Bax was used to evaluate Bax expression in the developing brain. As shown in Figure 4, ethanol increased the expression of the active form of Bax in the cerebral cortex; more importantly, pretreatment of lithium blocked ethanol-induced up-regulation of active Bax.

Overexpression of Wild-Type and S9A Mutant GSK3 β Sensitizes Cells to Ethanol Neurotoxicity

The results obtained from animal studies suggest that GSK3 β may mediate ethanol neurotoxicity. However, lithium also inhibits GSK3 α and modulates the activity of other protein kinases/phosphatases, such as PKA and PP2A (Jope, 2003; Sasaki et al., 2006; Liang et al., 2008). To determine the role of GSK3 β in ethanol neurotoxicity, we took advantage of an in vitro system (SK-N-MC neuroblastoma cells) in which the expression levels of GSK3 β can be manipulated (Ma et al., 2008). We overexpressed wild-type (WT), S9A mutant, or kinase-deficient (KD) GSK3 β in SK-N-MC cells. The KD GSK3 β functions as a dominant negative protein and specifically inhibits GSK3 β activity (Ma et al., 2007, 2008). The overexpression of WT, S9A, and KD GSK3 β in SK-N-MC cells was verified by the presence of a V5 tag and high levels of GSK3 β (Fig. 5A). With these cell lines, we demonstrated that overexpression of WT or S9A mutant GSK3 β promoted ethanol-induced cell death of SK-N-MC cells, whereas overexpression of KD GSK3 β conferred resistance to ethanol neurotoxicity.

We first determined the effect of ethanol on cell viability by MTT assay. As shown in Figure 5B, WT and S9A cells were much more sensitive to ethanol-induced cell death than vector cells. In KD cells, however, ethanol failed to reduce cell viability. Consistently, ethanol-induced activation of caspase-3 in WT and S9A cells was much stronger than in vector control cells; ethanol had little effect on caspase-3 in KD cells. We used an annexin V assay to quantify ethanol-induced apoptosis. In untreated parental SK-N-MC cells, the basal annexin V-positive cells ranged from 3% to 5%. Ethanol exposure (400 mg/dl, 24 hr) increased annexin V-positive cells to approximately 13% in parental and vector-transfected cells. As shown in Figure 5D, after treated with ethanol for 24 hr, annexin V-positive cells were $13.2\% \pm 1.7\%$, $28.5\% \pm 2.4\%$, $35.2\% \pm 2.1\%$, and $7.2\% \pm 1.6\%$ in vector control, WT, S9A, and KD cells, respectively.

To verify that the effect was mediated by GSK3 β , we used GSK3 β inhibitors (lithium and TDZD-8) to determine whether they were able to abolish hypersensitivity to ethanol caused by overexpression of WT and S9A GSK3 β . TDZD-8 is a highly selective, non-ATP competitive inhibitor of GSK3 β ; it binds to the active site of GSK3 β (Martinez et al., 2002). As shown in Figure 6, these GSK3 β inhibitors effectively reduced ethanol-induced cell death in parental SK-N-MC cells and cells overexpressing WT and S9A GSK3 β . Because our results obtained from animal study suggest that ethanol-induced Bax activation is dependent on GSK3 β (Fig. 4), we sought to determine whether the status of GSK3 β expression affects ethanol-mediated Bax activation. As shown in Figure 7, WT and S9A cells were much more sensitive to ethanol-induced Bax activation than vector control cells. Contrarily, ethanol failed to induce Bax activation in KD cells.

