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Pharmacological inhibition of Glycogen Synthase Kinase 3 increases operant alcohol self-administration in a manner associated with altered pGSK-3 β , PICK1 and GluA2 protein expression in the reward pathway of male C57BL/6J mice

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Abstract

Glycogen synthase kinase 3 (GSK-3) is a constitutively active serine-threonine kinase that regulates numerous signaling pathways and has been implicated in neurodegenerative and neuropsychiatric diseases including Alzheimer's, Parkinson's, schizophrenia, and bipolar disorder. Evidence indicates that alcohol exposure increases GSK-3 β (ser9) phosphorylation (pGSK-3 β); however, few studies have investigated whether GSK-3 regulates the positive reinforcing effects of alcohol, which drive repetitive drug use. This study aimed to address the potential role of GSK-3 in alcohol self-administration by investigating whether direct pharmacological inhibition of GSK-3 alters the positive reinforcing effect of alcohol in mice. Male C57BL/6J mice were trained to lever press on a fixed-ratio 4 schedule of sweetened alcohol or sucrose-only reinforcement in operant conditioning chambers. The GSK-3 inhibitor CHIR 99021 trihydrochloride (0–10 mg/kg, ip) was injected 45-min prior to self-administration sessions in a counterbalanced design. After completion of the self-administration dose-effect curve, potential locomotor effects of the GSK-3 inhibitor were assessed. To determine molecular efficacy, CHIR 99021 (10 mg/kg, ip) was evaluated on pGSK-3 β , GSK-3 β PICK1, and AMPA receptor GluA2 subunit protein expression in amygdala, nucleus accumbens (NAcb), and frontal cortex in the absence of alcohol. CHIR 99021 (10 mg/kg) dose-dependently increased alcohol reinforced responding with no effect on sucrose self-administration or locomotor activity. CHIR 99021 (10 mg/kg) significantly decreased pGSK-3 β expression in all brain regions tested, reduced PICK1 and increased GluA2 total expression only in the NAcb. We conclude that GSK-3 inhibition increased the reinforcing effects of alcohol in mice. This was associated with reduced pGSK-3 β and PICK1, and increased GluA2 protein expression. Given prior results showing that AMPA receptor activity regulates alcohol self-administration, we propose that signaling through the GSK-3 / PICK1 / GluA2 molecular pathway drives the positive reinforcing effects of the drug, which are required for abuse liability.

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Keywords

ethanol; alcohol drinking; self-administration; reinforcement; reward; GluA2; GSK3; mouse

Introduction

Glycogen synthase kinase 3 (GSK-3) is an abundant serine-threonine kinase that is found in almost all eukaryotic organisms. GSK-3 is expressed in the mammalian central nervous system in two known isoforms, GSK-3 α and GSK-3 β . GSK-3 β is more abundant in the brain than GSK-3 α (Embi et al., 1980; Woodgett, 1990; Leroy and Brion, 1999), but both isoforms are expressed throughout the neural axis. GSK-3 α activity is inhibited when phosphorylation occurs at Ser-21 and GSK-3 β activity is inhibited when phosphorylation occurs at Ser9. Although initially characterized as an enzyme that regulates glycogen synthesis in response to insulin (Embi et al., 1980), GSK-3 phosphorylates numerous other substrates, including ATP citrate lyase, c-Jun, c-Myc, CRMP2, Tau, PKA, and CREB, that regulate a wide array of endocrine, growth, immune, and neurobiological functions and diseases (Hur and Zhou, 2010; Sutherland, 2011).

Dysregulated GSK-3 activity is associated with several neurological and neuropsychiatric diseases including schizophrenia, Alzheimer's disease, depression, and addiction (Li et al., 2004; Beaulieu et al., 2008; Cai et al., 2012; Emamian, 2012; Llorens-Martin et al., 2014; Huang et al., 2015; Kirouac et al., 2017). Accordingly, home-cage alcohol drinking is associated with increased GSK-3 β (Ser9) phosphorylation in the rat nucleus accumbens (Liu et al., 2017) and mouse dorsomedial striatum (Cheng et al., 2017). Moreover, adult mice exposed to prenatal alcohol via maternal drinking show elevated GSK-3 β (Ser9) phosphorylation in the hippocampus, which suggests long-term disruption of kinase signaling (Cunningham et al., 2017). Although the molecular mechanism by which alcohol upregulates GSK phosphorylation is not known, CaMKII had been shown to phosphorylate GSK-3 α (Ser-21) and GSK-3 β (Ser9) in cerebellar cells (Song et al., 2010). Our work has shown that alcohol self-administration, or cue-induced reinstatement of alcohol-seeking behavior, is associated with increased CaMKII phosphorylation (activation) in reward-related brain regions of adult mice, including the nucleus accumbens, amygdala, and piriform cortex (Salling et al., 2016, 2017). This suggests that increased CaMKII activity following alcohol self-administration or relapse-like behavior may lead to downstream phosphorylation of GSK-3 and potential mechanistic regulation of alcohol self-administration. Indeed, inhibition of CaMKII (Faccidomo et al., 2016; Salling et al., 2016) and other upstream regulators of GSK-3, such as Akt and PI3K (Cuzzoli et al., 2009; Neasta et al., 2011), regulates alcohol drinking and/or self-administration in rats and mice.

