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Functional Regulation of PI3K-Associated Signaling in the Accumbens by Binge Alcohol Drinking in Male but not Female Mice

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Abstract

It is well established that binge alcohol consumption produces alterations in Group 1 metabotropic glutamate receptors (mGlu) and related signaling cascades in the nucleus accumbens (NAC) of adult male mice, but female and adolescent mice have not been examined. Thus, the first set of studies determined whether repeated binge alcohol consumption produced similar alterations in protein and mRNA levels of Group 1 mGlu-associated signaling molecules in the NAC of male and female adult and adolescent mice. The adult (9 weeks) and adolescent (4 weeks) C57BL/6J mice were exposed to 7 binge alcohol sessions every 3rd day while controls drank water. Repeated binge alcohol consumption produced sexually divergent changes in protein levels and mRNA expression for Group 1 mGlu and downstream signaling molecules in the NAC, but there was no effect of age. Binge alcohol intake decreased mGlu5 levels in females, whereas it decreased indices of phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), 4E-binding protein 1, and p70 ribosomal protein S6 kinase in males. Expression of genes encoding mGlu1, mGlu5, the NR2A subunit of the NMDA receptor, and Homer2 were all decreased by binge alcohol consumption in males, while females were relatively resistant (only phosphoinositide-dependent protein kinase 1 was decreased). The functional implication of these differences was investigated in a separate study by inhibiting mTOR in the NAC (via infusions of rapamycin) before binge drinking sessions. Rapamycin (50 and 100 ng/side) significantly decreased binge alcohol consumption in males, while consumption in females was unaffected. Altogether these results highlight that mTOR signaling in the NAC was necessary to maintain

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binge alcohol consumption only in male mice and that binge drinking recruits sexually divergent signaling cascades downstream of PI3K and presumably, Group 1 mGluR. Importantly, these findings emphasize that sex should be considered in the development of potential pharmacotherapeutic targets.

Keywords

Group 1 metabotropic glutamate receptor; homer2; rapamycin; SHAC procedure; sex differences; nucleus accumbens

1. Introduction

Alcohol use disorders, including binge alcohol consumption, have long been a public health concern. Binge alcohol consumption produces a blood alcohol concentration (BAC) of 80 mg% or higher; to achieve this level of intoxication, men on average consume more alcoholic beverages in a two-hour period compared to women (5 for men vs 4 for women; NIAAA, 2004). Additionally, while men are twice as likely to participate in problematic drinking (e.g., Meyer et al., 2000; Trillo et al., 2012), women are more susceptible to suffer liver injury and other risks when consuming equivalent doses (e.g., Frezza et al., 1990; Sugarman et al., 2009; Wiren, 2013). However, despite these known differences in the consumption rates and physiological consequences between the sexes, knowledge of sex-differences in the neurological underpinnings of binge alcohol consumption is not well established.

Alcohol alters glutamate neurotransmission throughout the extended amygdala (e.g., Dahchour and De Witte 2003; Griffin et al. 2014; Moghaddam and Bolinao 1994; Roberto et al. 2004; Rossetti and Carboni 1995; Selim and Bradberry 1996; but see Marty and Spigelman 2012), and a growing body of evidence demonstrates that glutamatergic signaling molecules in the nucleus accumbens (NAC) are sensitive to the neuroadaptive properties of alcohol (e.g., Ary et al., 2012; Besheer et al., 2010; Cozzoli et al., 2009, 2012, 2014a; Goulding et al., 2011; Marty and Spigelman, 2012; Neasta et al., 2010, 2011). In particular, the Group 1 metabotropic glutamate receptors (mGluR) are in position to regulate a variety of downstream signaling molecules, such as phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR), through their interaction with the Homer scaffolding protein (Daw et al., 2002; Klugmann and Szumlanski, 2008; Ronesi and Huber, 2008; Rong et al., 2003; Shiraishi-Yamaguchi and Furuichi, 2007). Specifically, Group 1 mGluR (mGlu1 and mGlu5) couple with PI3K enhancer to interact with PI3K directly through coiled-coil Homer scaffolding (Rong et al., 2003). This coupling then provides a structural and functional link between Group 1 mGluR and the PI3K signaling pathway as evident from *in vitro* studies showing that stimulation of Group 1 mGluR with the agonist dihydroxyphenylglycine increases phosphorylation of PI3K-associated signaling molecules, including phosphoinositide-dependent protein kinase 1 (PDK1), mTOR, 4E-binding protein 1 (4EBP1), and p70 ribosomal protein S6 kinase (p70S6K; Hou and Klann, 2004; Ronesi and Huber, 2008).

Previous studies in adult male mice with pharmacological antagonists have demonstrated that PI3K and Group 1 mGlu in the NAC play an important role in mediating binge alcohol consumption (e.g., Besheer et al., 2010; Cuzzoli et al., 2009, 2012; Lum et al., 2014; reviewed in Olive, 2010). Repeated bouts of binge alcohol drinking significantly increased the phosphorylation state of p85 α (a PI3K binding motif; Cuzzoli et al., 2009) and increased the activation of Akt (also known as protein kinase B), mTOR, and 4EBP1 in the NAC of adult male mice (Neasta et al. 2010, 2011). Additionally, up-regulation of PI3K signaling has been identified in pathway analysis of alcohol-induced changes in the NAC of adult male rats (McBride et al., 2009). However, no studies to date have investigated whether there is a sex-dependent role for Group 1 mGlu-associated signaling molecules in the NAC to influence binge drinking.