DISCUSSION

GSK3 β is an important regulator of neuronal apoptosis and promotes mitochondria-mediated apoptotic signaling induced by a broad range of insults, including conditions modeling a variety of neurodegenerative diseases (Beurel and Jope, 2006). GSK3 β is also an attractive potential target for reducing neuronal apoptosis in that it is inhibited by lithium, a drug with a long history of therapeutic use in humans for bipolar disorder (Jope, 2003). Lithium directly inhibits both GSK3 α and GSK3 β and also promotes inhibitory phosphorylation on Ser21 and Ser9 of GSK3 α and GSK3 β , respectively (Jope, 2003). Lithium has been shown to be beneficial in reducing neurodegeneration induced by various insults (Everall et al., 2002; Chen et al., 2004; Aghdam and Barger, 2007; Eom et al., 2007). Several studies, including this one, have explored the potential beneficial role of lithium in alleviating ethanol neurotoxicity.

With an animal model of developmental ethanol exposure, we demonstrate that pretreatment of lithium ameliorates an ethanol-induced increase in the proapoptotic index (active caspase-3 and Bax) in the cerebral cortex of 7-day-old mice. Although activation of caspase-3 and Bax is indicative of apoptosis, a direct verification of neuronal death is needed for future studies. Bax regulates mitochondria-associated apoptosis and is a substrate of GSK3 β ; GSK3 β -mediated Bax phosphorylation results in Bax activation (Linseman et al., 2004). Our study indicates that lithium is able to block ethanol-induced up-regulation of active Bax in the developing brain, suggesting that ethanol promotes apoptosis by promoting GSK3 β /Bax interaction. The protection of lithium against ethanol neurotoxicity is supported by two other independent studies. Zhong et al. (2006) also investigated the effect of lithium on cell death in the developing brain. They demonstrate that an intraperitoneal injection of lithium at 15 min after ethanol exposure provides protection against ethanol-induced apoptosis in the striatum and superficial frontal cortex of 7-day-old mice. They also show that ethanol-induced apoptosis and activation of caspase-3 and caspase-9 in cultured cerebellar granule neurons are prevented by lithium. A second study used a similar paradigm of ethanol exposure and lithium treatment (Chakraborty et al., 2008). This study reveals that exposure to lithium 15 min after ethanol injection blocks ethanol-induced caspase-3 activation in the forebrain of 7-day-old mice.

These studies clearly indicate that lithium is effective in protecting against ethanol-induced neurodegeneration. However, the role of GSK3 β in ethanol-induced neuronal death remains unclear, because lithium also inhibits GSK3 α and modulates the activity of other protein kinases/phosphatases, such as PKA and PP2A (Jope, 2003; Sasaki et al., 2006; Liang et al., 2008). The outcomes from studies using other GSK3 β inhibitors are contradicting. Takadera and Ohyashiki (2004) show that two GSK3 inhibitors (SB216763 and alsteropallone) completely eliminated ethanol-induced apoptosis of primary rat cortical neurons in culture. SB216763 inhibits GSK3 α and GSK3 β in an ATP-competitive manner (Coghlan et al., 2000). Alsteropallone is a potent inhibitor for GSK3 β and cyclin-dependent protein kinase 5 (CDK5; Leost et al., 2000). The authors provide further evidence that a specific CDK5 inhibitor fails to offer protection, concluding that GSK3 mediates ethanol neurotoxicity. It appears that lithium protects cerebellar granule neurons from ethanol-induced apoptosis but that SB415286 does not (Zhong et al., 2006). SB415286 is structurally distinct from SB216763 and also inhibits GSK3 α and GSK3 β in an ATP-competitive manner (Coghlan et al., 2000). The authors suggest that GSK3 β is not involved in ethanol-induced neurodegeneration.