A key downstream action of GSK-3 β is its ability to phosphorylate Protein Interacting with C kinase (PICK1) (Yagishita et al., 2015). PICK1 is a membrane bound PDZ domain protein that is highly expressed in neurons where it promotes internalization of GluA2 subunit-containing α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Xia et al., 1999; Cunningham et al., 2017). Reduced membrane expression of GluA2-containing AMPA receptors is associated with long-term depression (LTD) and cognitive decline

associated with neurodegenerative disorders, such as Alzheimer's disease (Luscher et al., 1999; Malenka, 2003; Volk et al., 2010; Jurado, 2017). We have shown that activation of AMPA receptors increases alcohol self-administration and cue-induced reinstatement of alcohol-seeking behavior (Cannady et al., 2013, 2017), suggesting that upregulation of AMPA receptor activity drives the reinforcing effects of alcohol (Salling et al., 2016). Interestingly, recent evidence shows that overexpression of GSK-3 β in mouse medial prefrontal cortex (mPFC) is associated with increased home-cage alcohol drinking in mice, and that systemic pharmacological inhibition of the kinase decreases alcohol self-administration in rats (van der Vaart et al., 2018). However, it remains unknown if GSK-3 mediated changes in the positive reinforcement function of alcohol are associated with altered phosphorylation at the alcohol-sensitive GSK-3 β (Ser9) site in the reward pathway.

To address this gap in knowledge, the present set of experiments was designed to first determine if inhibition of GSK-3 functionally alters the positive reinforcing effect of alcohol in mice using the pharmacological inhibitor of GSK-3, CHIR 99021 (Ring et al., 2003; Ye et al., 2012), in a well-characterized model of operant alcohol self-administration (Faccidomo et al., 2009, 2015, 2016). Given that GSK-3 phosphorylation is increased following alcohol drinking (Neasta et al., 2011), we hypothesized that pharmacological inhibition of GSK-3 activity might selectively increase the positive reinforcing effects of alcohol and promote escalated operant alcohol self-administration; thus, establishing a potential role for GSK-3 in alcohol addiction. Second, we sought to determine if systemic administration of the GSK-3 inhibitor is associated with altered GSK-3 β protein expression or GSK-3 β (Ser9) phosphorylation. Finally, we evaluated potential effects of GSK-3 inhibition on PICK1 and GluA2 protein expression as an index of downstream kinase activity.

Methods

Subjects

Ten-week-old male C57BL/6J mice (n=29) arrived from Jackson Laboratory and were group-housed upon arrival (4/cage) in clear polycarbonate cages (28 × 17 × 14 cm). Cages were lined with corn cob bedding and a cylindrical PVC pipe (8cm long x 5cm wide) and cotton nestlet were provided for environmental enrichment. Purina rodent chow and water were freely available. The vivarium was maintained on a 12h:12h reverse light/dark cycle (lights off at 0700) with temperature maintained at 21 ± 1°C and humidity maintained at 40 ± 2%. All experiments were approved by the Institutional Care and Use Committee at the University of North Carolina-Chapel Hill and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

Operant Alcohol or Sucrose Self-Administration

Mice were trained to lever press under conditions of sweetened alcohol or sucrose reinforcement using well-characterized procedures (Olive et al., 2000; Hodge et al., 2006; Salling et al., 2008, 2016; Agoglia et al., 2015b; Faccidomo et al., 2016). One week after arrival, mice (n=15) were given free access to either a 9% Alcohol (v/v)/ 2% Sucrose (w/v) solution (Alcohol Self-Administration Group) or a 2% Sucrose (w/v) solution (Sucrose Self-

Administration Group), along with water, in their home cage for two weeks. We have previously found that extended home cage access to the reinforcing solution familiarizes mice to the reinforcer and leads to reliable and consistent intake (Faccidomo et al. 2009, 2015, 20168).

After two weeks of home cage drinking, mice began training in operant conditioning chambers to lever press for a delivery of sweetened alcohol or sucrose. All self-administration sessions were conducted in operant conditioning chambers (Med Associates) that were each housed within a sound attenuating cubicle equipped with a 28V fan to provide ventilation and mask external noise. Opposite walls of the chamber each contained a stainless steel ultra-sensitive retractable lever located directly beneath a cue light.

Responding on only one of the levers (the “active” lever) was reinforced. Responses on the “inactive” lever were recorded but produced no programmed consequences. A drinking trough was located adjacent to the active lever and reinforcement delivery was accompanied by a brief illumination of the cue light (800ms) and pump sound, both of which served as secondary cues. A photobeam spanned the drinking trough to record head pokes into the trough. Number of head pokes per reinforcer was calculated as an index of consummatory behavior. Responses during the reinforcer delivery were measured but did not count towards the response requirement (time-out period). The chamber and pump were connected to an interface and computer that recorded the input and behavioral output of each mouse (MED-PC for Windows v.4.1). At the end of each session, drinking troughs were checked for fluid to verify consumption of the reinforcing solution.