Recent work found that mGlu5 antagonism decreased binge alcohol consumption in adult and adolescent male and female C57BL/6J mice, but that sex and age differences existed in the consequence of mGlu5 antagonism on later alcohol intake after a period of abstinence (Cuzzoli et al., 2014a). Based on this result and reports that alcohol consumption in adolescent rodents can increase alcohol intake during adulthood (e.g., Broadwater et al., 2013; Moore et al., 2010; Strong et al., 2010), it is possible that male and female adult and adolescent mice have similar sensitivity to mGlu5 antagonists at the receptor level while the signaling downstream of mGlu5 might differ. Therefore, the initial studies determined whether there were sex and age differences in the effect of repeated binge alcohol consumption on protein and mRNA levels of Group 1 mGlu-associated signaling molecules in the NAC of C57BL/6J mice. Because there was no effect of age and there were minimal changes in the signaling molecules in adult and adolescent female mice, a final study examined the functional ramifications of the sex-specific alterations that we observed in adult mice. Intra-NAC infusion of rapamycin was used to locally inhibit mTOR prior to binge alcohol drinking, with the prediction that females would be resistant to the ability of rapamycin to decrease binge alcohol intake.

2. Methods

2.1. Subjects

The present studies employed adult and adolescent male and female C57BL/6J mice (Jackson Laboratories-West, Davis, CA). All adolescent mice were obtained post-weaning (3 weeks), while adult mice were obtained at 8 weeks of age. Until the time of testing, mice were group housed (3-4 per cage, separated by sex and age) in clear polycarbonate cages (28 × 18 × 13 cm) on Ecofresh bedding. Mice were maintained on a 12-hr light/dark cycle (lights on 0600) in a temperature (22 ± 2°C) and humidity controlled environment. All experiments were conducted during the light phase of the light/dark cycle. Rodent chow (Labdiet 5001 rodent diet; PMI International, Richmond, IN) and water were available *ad libitum*, except for the Scheduled High Alcohol Consumption (SHAC) procedure where fluid availability was scheduled. At the start of the studies, adolescent mice were 4 weeks of age while adult mice were 9 weeks of age. All procedures complied with the National Institute of Health *Guidelines for the Care and Use of Laboratory Animals* and were

approved by the local Institutional Animal Care and Use Committee. All efforts were made to minimize distress and the number of animals used.

2.2. Experimental Procedures

2.2.1. Experiments 1 and 2: Influence of binge alcohol consumption on protein levels and gene expression within the NAC

2.2.1.a. SHAC Procedure: Two separate cohorts of animals were utilized for the assessment of protein levels and gene expression within the NAC. Adult and adolescent male and female mice were individually housed with nestlets and acclimated to the sipper tube prior to the start of the SHAC procedure (see Finn et al., 2005; Strong et al., 2010 for details). Briefly, mild fluid restriction was used to condition mice to drink their daily fluid requirement on a schedule, and animals were weighed daily. Animals had varying amounts of total fluid access per day, ranging from 4 – 10 hrs (slowly increased over time), with 30 min access to a 5% v/v alcohol (ethanol, 5E) solution in tap water every 3rd day. Following the 5E access period, water was provided for the remainder of the period of fluid availability. Water was the only fluid available on the intervening days. This 3-day cycle of fluid access was repeated (21 days total) so that “binge” animals received a total of 7 binge alcohol sessions, with the time of fluid access gradually increasing. Water “control” animals received the same total fluid access, but they drank only water.

2.2.1.b. Tissue dissection and sample preparation: Animals were decapitated at 24 hrs following the 7th binge alcohol session. Following decapitation, the brain was extracted, chilled on ice and sectioned (freehand, rather than using a brain mold) on a plate over ice. Briefly, the prefrontal cortex was removed to visualize the anterior commissure. Coronal sections (1-2 mm) were cut and the entire NAC was micropunched from the section containing the anterior commissure using a 16 gauge hollow needle, based on established anatomical coordinates from the mouse brain atlas (Paxinos & Franklin, 2001). All samples were placed in microcentrifuge tubes (1.5 ml), frozen immediately in dry ice, and then stored at -80°C until total RNA or protein isolation.

2.2.1.c. Protein isolation and Western blot analysis: Dissected tissue was homogenized in a medium consisting of 50 mM Tris (pH 7.5), 150 mM sodium chloride, 1% w/v sodium-deoxycholate, 1% w/v Tegitol-type NP-40, 0.5% sodium dodecyl sulfate. To inhibit phosphatases, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and Phosphatase Inhibitor Cocktail 3 (Sigma Aldrich, St. Louis, MO) was added to the solution, and 1 Complete Mini tablet (Roche Molecular Biochemicals, Indianapolis, IN) was also included to inhibit proteases. Samples were then subjected to centrifugation at $10,000 \times g$ for 20 min. Protein determinations were performed using the Thermo Scientific Pierce BCA Protein Assay Kit (Thermo Fischer Scientific Inc., Rockford, IL) according to the manufacturer's instructions. Homogenates were stored at -80°C until immunoblotting.

Normalized homogenates (10 µg protein/lane) were mixed with 1X Laemmli Sample Buffer (Bio-Rad Laboratories, Inc.) and 5% β-mercaptoethanol and boiled for 5 min. Due to the large number of treatment groups, each sex was examined separately (i.e., proteins from each sex were loaded on separate sets of gels to assess for age and treatment effects).