Our *in vivo* results support the involvement of GSK3 β in ethanol neurotoxicity, insofar as ethanol exposure induces dephosphorylation of GSK3 β at Ser9 in the developing brain. The dephosphorylation of GSK3 β at Ser9 suggests that ethanol can activate GSK3 β . To clarify

the role of GSK3 β in ethanol neurotoxicity, we use an in vitro model (SK-N-MC cells) to control confounding factors in the animal model, such as differentiating between the direct and the indirect effects of ethanol as well as the efficiency of knocking down GSK3 β in the brain. SK-N-MC cells were selected for the study because they are relatively insensitive to ethanol-induced apoptosis. At 400 mg/dl, ethanol produces only 13% annexin V-positive cells. We overexpressed WT or S9A mutant in SK-N-MC cells. Overexpression of WT or S9A does not significantly increase the basal apoptotic rate in SK-N-MC cells; this is probably a result of insufficient levels of overexpression to cause cytotoxicity. However, either WT or S9A overexpression drastically raises the apoptotic rate to 28% and 35%, respectively, indicating that GSK3 β indeed modulates cellular sensitivity to ethanol. We also observe a decrease of pGSK3 β (Ser9) following ethanol exposure in parental SK-N-MC cells, but not in S9A cells (data not shown). The effect of ethanol on GSK3 β phosphorylation is complex and may vary depending on the level of GSK3 β in a given cell. Further study will be needed to examine the effect of ethanol on SK-N-MC cells expressing various levels of WT or S9A GSK3 β . It is also important to investigate the effect of ethanol on the localization of phosphorylated GSK3 β among these cell lines. To demonstrate the role of GSK3 β in ethanol neurotoxicity further, we express a DN GSK3 β mutant in SK-N-MC cells. The DN GSK3 β specifically inhibits GSK3 β activity (Ma et al., 2007, 2008). SK-N-MC cells overexpressing DN GSK3 β mutant are resistant to ethanol-induced damage. The involvement of GSK3 β is further confirmed by showing that lithium and TDZD abolish hypersensitivity to ethanol caused by WT and S9A overexpression.

The importance of GSK3 β in mediating ethanol neurotoxicity is also signified by its role in growth factor or hormone signaling. For example, insulin and insulin-like growth factor (IGF) are trophic factors for the cerebellum and support the survival of cerebellar neurons. One mechanism by which insulin and IGF maintain neuronal survival is to stimulate the PI3K/AKT pathway, which results in GSK3 β phosphorylation at Ser9 and subsequently represses GSK3 β activity (Duarte et al., 2008). Gestational exposure to ethanol impairs insulin and IGF signaling and reduces neuronal viability in the cerebellum (de la Monde and Wands, 2002; Xu et al., 2003). Ethanol blocks insulin or IGF-mediated PI3K/Akt activation, causing GSK3 β dephosphorylation at Ser9 (de la Monde and Wands, 2002; Xu et al., 2003). These results indicate that ethanol may remove the repression of GSK3 β caused by neurotrophic growth factor or hormone.

Bax, a substrate of GSK3 β , is a proapoptotic protein. It has been demonstrated that GSK3 β directly phosphorylates Bax and promotes proapoptotic action of Bax (Linseman et al., 2004). Our results indicate that lithium blocks ethanol-induced activation of Bax in the developing brain. Furthermore, cells overexpressing WT or S9A GSK3 β were much more sensitive to ethanol-induced Bax activation than parental SK-N-MC cells. Contrarily, blocking GSK3 β activity with a DN GSK3 β mutant confers resistance to ethanol-induced Bax activation. Together these results indicate that Bax is downstream of GSK3 β and mediates ethanol neurotoxicity.

Our study has two important implications. First, the status of GSK3 β (expression levels/activity) in a cell will determine the sensitivity to ethanol. Although GSK3 β is necessary for normal cellular function, excessive GSK3 β activity is harmful. Overexpression of GSK3 β to specific regions of the brain in mice results in neuronal death in these regions (Lucas et al., 2001). It is noted that the S9A cells are more sensitive to ethanol than the WT cells. This probably is due to S9A cells already having higher GSK3 β activity, and ethanol-induced activation of endogenous GSK3 β makes GSK3 β activity reach the threshold that is harmful for cells. Brain regions display differential sensitivity to ethanol, and the vulnerability to ethanol is temporally and regionally dependent. Further study is needed to determine whether there is a correlation between the status of GSK3 β expression and the vulnerability

to ethanol in the developing CNS. Second, because GSK3 β is a potential mediator of ethanol neurotoxicity, developing a specific inhibitor that can cross the blood–brain barrier and efficiently block GSK3 β activity in the brain may be a new avenue for treating ethanol neurotoxicity.