The acquisition of operant alcohol and sucrose self-administration was established as previously described in (Salling et al., 2008; Faccidomo et al., 2009). Initially, mice were water restricted for 23 hours and were then placed into an operant conditioning chamber for a 16 hr overnight session during which they performed an operant response (lever press) that was reinforced with a delivery (.014 ml) of either alcohol or sucrose into the drinking trough. During the first overnight session, mice responded on a Fixed-Ratio 1 (FR 1) schedule of reinforcement. During the 2nd session, the response requirement was increased from FR 1 to FR 2 to FR 4 in increments of 25 (ie: after receiving the 25th reinforcement, the response requirement increased to FR 2, etc.). During the 3rd session, the response requirement was set at FR 4 for 16 hrs. After three 16 hr overnight sessions, all subsequent sessions were shortened to 1 hr in duration, behavior was maintained at a FR4 response requirement and sessions were conducted in the dark phase, between 12.00–16.00h, 5 days/week. We have previously reported that free-feeding C57BL/6J mice are motivated to lever press and consume a 9% alcohol + 2% sucrose solution. Using these parameters, we obtain reliable and stable levels of alcohol consumption (measured by checking the trough at the end of each session along with monitoring the number of head pokes into the drinking trough) in C57BL/6J mice. Our prior research has shown that these procedures result in pharmacologically relevant blood alcohol concentrations (BAC) of ~50 mg/dL at the end of 1-h sessions (Salling et al., 2008; Faccidomo et al., 2009).

Experiment 1: Effect of GSK inhibition on alcohol and sucrose self-administration

After stable levels of operant responding were obtained (ca. 45 days; Figure 1), alcohol (n=6) and sucrose (n=9) mice were habituated to i.p. injection of saline (0.9%) until levels of self-administration stabilized after injection (ca. 2–4 injections). Next, the selective GSK-3 inhibitor CHIR 99021 (0, 1, 3, or 10 mg/kg, i.p.; Tocris Bioscience) was injected 45 min prior to the start of the self-administration session. Drug dosing parameters were chosen based on work showing that CHIR 99021 (0 – 25 mg/kg, i.p.; 1-h pretreatment) dose-dependently attenuated amphetamine-induced hyperlocomotion in C57BL/6J mice (Pan et al., 2011). Doses were administered using a Latin Square design and a maximum of 2 drug injections per week were conducted to ensure that responding returned to baseline after drug administration.

Experiment 2: Effect of GSK inhibition on locomotor activity in an open field

Open field activity was measured in Plexiglas activity monitor chambers (27.9 cm²; ENV-510, Med Associates) as previously described (Stevenson et al., 2008; Faccidomo et al., 2009; Agoglia et al., 2016). Two sets of 16 pulse-modulated infrared photobeams recorded X-Y ambulatory movements. The mouse's position in the open field was assessed every 100ms to quantify the distance traveled (cm) throughout the session. Each open field chamber was connected to a computer that recorded the locomotor data for each session.

Initially, mice were habituated to the open field apparatus during a single 2-h session. One week later, mice with a history of alcohol (n=6) or sucrose self-administration (n=4) were given either an i.p. injection of NaCl or 10 mg/kg CHIR 99021. Forty-five min after injection, in accordance with the inject to test interval for Experiment 1, they were placed in the open field for 1 h. The next week, the alternate treatment was administered such that each mouse received both vehicle and drug prior to open field activity to account for inter-mouse variability in baseline activity level.

Experiment 3: Effect of GSK inhibition on, GSK-3, PICK1, and GluA2 expression in the brain

A second group of naïve male mice were injected, i.p., with either Vehicle (n=7) or 10 mg/kg CHIR 99021 (n=7). Forty-five minutes later they were rapidly decapitated, and brains were quickly removed and flash-frozen in cold (–40°C) isopentane (2-methylbutane; Sigma-Aldrich) for immunoblot analysis as previously described (Wilkie et al., 2007; Agoglia et al., 2015a; Agoglia et al., 2015b; Faccidomo et al., 2018).

Thick coronal sections (0.8mm for PFC, 1mm for NAC and AMYG) were taken on a cryostat (Leica Biosystems) and with consultation with a mouse brain atlas (Franklin and Paxinos, 2008), circular punches were used (1mm diameter) to collect tissue samples from the PFC, NAC and AMYG. These brain regions are known to regulate the reinforcing effects of alcohol (Hodge et al., 1992; Samson and Hodge, 1993; Hodge et al., 1996, 1997; Schroeder et al., 2003; Faccidomo et al., 2015; Salling et al., 2016; Cannady et al., 2017). Tissue from each region (n=5 for vehicle, n=7 for CHIR) was homogenized using an ultrasonifier in 100ul of a buffer containing SDS, protease and phosphatase inhibitors

(Sigma Aldrich). Next, a BCA assay (Thermo Fisher Scientific), was performed to quantify the total protein concentration in each sample.