Samples were electrophoresed on a 7.5% Mini-PROTEAN TGX Precast Gel (Bio-Rad Laboratories, Inc.), and the separated proteins were transferred to an Immun-Blot polyvinylidene-difluoride transfer membrane (Bio-Rad Laboratories, Inc.). Membranes were incubated for 1 hr in blocking TBS with 0.05% Tween-20 (TBS-T) with 5% BSA. The primary antibodies were applied in TBS-T with BSA at 4°C overnight. The following rabbit polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA): Phospho-PI3 Kinase p85-Tyr 458 (#4228, 1:500), PI3 Kinase p85 (#4292, 1:3000), Phospho-PDK1-Ser241 (#3061, 1:1000), PDK1 (#3062, 1:2000), Phospho-mTOR-Ser2448 (#2971, 1:1000), mTOR (#2972, 1:2000), Phospho-4E-BP1-Thr37/46 (#9459, 1:1500), 4E-BP1 (#9452, 1:500), and p70 S6 Kinase (#9202, 1:1000). The following monoclonal rabbit antibodies were used: mGlu5 (#EPR2425Y, Novus Biologicals, Littleton, CO) and Phospho-p70 S6 Kinase-Thr389 (#1175-1, Eptomics, Burlingame, CA), both at 1:1000. Monoclonal mouse antibody recognizing mGlu1 was purchased from BD Biosciences (#610965, San Jose, CA) and used at 1:1000. The α -tubulin antibody was a polyclonal rabbit antibody purchased from Cell Signaling Technology (#2144) and was used at 1:20000. After washing three times with TBS-T buffer, the membranes were incubated with horseradish peroxidase-linked goat anti-rabbit IgG antibody (Cell Signaling Technology) or goat anti-mouse IgG antibody (BD Biosciences), both at 1:10000 for 1 hr. Then, the bound antibodies were visualized by an enhanced chemiluminescence (ECL-Plus) detection system (Amersham Pharmacia Biotech, Piscataway, NJ) on GeneMate Blue Ultra autoradiographic film. Quantitative analysis of the proteins was performed in the linear range by volume densitometry using OptiQuant Software (Packard Instruments Company, Meriden, CT) after scanning of the film.

2.2.1.d. RNA isolation and quantitative reverse-transcriptase polymerase chain

reaction (qRT-PCR): Total RNA isolation and purification was performed according to the Absolutely RNA Microprep Kit (Agilent Technologies, Santa Clara, CA). The purity of the DNase treated RNA was confirmed by UV 260/280 ratios of 2.0. RNA integrity was confirmed using denaturing agarose gel electrophoresis followed by staining with SYBR Gold (Life Technologies, Grand Island, NY).

Real time qRT-PCR was performed with the iCycler IQ Real Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) using a one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) on DNase-treated total RNA (Hashimoto et al., 2004). The qRT-PCR reactions were carried out in 25 μ l with 20 ng of total RNA.

Real-time qRT-PCR efficiency was determined for each primer set using a five-fold dilution series of total RNA and did not differ significantly from 100%. Specificity of the PCR reaction was confirmed with melt curve analysis to ensure that only the expected PCR product was amplified. Relative expression of the RT-PCR product was determined using the comparative AACt method, after normalizing expression to total RNA measured with RiboGreen (Molecular Probes, Eugene, OR; Hashimoto et al., 2004). Primers were purchased pre-designed from Qiagen.

2.2.2. Experiment 3: Effect of intra-NAC inhibition of mTOR on binge alcohol consumption

2.2.2.a. Surgery: Under isoflurane anesthesia, adult male and female mice were placed into a stereotaxic apparatus (Cartesian Instruments) and implanted with 12 mm, 26 gauge stainless steel dual guide cannulae (Plastics One, Roanoke, VA, USA) 1 mm above the NAC. Implant coordinates were AP: +1.45 mm from bregma, L/M: ± 0.6 mm from the midsagittal suture, V: -3.3 mm from the skull surface according to the atlas of Paxinos and Franklin (2001). Once lowered, guide cannulae were secured to three skull screws using dental acrylic and were kept patent with 33 gauge stylets (Plastics One). Animals received the post-operative analgesic ketoprofen (1 mg/kg; Henry Schein Animal Health, Dublin, OH), and then were single-housed with nestlets and allowed 1 week for recovery.

2.2.2.b. Extended SHAC procedure: The SHAC procedure was identical to the one used above except in this study all animals were given access to 5E every third day and fluid access remained restricted to 4hr/day. All animals were given one binge access session prior to the onset of drug infusions.

2.2.2.c. Drug Infusions: Stainless steel microinjectors (13 mm long, Plastics One) were designed such that the tip extended 1 mm past the end of the cannulae, allowing for drug delivery directly into the NAC. Rapamycin (R&D Systems, Minneapolis, MN, USA) was initially dissolved in 100% DMSO and then diluted with water, resulting in a final vehicle solution of 20% DMSO in water. Doses of rapamycin (5, 50, and 100 ng/side) were chosen, based on previous studies conducted in the NAC (Neasta et al., 2010). On the day of the infusion, drug was administered 30 min before fluid access at a rate of 0.2 μ l/min for a total volume of 0.2 μ l/side using an automated syringe pump (Braintree Scientific Inc, Braintree, MA, USA), and the injectors remained in place for an additional 30 seconds. To assess for effects of intra-NAC inhibition of mTOR on alcohol consumption, mice received infusions of vehicle or rapamycin in a within-subjects design and then 5E was available for the 30-min access period followed by 3.5 hr access to water. Drug and vehicle administration occurred every third day (i.e., on each subsequent binge alcohol session) with access to only water occurring on the intervening days. To assess for nonspecific effects of intra-NAC inhibition of mTOR, animals were switched to having access to only water (i.e., no alcohol sessions every third day). After a 5 day period to acclimate, both vehicle and the maximally effective rapamycin dose (100 ng/side) was administered 30-min before fluid access on two separate water only days that were separated by 3 days.

2.2.2.d. Histology: Upon completion of the study, brains were removed and immediately frozen in isopentane on dry ice. Brains were sliced on a cryostat (40 μ m sections) and mounted on superfrost slides. Confirmation of cannulae placements in the NAC (i.e., placements in core and shell were considered as 'hits'; Figure 4F) were conducted by 2 individuals using a microscope at 1.25 X. Upon verification, 2 males and 2 females were removed from the study due to cannulae placements outside of the NAC.

2.3. Data analysis

For the SHAC data (experiments 1 and 2), the dependent variables were BAC, volume (in mLs) of water and alcohol consumed, dose of alcohol consumed (g/kg) and body weight. The effect of the period of fluid access as well as the drug treatment, age and sex of the animal on these dependent measures was assessed by analysis of variance (ANOVA). Data for the 7 binges were collapsed across time as there was no significant effect of day on alcohol intake.