Acknowledgments

Contract grant sponsor: National Institutes of Health; Contract grant number: AA015407; Contract grant sponsor: Ministry of Science and Technology of China; Contract grant number: 2007CB947100; Contract grant sponsor: National Natural Science Foundation of China; Contract grant number: 30870812, 30570580; Contract grant sponsor: Knowledge Innovation Program of the Chinese Academy of Sciences; Contract grant number: KSCX2-YW-R-115; Contract grant sponsor: Science and Technology Commission of Shanghai Municipality; Contract grant number: 07DJ104005; Contract grant sponsor: One Hundred Talents Program of the Chinese Academy of Sciences and Shanghai Pujiang Program (to Z.-J.K.).

REFERENCES

- Aghdam SY, Barger SW. Glycogen synthase kinase-3 in neurodegeneration and neuroprotection: lessons from lithium. *Curr Alzheimer Res.* 2007; 4:21–31. [PubMed: 17316163]
- Beurel E, Jope RS. The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways. *Prog Neurobiol.* 2006; 79:173–189. [PubMed: 16935409]
- Bhat RV, Budd SL. GSK3 β signalling: casting a wide net in Alzheimer's disease. *Neurosignals.* 2002; 11:251–261. [PubMed: 12566926]
- Chakraborty G, Saito M, Mao RF, Wang R, Vadasz C, Saito M. Lithium blocks ethanol-induced modulation of protein kinases in the developing brain. *Biochem Biophys Res Commun.* 2008; 367:597–602. [PubMed: 18190791]
- Chen G, Bower KA, Ma C, Fang S, Thiele CJ, Luo J. Glycogen synthase kinase 3 β (GSK3 β) mediates 6-hydroxydopamine-induced neuronal death. *FASEB J.* 2004; 18:1162–1164. [PubMed: 15132987]
- Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ, Rausch OL, Murphy GJ, Carter PS, Roxbee Cox L, Mills D, Brown MJ, Haigh D, Ward RW, Smith DG, Murray KJ, Reith AD, Holder JC. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem Biol.* 2000; 7:793–803. [PubMed: 11033082]
- Coyle-Rink J, Del Valle L, Sweet T, Khalili K, Amini S. Developmental expression of Wnt signaling factors in mouse brain. *Cancer Biol Ther.* 2002; 1:640–645. [PubMed: 12642687]
- Crowder RJ, Freeman RS. Glycogen synthase kinase-3 β activity is critical for neuronal death caused by inhibiting phosphatidylinositol 3-kinase or Akt but not for death caused by nerve growth factor withdrawal. *J Biol Chem.* 2000; 275:34266–34271. [PubMed: 10954722]
- de la Monte SM, Wands JR. Chronic gestational exposure to ethanol impairs insulin-stimulated survival and mitochondrial function in cerebellar neurons. *Cell Mol Life Sci.* 2002; 59:882–893. [PubMed: 12088287]
- Doble BW, Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci.* 2003; 116:1175–1186. [PubMed: 12615961]
- Duarte AI, Santos P, Oliveira CR, Santos MS, Rego AC. Insulin neuroprotection against oxidative stress is mediated by Akt and GSK3 β signaling pathways and changes in protein expression. *Biochim Biophys Acta.* 2008; 1783:994–1002. [PubMed: 18348871]
- Eom TY, Roth KA, Jope RS. Neural precursor cells are protected from apoptosis induced by trophic factor withdrawal or genotoxic stress by inhibitors of glycogen synthase kinase 3. *J Biol Chem.* 2007; 282:22856–22864. [PubMed: 17548347]
- Everall IP, Bell C, Mallory M, Langford D, Adame A, Rockenstein E, Masliah E. Lithium ameliorates HIV-gp120-mediated neurotoxicity. *Mol Cell Neurosci.* 2002; 21:493–501. [PubMed: 12498789]
- Grimes CA, Jope RS. The multifaceted roles of glycogen synthase kinase 3 β in cellular signaling. *Prog Neurobiol.* 2001; 65:391–426. [PubMed: 11527574]
- Hetman M, Cavanaugh JE, Kimelman D, Xia Z. Role of glycogen synthase kinase-3 β in neuronal apoptosis induced by trophic withdrawal. *J Neurosci.* 2000; 20:2567–2574. [PubMed: 10729337]