Protein samples were prepared by combining 10µg of sample, 1X reducing agent, 4X substrate buffer and sterile water (25µl total volume). Samples were vortexed, briefly warmed (70°C) and loaded into 4–15% Criterion TGX precast gels (Bio-Rad) with 1X Tris-Glycine-SDS running buffer (Bio-Rad). Molecular weight ladders (SeeBluePlus2; Thermo Fisher Scientific) were loaded into lanes 1 and 18 of the gel. Gel electrophoresis was conducted at 200V for approximately 35 min. Proteins were transferred to PVDF membranes using an iBlot® Dry blotting system (Thermo Fisher Scientific).

All membranes were initially blocked for 2 hours at room temperature in 3–5% normal goat serum (Vector Labs) and were incubated with the following primary antibodies overnight at 4°C: monoclonal rabbit anti-pGSK-3β (Ser9) (1:1000, Cell Signaling), monoclonal mouse anti-GSK-3 (1:2000, Millipore), monoclonal mouse anti-PICK1 (1:2000, NeuroMab), monoclonal rabbit anti-GluA2 (1:1500, Cell Signaling), monoclonal mouse anti-GAPDH (1:10,000, Advanced Immunochemical). Antibody specificity was independently confirmed by each vendor. The following day, membranes were washed immediately prior to a 1-hour incubation in a horseradish peroxidase-conjugated secondary antibody (rabbit anti-mouse [1:10,000] or goat anti-rabbit [1:10,000] in blocking buffer; Jackson ImmunoResearch, West Grove, PA). Next, the chemiluminescent signal was visualized and digitally captured using GE Healthcare Amersham ECL Prime and a digital imager (ImageQuant LAS 4000, GE Healthcare Life Sciences). The optical density of each band at was quantified using ImageQuant TL software and bands were normalized to the optical density of GAPDH (loading control) and expressed as a ratio (protein/GAPDH). Values that had poor expression of GAPDH or that exceeded 2SD from the mean, were excluded from analysis (n=1). Within each individual blot, data were expressed as percentage of NaCl.

Drugs

All alcohol solutions (v/v) were prepared by diluting 95% ethanol (Pharmco Products Inc.; Brookfield, CT) with water. The potent and selective GSK-3 inhibitor, CHIR 99021 (6-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1*H*-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile trihydrochloride (Tocris Bioscience; Ellisville, MO) was dissolved in 0.9% sodium chloride (NaCl) immediately prior to injection. All injections were conducted with a 27-gauge needle and a 1mL/100g injection volume.

Data Analysis

Two-way RM ANOVAs (DOSE x TIME) were used to assess dose- and time-dependent effects of CHIR 99021 (0 – 10 mg/kg) on operant response rate expressed as cumulative responses per 5-min time bins. Dose-effect curves for 1-h session totals of additional behaviors (number of reinforcements, inactive lever responses, dose consumed) were analyzed using a one-way RM ANOVA. Locomotor activity (total distance traveled/1h) was analyzed using a one-way RM ANOVA for each reinforcing solution. One-tailed t-tests were

conducted for all western blot analyses. Dunnett's multiple-comparison test was conducted when appropriate and the α -level was set at 0.05 for all statistical tests.

Results

Experiment 1: Effect of GSK inhibition on alcohol and sucrose self-administration

Alcohol—Systemic inhibition of GSK-3 with CHIR 99021 significantly increased alcohol-reinforced responding in both a time- and dose-dependent manner, and an interaction between the two factors was observed (Figure 1A). Specifically, alcohol-reinforced responding increased as a function of CHIR 99021 dose [$F(3, 12)=3.69$, $P<0.05$] and session time [$F(11, 44)=58.51$, $P<0.001$]. In addition, there was a significant interaction between CHIR 99021 dose and session time [$F(33, 132)=3.02$, $P<0.001$] indicating that the effect of GSK-3 inhibition depended on session time. Multiple comparison analysis revealed that CHIR 99021 (10 mg/kg) significantly increased the rate of alcohol-reinforced responding as compared to vehicle and that this effect emerged during and persisted throughout the second half of the 1-h session (Figure 1A). The lower doses of CHIR 99021 did not alter alcohol reinforced response rate at any point throughout the session.

As predicted by the effect of CHIR 99021 on response rate, the total number of reinforcers obtained per session for alcohol self-administering mice was significantly increased by CHIR 99021 [$F(3, 12)=4.81$, $P<0.025$] following the 10 (mg/kg) dose (Figure 1B). Likewise, alcohol intake followed a similar pattern after injection of vehicle and CHIR 99021. The average alcohol dose (g/kg) consumed increased as a function of CHIR 99021 ($F(3, 12)=4.86$, $P<0.02$). Multiple comparison analysis showed that a significant increase in alcohol intake after injection of CHIR 99021 (10 mg/kg, Figure 1D). Analysis of head pokes into the drinking trough showed that mice engaged in approximately two head pokes per reinforcer, regardless of the total number of reinforcers earned (Figure 1C). Inactive lever responding and head pokes per reinforcer were unaffected by drug treatment, and the percentage of responses on the active lever was maintained at about 89% regardless of drug dose.