For the Western blot and qRT-PCR data (experiments 1 and 2, respectively), all data were normalized to their respective loading controls for each sex. Initial analyses were conducted using a 2-way ANOVA (with age and treatment as factors); however, no significant effects of age or significant interactions between age and treatment were detected. Therefore, data were collapsed across age and re-analyzed. To this end, the ratios were then normalized to the mean for each protein or gene of the water controls for each individual gel (n=5-6/gel) or qPCR reaction. Independent t-tests were then conducted to assess for the effect of treatment on each protein and gene for each sex.

For the intra-NAC rapamycin study (experiment 3), the dependent variables were volume of water consumed, dose of alcohol consumed (g/kg), total fluid intake (in mLs), and body weight. The effect of the rapamycin was assessed by 2-way repeated measures ANOVA, followed by one-way repeated measures ANOVA for each sex. Post-hoc paired t-tests with Bonferonni corrections confirmed the effect of dose versus vehicle.

In all cases, data are presented as the mean $\pm$ SEM. The level of significance was set as p 0.05, and p 0.09 was considered a trend. Statistical analyses were conducted with SYSTAT (version 11, SYSTAT Software, Inc., Richmond, CA).

3. Results

3.1. Sex differences in the influence of binge alcohol consumption on levels of mGlu1, mGlu5, and PI3K-associated signaling proteins in the NAC

Male and female adult and adolescent mice underwent 7 binge alcohol sessions (SHAC groups) or consumed water (water groups). Averages of the binge alcohol consumption (in g/kg), the daily total fluid intake (in mLs and mL/kg), and the body weight (in grams) are listed in Table 1. A 2-way ANOVA of g/kg intake with age and sex as factors revealed a main effect of age [$F(1,32)=7.056$, $p<0.05$; adolescents > adults] and a trend for intake to be higher in male versus female mice [$F(1,32)=3.208$, $p<0.09$], but no interaction between sex and age. A 3-way ANOVA indicated that total fluid intake (in mLs) was significantly higher in male vs. female mice [$F(1,62)=29.081$, $p<0.001$] but was not influenced by age or treatment group (i.e., water vs. SHAC), and the 3-way interaction was not significant. When the analysis was conducted on total fluid intake that had been expressed as a function of body weight (mL/kg), total fluid intake was significantly higher in females versus males [$F(1,62)=50.207$, $p<0.001$] and in adolescent versus adult mice [$F(1,62)=140.510$, $p<0.001$], but it was not influenced by treatment group, and the 3-way interaction was not significant. Analysis of body weight, collapsed over the 21 days, indicated that body weight was higher in males versus females [$F(1,62)=295.631$, $p<0.001$] and in adult versus adolescent mice

[$F(1,62)=206.629$, $p<0.001$]. While there was a significant interaction between sex and age [$F(1,62)=23.063$, $p<0.001$], there was no effect of treatment group (i.e., water vs. SHAC) and no 3-way interaction. Thus, treatment group did not significantly alter total fluid intake or body weight in either sex or age group.

For the NAC Western blot data, each sex was analyzed separately for age and treatment effects. The initial analysis revealed that there was no significant effect of age or significant interaction between age and treatment. Therefore, data were collapsed across age and analyzed.

Analysis of the NAC of male mice revealed that withdrawal from binge alcohol consumption produced a 19% and 36% decrease in the phosphorylation of PI3K and mTOR respectively [p-PI3K: $t(30)=-2.092$, $p<0.05$, Figure 1B; p-mTOR: $t(32)=-2.934$, $p<0.01$, Figure 1D], and this decrease carried over to the activation index of both proteins [p-PI3K/PI3K Ratio: $t(30)=-1.850$, $p=0.07$ ($\downarrow$ 15%); p-mTOR/mTOR Ratio: $t(32)=-2.404$, $p<0.05$ ($\downarrow$ 31%)], although the PI3K ratio only trended toward significance. Additionally, binge alcohol intake resulted in a 23% and 14% decrease in levels of phosphorylated 4EBP1 and p70s6K respectively [p-4EBP1: $t(32)=-2.035$, $p=0.05$, Figure 1E; p-p70s6K: $t(32)=-2.868$, $p<0.01$, Figure 1F] as well as an 10% decrease in levels of p70s6K [$t(32)=2.128$, $p<0.05$]. However, no binge-induced alterations occurred in the protein levels of either mGlu1, mGlu5 (Figure 1A), or PDK-1 (Figure 1C). Altogether these data suggest that 24-hr withdrawal from binge alcohol consumption alters the activation of PI3K and several associated signaling molecules in the NAC of male mice.

In contrast, analyses of the NAC of female mice revealed that binge alcohol consumption resulted in a 48% decrease in mGlu5 protein levels [$t(26)=-3.230$, $p<0.01$; Figure 2A] without affecting levels of any of the other proteins examined (Figure 2B-F). These data suggest that the NAC of female mice is resistant to alterations in the mGlu5 and PI3K signaling cascade following 24-hr withdrawal from binge alcohol consumption.

3.2. Sex differences in the influence of binge alcohol consumption on expression of Group 1 mGlu-associated genes in the NAC

As Western blot analysis revealed sex-specific differences in the binge-induced alterations of protein levels of mGlu1, mGlu5, and PI3K-associated signaling molecules following 24 hr withdrawal, we assessed mRNA transcription of a panel of specific Group 1 mGlu/Homer2-associated genes as a possible mechanism leading to the protein alterations. To this end, a separate cohort of mice (males and females, adult and adolescent) underwent 7 binge alcohol sessions or consumed water. Averages of binge consumption, total fluid consumption, and body weight are presented in Table 2, and these values did not differ statistically from those in the first experiment (t-tests, $ps>0.05$). Nonetheless, subsequent analyses revealed that binge alcohol intake was significantly higher in adolescent versus adult mice [$F(1,31)=11.892$, $p<0.01$] and in male versus female mice [$F(1,31)=21.974$, $p<0.001$], with a significant interaction due to the higher intake in adolescent versus adult mice only in male mice [$F(1,31)=7.767$, $p<0.01$]. Total fluid intake (mLs) was significantly higher in male versus female mice [$F(1,61)=8.003$, $p<0.01$], but was not influenced by age or treatment, and the 3-way interaction was not significant. Total fluid intake as a function of