- Joje RS. Lithium and GSK-3: one inhibitor, two inhibitory actions, multiple outcomes. *Trends Pharmacol Sci.* 2003; 24:441–443. [PubMed: 12967765]
- Kaytor MD, Orr HT. The GSK3 beta signaling cascade and neurodegenerative disease. *Curr Opin Neurobiol.* 2002; 12:275–278. [PubMed: 12049933]
- Ke ZJ, Calingasan NY, Karuppagounder SS, DeGiorgio LA, Volpe BT, Gibson GE. CD40L deletion delays neuronal death in a model of neurodegeneration due to mild impairment of oxidative metabolism. *J Neuroimmunol.* 2005; 164:85–92. [PubMed: 15904977]
- Leost M, Schultz C, Link A, Wu YZ, Biernat J, Mandelkow EM, Bibb JA, Snyder GL, Greengard P, Zaharevitz DW, Gussio R, Senderowicz AM, Sausville EA, Kunick C, Meijer L. Paullones are potent inhibitors of glycogen synthase kinase-3beta and cyclin-dependent kinase 5/p25. *Eur J Biochem.* 2000; 267:5983–5994. [PubMed: 10998059]
- Leroy K, Brion JP. Developmental expression and localization of glycogen synthase kinase-3beta in rat brain. *J Chem Neuroanat.* 1999; 16:279–293. [PubMed: 10450875]
- Liang MH, Wendland JR, Chuang DM. Lithium inhibits Smad3/4 transactivation via increased CREB activity induced by enhanced PKA and AKT signaling. *Mol Cell Neurosci.* 2008; 37:440–453. [PubMed: 18077182]
- Linseman DA, Butts BD, Precht TA, Phelps RA, Le SS, Laessig TA, Bouchard RJ, Florez-McClure ML, Heidenreich KA. Glycogen synthase kinase-3beta phosphorylates Bax and promotes its mitochondrial localization during neuronal apoptosis. *J Neurosci.* 2004; 24:9993–10002. [PubMed: 15525785]
- Lucas JJ, Hernandez F, Gomez-Ramos P, Moran MA, Hen R, Avila J. Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. *EMBO J.* 2001; 20:27–39. [PubMed: 11226152]
- Luo J. Glycogen synthase kinase 3beta (GSK3beta) in tumorigenesis and cancer chemotherapy. *Cancer Lett.* 2009; 273:194–200. [PubMed: 18606491]
- Luo J, Lindström CL, Donahue A, Miller MW. Differential effects of ethanol on the expression of cyclo-oxygenase in cultured cortical astrocytes and neurons. *J Neurochem.* 2001; 76:1354–1363. [PubMed: 11238720]
- Ma C, Wang J, Gao Y, Gao T, Chen G, Bower KA, Odetallah M, Ding M, Ke ZJ, Luo J. The role of glycogen synthase kinase 3 beta in the transformation of epidermal cells. *Cancer Res.* 2007; 67:7756–7764. [PubMed: 17699780]
- Ma C, Bower KA, Chen G, Shi X, Ke ZJ, Luo J. Interaction between ERK and GSK3beta mediates basic fibroblast growth factor-induced apoptosis in SK-N-MC neuroblastoma cells. *J Biol Chem.* 2008; 283:9248–9256. [PubMed: 18263590]
- Martinez A, Alonso M, Castro A, Pérez C, Moreno FJ. First non-ATP competitive glycogen synthase kinase 3 beta (GSK-3beta) inhibitors: thiadiazolidinones (TDZD) as potential drugs for the treatment of Alzheimer's disease. *J Med Chem.* 2002; 45:1292–1299. [PubMed: 11881998]
- May PA, Gossage JP. Estimating the prevalence of fetal alcohol syndrome. *Alcohol Res Health.* 2001; 25:159–167. [PubMed: 11810953]
- Olney JW, Tenkova T, Dikranian K, Muglia LJ, Jermakowicz WJ, D'sa C, Roth KA. Ethanol-induced caspase-3 activation in the in vivo developing mouse brain. *Neurobiol Dis.* 2002; 9:205–219. [PubMed: 11895372]
- Riley EP, McGee CL. Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior. *Exp Biol Med.* 2005; 230:357–365.
- Sasaki T, Han F, Shioda N, Moriguchi S, Kasahara J, Ishiguro K, Fukunaga K. Lithium-induced activation of Akt and CaM kinase II contributes to its neuroprotective action in a rat microsphere embolism model. *Brain Res.* 2006; 1108:98–106. [PubMed: 16843447]
- Song L, De Sarno P, Joje RS. Central role of glycogen synthase kinase-3beta in endoplasmic reticulum stress-induced caspase-3 activation. *J Biol Chem.* 2002; 277:44701–44708. [PubMed: 12228224]
- Stratton, K.; Howe, C.; Battagila, F., editors. Fetal alcohol syndrome: diagnosis, epidemiology, prevention, and treatment. National Academy Press; Washington, DC: 1996.