Sucrose control—To assess potential alcohol specificity, effects of CHIR 99021 (0 – 10 mg/kg) were tested in a parallel group of behavior-matched sucrose-only controls. Systemic administration of CHIR 99021 had no effect on sucrose-reinforced responding as measured by response rate, number of reinforcers, and head pokes per reinforcer were unchanged by CHIR 99021 (0 – 10 mg/kg; Figure 2A – C). The percentage of responses on the active lever was maintained at 80% regardless of CHIR 99021 dosage, showing a high degree of specificity for the active lever and preference for sucrose. Furthermore, there was no effect on responses on the inactive lever (data not shown). Thus, under the present experimental conditions, the pharmacological effect of CHIR 99021 appears to be specific to alcohol-reinforced responding.

Experiment 2: Effect of GSK inhibition on locomotor activity in an open field

Neither experimental group (alcohol or sucrose controls) showed altered open field activity after injection of 0 or 10 mg/kg CHIR 99021 (Figure 3), indicating that the effect of GSK-3

inhibition on operant alcohol reinforced responding was not associated altered motor activity.

Experiment 3: Effect of CHIR 99021 on GSK-3 β phosphorylation and total expression

Expression of pGSK-3 β (ser9) and total GSK-3 β protein were quantified via immunoblot in whole-cell lysates taken from the prefrontal cortex, nucleus accumbens and amygdala, after systemic injection of the GSK-3 inhibitor CHIR 99021 (0 or 10 mg/kg, i.p.). A significant decrease in the level of pGSK-3 β /GAPDH expression was observed in all three brain regions after injection of CHIR 99021 [PFC: $t(11)=4.75$, $P<0.001$; NAC: $t(10)=2.04$, $p<0.05$; AMYG: $t(9)=2.88$, $p<0.01$; Figure 4A–C, left panels] as compared to vehicle control. Total GSK-3 β protein expression remained unchanged (Figure 4A–C, middle panels) and consequently, the protein ratios of phosphorylated / total GSK-3 β were significantly reduced by the GSK-3 inhibitor [PFC: $t(11)=4.82$, $P<0.001$; AMYG: $t(9)=3.24$, $p<0.01$; Figure 4A–C, right panels]. The protein ratio in the NAC approached significance [$t(10)=1.64$, $p=0.067$].

Experiment 4: Effect of CHIR 99021 on PICK1 and GluA2 protein expression

Using the same tissue samples, expression of the GluA2 subunit of the AMPA receptor and PICK1 were quantified via immunoblot. CHIR 99021 (0 or 10 mg/kg) significantly increased GluA2 protein expression in the nucleus accumbens [$t(10)=1.91$, $P<0.05$; Figure 5A] in the absence of significant effects in the prefrontal cortex and amygdala (data not shown). In contrast, protein expression of PICK1, a protein known to facilitate trafficking and internalization of AMPA receptors, was significantly reduced in the nucleus accumbens [$t(12)=2.25$, $P<0.025$; Figure 5B], with no changes seen in amygdala or frontal cortex (data not shown).

Discussion

The fundamental behavioral process of reinforcement reflects the tendency of all animals, human and non-human, to repeat responses that produce a desired outcome. Accordingly, reinforcement mechanisms are required for the repetitive nature of alcohol seeking-behavior during both the initial binge/intoxication and subsequent stages of addiction (Stolerman, 1992; Wise and Koob, 2014). The goal of these preclinical experiments was to determine if GSK-3 regulates the positive reinforcing effects of alcohol in non-dependent male C57BL/6J mice. The results showed that systemic administration of the potent and selective GSK-3 inhibitor CHIR 99021 dose-dependently and selectively increased operant alcohol self-administration that was maintained on a fixed-ratio 4 schedule of sweetened alcohol reinforcement. Overall, these results suggest that GSK-3 plays a functional role in the reinforcing effects of alcohol, which drive repetitive drug-seeking behavior in addiction.

Evidence indicates that intermittent home-cage alcohol drinking is associated with an increase in pGSK-3 β (ser9) in rat nucleus accumbens (Liu et al., 2017) and mouse dorsomedial striatum (Cheng et al., 2017). This raises the possibility that GSK-3 β phosphorylation is a target of self-administered alcohol that regulates the reinforcing effects of the drug via simultaneous activity in multiple reward-related brain regions. To address

this hypothesis, we tested effects of systemic administration of the selective ATP competitive GSK-3 inhibitor CHIR 99021 (0 – 10 mg/kg, i.p.) on operant alcohol or sucrose self-administration sessions in separate groups of mice. Analysis of alcohol- and sucrose-reinforced response rate, a direct measure of reinforcement function, showed that the highest dose of CHIR 99021 selectively increased response rate during the 30 – 60 min interval of the session, indicating that behavioral effects of the inhibitor emerged during the maintenance of self-administration (Samson and Hodge, 1996). This pattern of alcohol-reinforced responding resulted in significant increases in the number of alcohol reinforcers obtained and self-administered alcohol dosage (g/kg/1-hr). No changes were observed in sucrose-reinforced responding or on spontaneous locomotor activity in an open field. Complementary evidence showed that GSK-3 β knockdown in the nucleus accumbens increased cocaine self-administration (Crofton et al., 2017), supporting a general role for the kinase in alcohol and drug reinforcement. Thus, GSK-3 may represent a common neural mechanism that drives repetitive use of alcohol and cocaine.