body weight (mL/kg) was significantly higher in female versus male mice [$F(1,61)=29.204$, $p<0.001$] and in adolescent versus adult mice [$F(1,61)=49.084$, $p<0.001$], but it was not influenced by treatment, and the 3-way interaction was not significant. Body weight was significantly higher in male versus female mice [$F(1,61)=339.599$, $p<0.001$] and in adult versus adolescent mice [$F(1,61)=282.058$, $p<0.001$]. Although there was a significant interaction between age and sex [$F(1,61)=41.824$, $p<0.001$], there was no effect of treatment and no 3-way interaction. Overall, the ANOVA results are very similar to those from the first experiment, and again, treatment group did not significantly alter total fluid intake or body weight in either sex or age group.

The initial analysis of the gene expression data revealed that there was no significant effect of age or interaction with age. As a result, data were collapsed across age and analyzed.

As shown in Figure 3A, withdrawal from binge alcohol consumption significantly decreased mRNA expression of mGlu1 and mGlu5 in the NAC of male mice [mGlu1 (*Grm1*): $t(24)=-2.487$, $p<0.05$ ($\downarrow$ 29%); mGlu5 (*Grm5*): $t(24)=-2.857$, $p<0.01$ ($\downarrow$ 36%)]. There also was a 38% binge-induced decrease in mRNA expression of the NR2A subunit of the NMDA receptor [*Grin2A*; Figure 3B; $t(22)=-2.286$, $p<0.05$] but the 48% decrease in expression of the NR2B subunit of the NMDA receptor (*Grin2B*) did not reach significance. Withdrawal from binge alcohol consumption tended to decrease mRNA expression of Homer2 by 28% [Figure 3C; $t(23)=-1.803$, $p=0.08$], while expression of PDK-1 (*Pdkp1*) was unchanged. These results suggest that alterations in mRNA expression of glutamatergic genes could be one mechanism underlying the binge-induced changes in PI3K-associated signaling proteins in the NAC of male mice.

Consistent with the Western blot data, the qRT-PCR results from the NAC of female mice revealed that the effect of binge alcohol consumption on PI3K-associated signaling molecules was profoundly sex-specific. In fact, withdrawal from binge alcohol consumption failed to alter the mRNA expression of all genes examined in female mice, with the exception of a 13% decrease in PDK-1 [*Pdpk1*; Figure 3F; $t(26)=-2.066$, $p<0.05$], which was unaltered in male mice. Altogether, female mice are relatively resistant to the effect of repeated binge alcohol intake on PI3K-associated signaling molecules in the NAC.

3.3. Sex specific differences in the ability of intra-NAC inhibition of mTOR to decrease binge alcohol consumption

To test the functional importance of the sex-specific alterations in protein levels described above, male and female adult mice were pretreated with intra-NAC rapamycin to locally inhibit mTOR signaling and determine the influence on binge drinking. The effect of rapamycin on binge alcohol intake was dependent on sex [Sex $\times$ Drug: $F(3,39)=3.25$, $p<0.05$], with rapamycin reducing intake in males [$F(3,21)=5.13$, $p<0.01$] but not females ($p>0.05$; Figure 4A). Post-hoc analysis confirmed that both the 50 ng and 100 ng rapamycin doses significantly reduced alcohol intake in males ($p<0.05$) by 18% and 28%, respectively. Importantly, intra-NAC rapamycin did not alter subsequent water intake or total fluid intake on the day of the test in either sex ($p>0.05$; Figure 4B-C). These results demonstrated that administration of intra-NAC rapamycin selectively reduced binge alcohol intake in male mice.

We also wanted to confirm that intra-NAC rapamycin would not influence water consumption on a day when animals only had access to water during the fluid access period. Therefore as a final test, we administered vehicle and 100 ng/side rapamycin on water only days. Under these conditions, intra-NAC rapamycin did not alter water intake during the first 30 minutes of access or during the 4 hr fluid access period ($p>0.05$; Figure 4D-E).

4. Discussion

The current studies provide important evidence for sexually divergent alterations in mGlu1, mGlu5, and PI3K-related signaling and in function of the mTOR pathway in the NAC following repeated binge alcohol consumption. Binge alcohol intake was consistent across studies (i.e., compare Tables 1 and 2), and a separate study with comparable mean alcohol intake and corresponding mean BACs [adult male (2.3 g/kg; 1.4 mg/ml), adolescent male (3.0 g/kg; 1.7 mg/ml), adult female (2.4 g/kg; 1.4 mg/ml), adolescent female (2.7 g/kg, 1.8 mg/ml)] document that the present results were not due to sex differences in binge alcohol intake or BAC achieved. And even though binge alcohol intake and BACs were higher in the adolescent versus adult animals (mainly due to the higher intake in adolescent males), age was not a factor in the current study with the results in adolescent animals paralleling the adult animals in both sexes.

It is possible that the lack of differential response to repeated binge alcohol intake in adolescent versus adult mice was due to the timing of the exposure (early – mid adolescence in the present study), as different patterns of consequences resulting from binge alcohol administration have been reported after exposure during early – mid adolescence versus late-adolescence/emerging adulthood (reviewed in Spear, 2015). Nonetheless, the sex difference for binge-alcohol effects on PI3K signaling molecules in adolescent animals is consistent with the sex-specific association of polymorphisms within *PIK3RI*, which encodes the 85 kD regulatory subunit of PI3K, and patterns of risky alcohol consumption in male but not female adolescent humans (Desrivieres et al., 2008).