- Takadera T, Ohyashiki T. Glycogen synthase kinase-3 inhibitors prevent caspase-dependent apoptosis induced by ethanol in cultured rat cortical neurons. *Eur J Pharmacol.* 2004; 499:239–245. [PubMed: 15381045]
- Wang X, Fan Z, Wang B, Luo J, Ke ZJ. Activation of double-stranded RNA-activated protein kinase by mild impairment of oxidative metabolism in neurons. *J Neurochem.* 2007; 103:2380–2390. [PubMed: 17953670]
- Xu J, Yeon JE, Chang H, Tison G, Chen GJ, Wands J, de la Monde S. Ethanol impairs insulin-stimulated neuronal survival in the developing brain: role of PTEN phosphatase. *J Biol Chem.* 2003; 278:26929–26937. [PubMed: 12700235]
- Zhong J, Yang X, Yao W, Lee W. Lithium protects ethanol-induced neuronal apoptosis. *Biochem Biophys Res Commun.* 2006; 350:905–910. [PubMed: 17045245]

Fig. 1. Ethanol-induced caspase-3 activation in the cerebral cortex. Mice were injected with ethanol (2.5 g/kg, 20% solution in saline) or saline for specified times as described in Materials and Methods. **A:** The expression of active caspase-3 in the cerebral cortex was analyzed via immunohistochemistry. **B:** The cerebral cortex was dissected, and protein was extracted. The expression of active caspase-3 was then determined with immunoblotting. The experiment was replicated three times. Scale bar = 100 μ m.

Fig. 2.