To confirm that the GSK-3 inhibitor targeted the alcohol-sensitive 3 β isoform in brain, we evaluated effects of CHIR 99021 (0 or 10 mg/kg, i.p.) on p-GSK-3 β (ser9) and total GSK-3 β protein expression in the amygdala, nucleus accumbens, and frontal cortex, three reward-related brain regions that are known to regulate the positive reinforcing effects of alcohol (Hodge et al., 1992, 1996, 1997; Samson and Hodge, 1993; Schroeder et al., 2003; Besheer et al., 2010; Faccidomo et al., 2016; Cannady et al., 2017). Immunoblot results showed that CHIR 99021 decreased pGSK-3 β (ser9) in all three brain regions without altering total GSK-3 β protein expression, which confirmed bioavailability and efficacy of the inhibitor following systemic administration. These results were somewhat unexpected, however, since a variety of studies indicate that GSK-3 inhibition is associated with increased phosphorylation of GSK-3 β (ser9) (Doble and Woodgett, 2003). However, our data agree with other evidence showing that ATP-competitive inhibitors, such as CHIR 99021, reduce GSK-3 β (ser9) phosphorylation in cortical neurons (Liang and Chuang, 2007). The reason for this discrepancy is unclear; however, GSK-3 β activation varies as a function of its distribution in cellular compartments including the cytosol, nucleus, and mitochondria (Bijur and Jope, 2003). In the present study, we evaluated GSK-3 β (ser9) phosphorylation and expression in whole-cell lysates derived from mouse brain tissue. Future work evaluating potential subcellular effects could clarify specific mechanism(s) of action of GSK-3 inhibitors. Overall, results from the present study indicate that systemic administration of an ATP-competitive inhibitor reduces GSK-3 β (ser9) phosphorylation in reward-related brain regions and suggest that this inhibition may drive the positive reinforcing effects of alcohol.

Increases in drug-reinforced responding can arise from blockade of the pharmacological effects of the drug; thus, more drug is self-administered under voluntary conditions to produce the same pharmacological effect. This is consistent with a large body of evidence regarding mechanisms of drug self-administration. For example, a primary pharmacological effect of cocaine is to block the dopamine transporter, which increases synaptic dopamine and activates dopamine receptors. Accordingly, administration of dopamine D1-like or D2-like receptor antagonists increases cocaine reinforced responding (Ettenberg et al., 1982; Woolverton, 1986; Koob et al., 1987). Similarly, low doses of the opiate antagonist naloxone

increase morphine (Woods et al., 1975; Weeks and Collins, 1976) and heroin (Ettenberg et al., 1982; Koob et al., 1984) self-administration. We have found that alcohol self-administration increases ERK MAP kinase phosphorylation in the frontal cortex, and that ERK inhibition in this brain region increases the reinforcing effects of alcohol (Faccidomo et al., 2015). Since alcohol drinking increases pGSK-3 β (ser9) in rodent brain (Cheng et al., 2017; Liu et al., 2017), inhibition of this target would be expected to increase alcohol self-administration if kinase activity is functionally related to drug-seeking behavior. Thus, results of the present study showing that CHIR 99021 administration reduced GSK-3 β phosphorylation and increased alcohol reinforced responding suggest that increased GSK-3 β phosphorylation is a primary pharmacological effect of alcohol self-administration that regulates the positive reinforcing effects of the drug.

Interestingly, the finding that the CHIR 99021-induced increase in alcohol-reinforced responding emerged after approximately 30-min of self-administration raises questions regarding the pharmacological timecourse of the inhibitor and potential interactions with alcohol. Evidence indicates that a similar dose range of CHIR 99021 attenuated acute amphetamine-induced locomotion following a 1-h pretreatment interval in C57BL/6J mice (Pan et al., 2011). When considered in the context of the present data, where behavioral effects emerged 75-min following CHIR 99021 administration, this suggests that the inhibitor has a slow timecourse of action. However, the results also show that CHIR 99021 (10 mg/kg) significantly inhibited pGSK-3 β expression in the PFC, NAcb and amygdala at 45-min post administration, which corresponds to the start of self-administration sessions and argues against emergence of effects due to slow pharmacological timecourse unless additional downstream neural targets exerted behavioral control. Thus, it is also plausible that the behavioral effect of CHIR 99021 required the presence of self-administered alcohol. That is, since the behavioral effect of CHIR 99021 emerged only after alcohol self-administration reached approximately 50% of control level, the inhibitor may have blocked the ability of self-administered alcohol to increase pGSK-3 β (ser9) expression. If elevated GSK-3 β (ser9) phosphorylation is a direct pharmacological effect of self-administered alcohol that regulates positive reinforcement, as suggested above, then it is plausible that alcohol-reinforced responding increased in a compensatory manner. Resolving these questions will require additional research that measures GSK-3 β (ser9) phosphorylation after alcohol self-administration, evaluates the potential impact of CHIR 99021 following a longer pretreatment interval, and integrates alcohol pretreatment methods to assess pharmacological interaction.