Western blot analyses confirmed the sex-specific alcohol-induced alterations in PI3K-related signaling, which we presumed was downstream of mGlu1/5. Notably, 24-hr withdrawal from repeated binge alcohol consumption decreased phosphorylation of PI3K, mTOR, 4EBP1, and p70S6K without altering mGlu1 or mGlu5 protein levels in males, while only mGlu5 levels were decreased in females. The lack of effect of binge alcohol intake on mGlu1/5 levels in the NAC of males in the current study is consistent with previous results in male NAC using the SHAC procedure (Cozzoli et al., 2009) as well as a recent report that densities of mGlu1/5 binding in the NAC did not differ in alcoholic versus control subjects (Kupila et al., 2013). Regardless, the present findings indicate that a double dissociation is observed between binge-alcohol effects at the receptor versus the intracellular signaling molecules (i.e., no receptor change but signaling change in males; receptor change but no signaling change in females). One potential explanation is that withdrawal from repeated binge alcohol consumption produced an alteration in the function of mGlu1/5 that was reflected in the decrease in the phosphorylation of downstream signaling molecules (for more detailed discussion, see Cozzoli et al. 2014b). However, another possible explanation is that the present results argue against a link between mGlu1/5 and PI3K in terms of binge

alcohol effects, given that PI3K is a common transduction pathway for receptors such as tyrosine kinase B (TrkB), insulin-like growth factor-1 (IGF-1), and serotonin 1A (5HT_{1A}; see Figure 1 in Banerjee et al., 2012; Li and Jope, 2010), which are all localized in the NAC (e.g., Araujo et al., 1989; Clissold et al., 2013; Li et al., 2012). Thus, it should be considered that binge alcohol produced an effect on PI3K signaling in males in the present study that was independent of an effect on mGlu1/5.

When the transcription of genes in the NAC was examined at 24-hr withdrawal from repeated binge alcohol consumption, these results again confirmed that females were relatively resistant to alcohol-induced alterations, with only a small (but significant) reduction in *Pdpk1*. In NAC tissue from male mice, gene transcription of mGlu1 and 5 (*Grm1* and 5), the 2A subunit of the NMDA receptor (*Grin2A*), and Homer2 (*Homer2*) was down regulated. This alcohol-induced down regulation of *Grm1* is consistent with data from microarray analysis of the NAC of inbred alcohol-preferring rats after 24 hr withdrawal from operant self-administration (Rodd et al., 2008). There was some discordance between mRNA and protein levels of mGlu1/5 and downstream signaling molecules following repeated binge alcohol consumption in male and female mice, perhaps due to post-translational or allosteric regulation of mGlu1/5 by binge alcohol, in addition to transcriptional regulation. Collectively, the current data extend the importance of PI3K signaling in the NAC of male mice to the downstream signaling molecules PDK1, mTOR, p70S6K, and 4EBP1. As these molecules are critical in driving the translation of 5'-TOP mRNAs and protein synthesis (see Neasta et al., 2014), the ability of alcohol to alter these proteins could directly influence signaling differently in the NAC of male and female mice.

Interestingly, while the current work confirms the importance of this signaling cascade in the neuroadaptive properties of binge alcohol consumption in male mice, the down regulation in phosphorylation is in direct contrast to previous work which indicated an alcohol-induced increase in the phosphorylation of members of the Group 1 mGlu signaling cascade following alcohol consumption in male rodents (e.g., Cozzoli et al., 2009, 2012; Neasta et al., 2010, 2011). Although the mechanism for this effect is not completely understood, we note that in one study alcohol injection did not change the phosphorylation state of the same proteins (Goulding et al., 2011) and that consumption of ethanol in a liquid diet for 16 weeks (Li and Ren, 2007) or 20 – 26 weeks (Vary et al., 2008) produced a decrease in phosphorylation state of the same proteins. Based on evidence that the activation of mTOR appears to be transient following injection of drugs such as cocaine, tetrahydrocannabinol and methamphetamine (reviewed in Neasta et al., 2014), it is likely that the reported binge alcohol-induced activation of the PI3K pathway in male mice occurred at an earlier time point than what was measured in the present study. Perhaps related to the time course for activation of the PI3K pathway, the timing of the binge alcohol exposure during the animal's circadian cycle may also contribute to the directionality of the effect. Light activation of the mTOR pathway in the suprachiasmatic nuclei is phase-restricted to the nighttime (Cao and Obrietan, 2010), but additional studies are necessary to determine whether there is a causal relationship between circadian cycle and mTOR-associated signaling specifically in the NAC. A comparison of studies that examined the phosphorylation state of PI3K and downstream signaling molecules during withdrawal from binge drinking suggest that activation was seen when binge alcohol consumption occurred during the circadian dark

phase (Cuzzoli et al., 2009, 2012; Neasta et al., 2010 – but see Neasta et al., 2011), whereas in the present study, binge alcohol consumption occurred during the circadian light phase. It is hard to know whether timing of the binge alcohol exposure during the circadian cycle or the total dose of alcohol consumed contributed to the directionality of the effect between studies. Another possibility is that variations in circulating corticosterone levels between studies contributed to the differences in the directionality of the effects, as 7 days of corticosterone exposure in the drinking water reduced mGlu5 expression in the NAC of male rats (Besheer et al., 2014). Regardless, the current work confirms the alcohol-induced alteration of PI3K-related signaling in the NAC of male but not female mice. This result is consistent with the observation that neuroadaptive changes observed in the medial prefrontal cortex following chronic intoxication indicate a distinct biology between males and females, with different signaling pathways affected during exposure and early withdrawal (Hashimoto and Wren, 2008; Wilhelm et al., 2014).

In addition to the molecular data, the current work establishes for the first time that local infusion of rapamycin into the NAC did not alter binge alcohol consumption in female mice, whereas the reduction in binge alcohol consumption in male mice is consistent with prior work (e.g., Neasta et al., 2010, 2011). Rapamycin inhibits mTOR signaling by complexing with FKBP12, which in turn binds to the FRB domain of mTOR to prevent the association of its catalytic sites with its substrates (Dowling et al., 2010; Yip et al., 2010). To this end, rapamycin is capable of reducing the activation of mTOR-related substrates without directly altering mTOR phosphorylation (Oshiro et al., 2004). This allosteric mechanism of action for rapamycin supports the ability to further deactivate mTOR despite the possible reduction in phosphorylation via withdrawal from binge alcohol consumption. However, an alternate and more parsimonious explanation is that rapamycin blocked a binge alcohol-induced activation of mTOR, because it was administered prior to binge alcohol consumption, and that the alcohol-induced activation of mTOR was more transient than what was reported in other studies and not detectable at the withdrawal time point examined in the present study.