Effect of LiCl on caspase-3 activation. **A:** Mice were injected with LiCl (40 mg/kg body weight; 4 mg/ml) or saline. Twelve hours after injection, the cerebral cortex was dissected, and the expression of active caspase-3 was analyzed via immunohistochemistry as described in Materials and Methods. **B:** Mice were injected with LiCl (40 mg/kg body weight; 4 mg/ml) 30 min prior to ethanol exposure. Mice were injected subcutaneously with ethanol (2.5 g/kg, 20% solution in saline), and, at specified times after ethanol injection, the expression of active caspase-3 was analyzed via immunohistochemistry. **C:** Mice were injected with ethanol with/without lithium as described above. The cerebral cortex was dissected, and protein was extracted. The expression of active caspase-3 was then determined via immunoblotting. The experiment was replicated three times. Scale bars = 100 μ m.

Fig. 3.

Effect of ethanol on GSK3β. **A:** Mice were injected with ethanol (2.5 g/kg) or saline for specified times. The cerebral cortex was dissected, and protein was extracted. The expression of p-GSK3β(Ser9), p-GSK3β(Tyr216), GSK3β and tubulin was determined via immunoblotting as described in Material and Methods. **B:** The relative amounts of p-GSK3β(Ser9) were quantified microdensitometrically and normalized to the expression of total GSK3β. ***Significant difference from matched untreated controls, $P < 0.01$. The experiment was replicated three times.

Fig. 4.

Effect of ethanol and LiCl on Bax expression. **A:** Mice were injected with LiCl (40 mg/kg body weight; 4 mg/ml) 30 min prior to ethanol exposure. At specified times after ethanol injection, the cerebral cortex was dissected and the expression of active Bax was examined via immunoblotting as described in Materials and Methods. **B:** The relative amounts of active Bax were quantified microdensitometrically and normalized to the expression of tubulin. The expression was replicated three times.

Fig. 5.

The status of GSK3β and sensitivity to ethanol-induced cell death. **A:** SK-N-MC cells stably overexpressing WT, S9A, and KD GSK3β were established as described in Materials and Methods. The expression of the V5 tag, GSK3β, and cyclin D1 was determined via immunoblotting. **B:** SK-N-MC cells stably overexpressing WT, S9A, and KD GSK3β were exposed to ethanol (0 or 400 mg/dl) for 48 hr. Cell viability was determined by MTT assay as described in Material and Methods, and a relative MTT reading was calculated.

*Significant difference from matched untreated controls. [#]Significant difference from ethanol-treated vector cells. [&]Significant difference from ethanol-treated WT cells. **C:** SK-N-MC cells stably overexpressing WT, S9A, and KD GSK3β were exposed to ethanol (0 or 400 mg/dl) for 12 hr. The expression of active caspase-3 was determined via immunoblotting. Lanes 1, 3, 5, 7: Controls. Lanes 2, 4, 6, 8: Ethanol-treated groups. **D:** SK-N-MC cells stably overexpressing WT, S9A, and KD GSK3β were exposed to ethanol (400 mg/dl) for 12 hr. Annexin V-positive cells were determined via flow cytometry as described in Materials and Methods. The percentage of annexin V-positive cells was calculated and shown in the figure. The experiment was replicated three times.

Fig. 6.

Effect of GSK3 β inhibitors on cell viability. SK-N-MC cells stably overexpressing WT, S9A, and KD GSK3 β were pretreated with GSK3 β inhibitors (lithium 20 mM and TDZD-8 10 μ M) for 30 min, then exposed to ethanol (0 or 400 mg/dl) for 48 hr. Cell viability was determined via MTT assay. The relative MTT reading was expressed as percentage of the untreated controls. The experiment was replicated three times. *Significant difference from ethanol-treated cells without treatment of GSK3 β inhibitors.

Fig. 7.

Effect of ethanol on the expression of active Bax in SK-N-MC cells stably overexpressing WT, S9A, and KD GSK3 β . **A:** SK-N-MC cells stably overexpressing WT, S9A, and KD GSK3 β were exposed to ethanol (0 or 400 mg/dl) for 6 or 12 hr. The expression of active Bax was determined via immunoblotting. **B:** The relative amounts of active Bax were quantified microdensitometrically and normalized to the expression of actin. The experiment was replicated three times.