Importantly, the present study evaluated potential nonspecific effects of GSK-3 inhibition using three separate controls, all of which showed no effect. First, both alcohol and sucrose self-administration sessions were conducted using a two-lever procedure where responding was reinforced on an “active” lever and nonspecific exploratory or motor behavior was recorded on an “inactive” lever that produced no programmed consequence. The results showed that mice emitted an average of about 10 responses on the inactive lever per session, which was unaltered by CHIR 99021. This suggests that the increase in alcohol self-administration observed after GSK-3 β inhibition was not related to nonspecific changes in activity during self-administration sessions. Second, since the alcohol solution was sweetened, it was imperative to evaluate drug effects in a parallel behavior-matched sucrose

self-administration procedure, which showed no effect of CHIR 99021 on sucrose reinforced responding and suggests that alterations in alcohol self-administration were not due to general effects on motivated behavior, or general reward mechanisms. Lack of alcohol specificity would not be surprising given the effects of GSK-3 β knockdown on cocaine self-administration mentioned above, which may reflect general involvement of GSK-3 signaling in reward and reinforcement processes. Finally, the effective dose of CHIR 99021 (10 mg/kg, i.p.) was devoid of effects on spontaneous open-field locomotor activity, indicating the absence of locomotor activation that might alter alcohol self-administration behavior. Overall, these control conditions lend support to the conclusion that GSK-3 inhibition increased the positive reinforcing effects of alcohol.

In contrast to the present study, GSK-3 β overexpression in mPFC was recently shown to be associated with increased home-cage alcohol drinking in mice (van der Vaart et al., 2018). This suggests that the magnitude of GSK-3 β gene expression in the mPFC is positively correlated with alcohol drinking. In the same study, systemic injection of the non-ATP competitive GSK-3 β inhibitor TDZD-8 decreased operant alcohol and sucrose reinforced responding in rats, which was interpreted by the authors as a nonspecific reduction in reward-related behavior. The authors did not determine, however, if increased gene expression in mouse mPFC or pharmacological inhibition in rats altered GSK-3 phosphorylation or activity. As noted above, we found that the ATP competitive GSK-3 inhibitor CHIR 99021 selectively increased alcohol reinforced responding in a manner associated with reduced GSK-3 β phosphorylation in the frontal cortex, amygdala and nucleus accumbens. We observed no changes in sucrose-reinforced responding or locomotor activity suggesting specificity regarding alcohol self-administration. Although future research is needed to clarify these seemingly discrepant findings, several methodological differences such as potential interspecies (rat vs. mouse) difference in response to systemic GSK-3 pharmacological inhibition, different GSK-3 β selectivity of test compounds, and different mechanism of action of GSK-3 inhibitors (ATP competitive vs ATP non-competitive) may be contributing factors. It would be especially interesting in future research to test these compounds in parallel and determine if TDZD-8 alters GSK-3 phosphorylation as we observed following CHIR 99021.

Although the mechanism(s) by which alcohol drinking increases pGSK-3 β (ser9) remains to be determined, evidence indicates that calmodulin-dependent protein kinase II (CaMKII) phosphorylates GSK-3 β (ser9) (Song et al., 2010). We have shown that alcohol self-administration increases CaMKII-T286 immunoreactivity (activation) (Salling et al., 2016) and that CaMKII inhibition in the frontal cortex increases alcohol self-administration (Faccidomo et al., 2016) in a manner like that reported in the present study after systemic injection of the GSK-3 inhibitor. CaMKII-T286 phosphorylation is also increased in reward-related brain regions by exposure to cues that were previously paired with alcohol (Salling et al., 2017). Thus, future research might focus on CaMKII signaling as a potential upstream mechanism by which alcohol drinking increases GSK-3 β (ser9) phosphorylation.

In addition, many of the biochemical, physiological, and behavioral effects of alcohol are mediated by excitatory glutamate AMPA receptor neurotransmission (Tabakoff and Hoffman, 2013). AMPA receptor activity is modulated by postsynaptic protein complexes

that regulate receptor localization. For example, the PDZ domain-containing protein PICK1 is co-localized with AMPA receptors in the brain where it regulates synaptic clustering, and function, at excitatory synapses. Specifically, PICK1 binds to the C-terminus of the GluA2 subunit to facilitate internalization of GluA2-containing AMPA receptors, which occurs during LTD (Xia et al., 1999). Accumulating evidence indicates that GSK-3 phosphorylates PICK1, which promotes binding of PICK1 to GluA2, removal of GluA2 from the synaptic membrane, and downregulation of receptor activity (Iwakura et al., 2001; Yagishita et al., 2015). Thus, GSK-3 inhibition would be predicted to upregulate AMPA receptor function via reduced PICK1 activity or expression.

In support of this prediction, the present results show that systemic administration of the GSK-3 inhibitor CHIR 99021 was associated with reduced PICK1 and increased AMPA receptor GluA2 subunit protein expression in the nucleus accumbens. No changes in PICK1 or GluA2 expression were seen in the amygdala or frontal cortex. Our prior research has shown AMPA receptor activity is required for the reinforcing effects of alcohol (Salling et al., 2016) and that systemic pharmacological activation of glutamate AMPA receptor activity is associated with escalated alcohol self-administration and relapse-like behavior (Cannady et al., 2013, 2017). Thus, it is plausible that CHIR 99021 increased alcohol self-administration via downstream inhibition of PICK1, which promoted increased GluA2-containing AMPA receptor expression in the nucleus accumbens. Additional research is required to address this hypothesis and evaluate potential mechanistic regulation of alcohol self-administration by PICK1 or GluA2 within the nucleus accumbens.