While the current work confirms the importance of PI3K-related signaling following binge alcohol consumption in the NAC of male mice, it is the first to indicate that this effect is robustly sex-specific. Female mice were relatively resistant to alcohol-induced changes in both protein levels and gene expression, and this sex difference corresponded to the functional analysis, where again females were resistant to the ability of rapamycin to decrease binge alcohol consumption. Importantly, the range of rapamycin doses administered into the NAC in the present study (5 – 100 ng/side) exceeded doses than were shown to significantly decrease alcohol intake in prior work where a 5 ng/side dose of rapamycin significantly decreased 30 min alcohol intake in males (Neasta et al., 2010), making it unlikely that the dose response curve for rapamycin was simply shifted to the right in females. Collectively, the results provide strong functional evidence that targeting a molecule that is not affected by binge drinking in females will not disrupt binge alcohol intake.

There are several important biological implications of these findings. The mTOR signaling pathway is important for the formation of long-term fear memories (e.g., Parsons et al., 2006), and it was recently shown to be critical in the reconsolidation of alcohol-related

memories (Barak et al., 2013). Rapamycin disrupted the reconsolidation of alcohol-related cues and suppressed cue-induced relapse in male rats (Barak et al., 2013), but the present results suggest that a similar strategy to reduce relapse in females would be ineffective. Another consideration is that Group 1 mGluR can regulate the activity of multiple DNA transcription factors and thereby alter gene expression at both the transcriptional and translational levels (e.g., Wang and Zhuo, 2012). Thus, the sex differences in the binge alcohol-induced down regulation in mGlu5 mRNA and protein might differentially alter the expression of multiple genes beyond those implicated in glutamatergic signaling. Related to this point, female mice were sensitive to the ability of an mGlu5 antagonist to decrease binge alcohol consumption (Cuzzoli et al., 2014a), but they were insensitive to the ability of an antagonist of a molecule in the PI3K signaling cascade (i.e., rapamycin; mTOR inhibitor) to decrease binge drinking (present findings). These findings indicate that in female mice, an alternate signaling pathway that is independent of PI3K and that links group 1 mGluR to transcriptional changes in the nucleus (i.e., protein kinase A, calcium calmodulin dependent protein kinase, or mitogen-activated protein kinase (MAPK); see Wang and Zhuo, 2012) is influenced by repeated binge alcohol consumption. Related to this point, estradiol acting at a membrane estrogen receptor (mERa) facilitated mGlu1a signaling that resulted in phosphorylation of cAMP response element binding protein (CREB) through phospholipase C regulation of MAPK in hippocampal neurons (Boulware et al., 2005). In female striatal neurons, mERa activates mGlu5 signaling to influence MAPK-dependent CREB phosphorylation (Grove-Strawser et al., 2010). Thus, coupling of mERa to mGluR can initiate independent signal transduction pathways (reviewed in Meitzen and Mermelstein, 2011).

In summary, the present results highlight sex differences in the influence of repeated binge alcohol consumption on PI3K-related signaling in the NAC of male and female mice. Additionally, mTOR signaling in the NAC was necessary to maintain binge alcohol consumption only in male mice. Because mTOR complex 1 is proposed to be a common point of neuronal signaling that is important for drug-induced plasticity, the resistance of female mice to the ability of rapamycin to decrease binge alcohol intake emphasizes the sex specificity of potential pharmacotherapeutic targets for alcohol use disorders. Consistent with this idea, expression profiling following chronic intoxication in male and female mice revealed a divergent neuroadaptive response in the prefrontal cortex that would result in dysregulation of distinct biological pathways between the sexes (Hashimoto and Wren, 2008; Wilhelm et al., 2014). Thus, an increased understanding of sexually dimorphic molecular pathways influenced by repeated binge alcohol intoxication can lead to novel treatment options for males and females.

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Bullet Points/Highlights

Binge drinking recruits sexually divergent signaling cascades in the accumbens

Intra-accumbens rapamycin decreases binge drinking only in male mice

Binge drinking decreased PI3K signaling in the accumbens only in male mice

Age did not alter sex differences in accumbens signaling after binge drinking

Figure 1. Withdrawal from repeated binge alcohol consumption significantly altered protein levels of phosphoinositide 3-kinase (PI3K) and downstream signaling molecules without altering metabotropic glutamate receptor 1 or 5 (mGlu1 or mGlu5) in the nucleus accumbens (NAC) of male mice

Withdrawal (24 hr) from repeated binge alcohol consumption (SHAC) did not alter (A) mGlu1 or mGlu5 or (C) phosphoinositide-dependent protein kinase 1 (PDK1), but it significantly decreased the phosphorylation or ratio (index of activation level) of (B) PI3K, (D) mammalian target of rapamycin (mTOR), (E) 4E-binding protein 1 (4EBP1), and (F) p70 ribosomal protein S6 kinase (p70s6K), when compared with water drinking control

animals (Water). Values are collapsed across age and represent the mean $\pm$ SEM for each group (Water: n=15-16; SHAC: n=17-18). All levels were initially normalized to α -tubulin. Fold regulation was then determined by normalizing all values to the mean of the relative expression of the Water group. Representative immunoblots for each protein are included beneath each graph. ⁺ $p<0.09$, * $p<0.05$ vs. water.

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Figure 2. Withdrawal from repeated binge alcohol consumption significantly altered protein levels of metabotropic glutamate receptor 5 (mGlu5) without altering phosphoinositide 3-kinase (PI3K) or downstream signaling molecules in the nucleus accumbens (NAC) of female mice. Withdrawal (24 hr) from repeated binge alcohol consumption (SHAC) significantly decreased (A) mGlu5, but not mGlu1, and it did not alter indices of (B) PI3K, (C) phosphoinositide-dependent protein kinase 1 (PDK1), (D) mammalian target of rapamycin (mTOR), (E) 4E-binding protein 1 (4EBP1), or (F) p70 ribosomal protein S6 kinase (p70s6K), when compared with water drinking control animals (Water). Values are collapsed across age and represent the mean $\pm$ SEM for each group (Water: n=16-18; SHAC: n=16-17;

except for p-4EBP1, 4EBP1, and mGlu5 where Water: n=9-14 and SHAC: n=8-13). All levels were initially normalized to α -tubulin. Fold regulation was then determined by normalizing all values to the mean of the relative expression of the Water group. Representative immunoblots for each protein are included beneath each graph. * $p<0.05$ vs. water.

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Figure 3. Sex differences in the effect of withdrawal from repeated binge alcohol intake on RNA expression of glutamatergic genes in the nucleus accumbens (NAC)

Withdrawal (24 hr) from repeated binge alcohol consumption (SHAC) significantly decreased the expression of (A) *Grm1* & *Grm5* (mGlu1 and 5 gene), (B) *Grin2A* (NR2A subunit of the NMDA receptor gene), and (C) *Homer2* (homer homolog 2 gene) without altering expression of (B) *Grin2B* (NR2B subunit of the NMDA receptor gene) or (C) *Pdkp1* (3-phosphoinositide dependent protein kinase 1 gene) in the NAC of male mice, when compared with controls (Water). Conversely, withdrawal from repeated binge alcohol intake (SHAC) did not alter (D) *Grm1* & *Grm5*, (E) *Grin2A* or (F) *Homer2*, but it decreased the expression of (F) *Pdkp1* in the NAC of female mice, when compared with controls (Water). Values are collapsed across age and represent the mean $\pm$ SEM for each group (Water: n=11-14; SHAC: n=7-14). Fold regulation was determined by normalizing all values to the mean of the relative expression of the Water group. + $p < 0.09$, * $p < 0.05$ vs. water. N.D. = not detectable.

Figure 4. Inhibition of mammalian target of rapamycin (mTOR) in the nucleus accumbens (NAC) decreases binge alcohol consumption in male but not female mice without altering water consumption

(A) Rapamycin dose-dependently decreased alcohol intake during a 30-min binge alcohol session in male but not in female mice. (B) Summary of the effects of the intra-NAC doses of rapamycin on the subsequent 3.5 hr water access period as well as (C) the total intake (in mLs) from the entire 4 hr fluid access period. (D) Intra-NAC infusion of vehicle or 100 ng/side rapamycin did not alter 30 min water intake or (E) 4 hr total water intake on days when animals only had access to water, demonstrating selectively to reduce binge alcohol intake in male mice. (F) Schematic representation of microinjection placements in the NAC for the male (black circles) and female (gray circles) animals included in the study. Values represent the mean $\pm$ SEM of each group (Males: $n=8$; Females: $n=6-7$). * $p<0.05$, ** $p<0.01$ vs. respective vehicle infusion; ** $p<0.01$ ANOVA.

Average binge alcohol intake, total fluid intake, and body weight of animals used for Western blot analysis

Table 1

Data are the mean $\pm$ SEM for the number of mice in parentheses. Total fluid intake (as mL/kg and mL) and body weight (g) reflect data from both the binge alcohol (SHAC) and water control groups, as there was no effect of treatment on these dependent variables.

Sex	Age	Alcohol Intake (g/kg)	Total Fluid Intake (mL/kg)	Total Fluid Intake (mL)	Body Weight (g)
Male	Adult	2.3 $\pm$ 0.1 (9)	161.2 $\pm$ 1.9 (16)	3.8 $\pm$ 0.1 (16)	23.2 $\pm$ 0.4 (16)
	Adolescent	2.7 $\pm$ 0.1 (9)	222.5 $\pm$ 3.9 (18)	3.9 $\pm$ 0.1 (18)	17.6 $\pm$ 0.2 (18)
Female	Adult	2.3 $\pm$ 0.1 (9)	202.5 $\pm$ 5.1 (18)	3.4 $\pm$ 0.1 (18)	16.8 $\pm$ 0.3 (18)
	Adolescent	2.4 $\pm$ 0.1 (9)	240.5 $\pm$ 5.1 (18)	3.4 $\pm$ 0.1 (18)	14.1 $\pm$ 0.2 (18)

Table 2
Average binge alcohol intake, total fluid intake, and body weight of animals used for qRT-PCR analysis

Data are the mean $\pm$ SEM for the number of mice in parentheses. Total fluid intake (as mL/kg and mL) and body weight reflect data from both the binge alcohol (SHAC) and water control groups, as there was no effect of treatment on these dependent variables.

Sex	Age	Alcohol Intake (g/kg)	Total Fluid Intake (mL/kg)	Total Fluid Intake (mL)	Body Weight (g)
Male	Adult	2.4 $\pm$ 0.1 (9)	164.8 $\pm$ 3.2 (16)	3.9 $\pm$ 0.1 (16)	23.5 $\pm$ 0.4 (16)
	Adolescent	2.9 $\pm$ 0.1 (9)	228.3 $\pm$ 6.0 (18)	3.9 $\pm$ 0.1 (18)	17.4 $\pm$ 0.2 (18)
Female	Adult	2.2 $\pm$ 0.1 (9)	217.9 $\pm$ 8.4 (18)	3.7 $\pm$ 0.1 (18)	16.9 $\pm$ 0.2 (18)
	Adolescent	2.3 $\pm$ 0.1 (8)	245.9 $\pm$ 6.3 (17)	3.5 $\pm$ 0.1 (17)	14.2 $\pm$ 0.2 (17)