In conclusion, GSK-3 is a multi-functional serine/threonine kinase that subserves numerous functions in the central nervous system, including modulation of activity-dependent plasticity, various neurological diseases, and addiction. The results of this study show that systemic administration of the GSK-3 inhibitor CHIR 99021 promotes escalated operant alcohol self-administration in mice, which is direct evidence for increased positive reinforcing effects of the drug. This behavioral effect of the GSK-3 inhibitor was associated with reduced GSK-3 β phosphorylation in mesocorticolimbic brain regions that are known to regulate the reinforcing effects of alcohol. Evaluation of the downstream GSK-3 substrate, PDZ-domain containing PICK1, showed that it was downregulated selectively in the nucleus accumbens in a manner associated with increased AMPA receptor GluA2 subunit expression. Thus, we raise the hypothesis that GSK-3 inhibition may have increased alcohol self-administration, and reinforcement function, via enhanced glutamate activity at GluA2-containing receptors in the nucleus accumbens, which can be tested in future studies targeting GluA2 activity specifically in this brain region.

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Figure 1.

Effect of the GSK-3 inhibitor CHIR 99021 on lever press responding reinforced by sweetened alcohol. **(A)** Dose-effect curve for CHIR 99021 on alcohol-reinforced response rate expressed as cumulative responses per 5-min interval on the active lever. Symbols represent means for all doses and vertical error bars represent SEM. Open circles represent responding 45 min after an i.p. injection of NaCl. Ascending doses of the inhibitor are represented by squares (1 mg/kg), upright triangle (3 mg/kg) and upside-down triangle (10 mg/kg), respectively. *Asterisks* indicate significant difference from vehicle control at corresponding time point, $p < 0.05$, 2-way RM ANOVA, Dunnett's multiple comparison test. **(B – D)** Total number of alcohol reinforcers **(B)**, head pokes per reinforcer **(C)**, and alcohol dosage consumed **(D)** plotted as a function of CHIR 99021 dosage. *White bars* represent means for NaCl vehicle and *black bars* represent means of doses of CHIR 99021. *Vertical error bars* represent SEM. *Asterisks* indicates significance from NaCl, $P < 0.05$, 1-way RM ANOVA followed by Dunnett's multiple comparison test.

Figure 2.

Effect of the GSK-3 inhibitor CHIR 99021 on sucrose reinforced responding.

(A) Sucrose reinforced response rate plotted as a function of session time. Symbols represent individual doses of CHIR 99021 (0 – 10 mg/kg). (B – C) Total number of reinforcers (B) and head pokes per reinforcer (C) plotted as a function of CHIR 99021 dosage. *White bars* represent means for NaCl vehicle and *black bars* represent means of doses of CHIR 99021. *Vertical error bars* represent SEM.

Figure 3.

Effect of the GSK-3 inhibitor, CHIR 99021, on open field activity in alcohol- and sucrose-trained mice. Spontaneous locomotor activity of (A) alcohol-trained and (B) sucrose-trained mice. Distance traveled was measured in an open field 45 min after an i.p. injection of NaCl (*white bars*) or 10 mg/kg CHIR 99021 (*black bars*). Vertical error bars represent SEM.

A. Prefrontal Cortex **B. Nucleus Accumbens** **C. Amygdala** **Figure 4.**

Effect of the GSK-3 inhibitor CHIR 99021 on pGSK-3β (ser9) and tGSK-3β protein expression in reward-related corticolimbic brain regions. Representative immunoblots (pGSK-3β (ser9), tGSK-3β, pGSK-3β/tGSK-3β, *left, center, right panels*, respectively) and quantitative analyses (*bar graphs*) showing systemic administration of the 10 mg/kg CHIR 99021 (*black bars*), on pGSK-3β (*left graphs*), tGSK-3β (*center graphs*), pGSK-3β/tGSK-3β (*right graphs*) immunoreactivity relative to NaCl (*white bars*) in the (A) prefrontal

cortex, (B) nucleus accumbens and (C) amygdala. All data represent group MEAN $\pm$ SEM and are plotted relative to GAPDH. *Asterisks* indicate statistical significance, p 0.05, t-test.

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A. GluA2 **B. PICK1** **Figure 5.**

Effect of the GSK-3 inhibitor CHIR 99021 on GluA2 and PICK1 protein expression in the nucleus accumbens. Representative immunoblots (GluA2 and PICK1, *left and right panels*, respectively) from nucleus accumbens mouse tissue and quantitative analyses (*bar graphs*) showing systemic administration of 10 mg/kg CHIR 99021 (*black bars*), on (A) GluA2 and (B) PICK1 immunoreactivity relative to NaCl (*white bars*). All data represent group MEAN $\pm$ SEM and are plotted relative to GAPDH. *Asterisks* indicate statistical significance, $p < 0.05$, t-test.