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CaMKIIα-GluA1 activity underlies vulnerability to adolescent binge alcohol drinking

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Abstract

Background—Binge drinking during adolescence is associated with increased risk for developing alcohol use disorders (AUDs); however, the neural mechanisms underlying this liability are unclear. In this study, we sought to determine if binge-drinking alters expression or phosphorylation of two molecular mechanisms of neuroplasticity, calcium/calmodulin dependent kinase II alpha (CaMKIIα) and the GluA1 subunit of AMPA receptors (AMPAR) in addiction-associated brain regions. We also asked if activation of CaMKIIα-dependent AMPAR activity escalates binge-like drinking.

Methods—To address these questions, $CaMKII\alpha_{T286}$ and $GluA1_{S831}$ protein phosphorylation and expression were assessed in the amygdala and striatum of adolescent and adult male C57BL/6J mice immediately after voluntary binge-like alcohol drinking (blood alcohol > 80mg/dL). In separate mice, effects of the CaMKIIα-dependent $pGluA1_{S831}$-enhancing drug tianeptine were tested on binge-like alcohol consumption in both age groups.

Results—Binge-like drinking decreased $CaMKII\alpha_{T286}$ phosphorylation ($pCaMKII\alpha_{T286}$) selectively in adolescent amygdala with no effect in adults. Alcohol also produced a trend for reduced $pGluA1_{S831}$ expression in adolescent amygdala but differentially increased $pGluA1_{S831}$ in adult amygdala. No effects were observed in the nucleus accumbens or dorsal striatum. Tianeptine increased binge-like alcohol consumption in adolescents but decreased alcohol consumption in adults. Sucrose consumption was similarly decreased by tianeptine pretreatment in both ages.

Conclusions—These data show that the adolescent and adult amygdalae are differentially sensitive to effects of binge-like alcohol drinking on plasticity-linked glutamate signaling molecules. Tianeptine-induced increases in binge-like drinking only in adolescents suggest that differential CaMKIIα-dependent AMPAR activation may underlie age-related escalation of binge drinking.

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Keywords

Adolescence; alcohol; binge; CaMKII; GluA1

Introduction

Adolescence is a distinct developmental period that in humans occurs from the early teens to the early twenties. This developmental stage is characterized by increased exploration and risk-taking, and is the time at which experimentation with drugs of abuse, including alcohol, is usually initiated (Spear, 2000). Alarming data show that 25 – 37% of U.S. high school students engage in heavy episodic or binge drinking (Miller et al., 2007) at more than twice the rate of adults (Nelson et al., 2009), and a recent survey revealed that approximately 22% of 12th grade students had engaged in binge drinking in the last two weeks (Johnston LD, 2012). Binge drinking during adolescence is particularly troubling in light of evidence that individuals who initiate alcohol use during early adolescence are at substantially higher risk for the development of alcohol use disorders (AUDs) than those who initiate alcohol use as young adults (Hingson et al., 2006, Grant and Dawson, 1998). These data suggest that adolescent alcohol users are uniquely vulnerable to the development of AUDs, and indicate that a significant portion of the population is subject to this increased risk. However, the precise neurobiological mechanisms that mediate this effect have yet to be determined (Schramm-Sapyta et al., 2008).

In humans and in rodent models, adolescence is characterized by increased neuronal plasticity as the brain matures from childhood to adulthood (Selemon, 2013). This suggests that adolescents may be more vulnerable to the effects of alcohol on plasticity-linked cellular functions. Indeed, evidence indicates that adolescent rodents are more sensitive to alcohol-induced disruption of long-term potentiation (Pyapali et al., 1999), which is a cellular mechanism that underlies behavioral plasticity. Similarly, we have shown that adolescent mice are more sensitive than adults to acute alcohol-induced changes in ERK MAP kinase activation in the amygdala (Spanos et al., 2012) which is required for LTP in this region (Apergis-Schoute et al., 2005). Further, alcohol exposure blunts LTP in the amygdala (Stephens et al., 2005) where plasticity-linked proteins regulate alcohol-seeking behavior (Salling et al., 2014, Schroeder et al., 2008). Thus, increased understanding of alcohol-induced changes in signaling systems that regulate cellular plasticity may be of interest to identify mechanisms underlying adolescent vulnerability to development of AUDs.

Glutamate is the primary excitatory neurotransmitter in the mammalian brain and its receptors and cellular signaling pathways are required for adaptive plasticity. Calcium/calmodulin-dependent protein kinase II (CaMKII) is a 12-subunit protein expressed primarily in glutamatergic synapses, where it has a well-established role in synaptic plasticity, learning and memory (Lisman et al., 2002). CaMKII has been shown to be involved in alcohol consumption and reward. Mice expressing autophosphorylation-deficient CaMKIIα have been shown to drink less alcohol than wild type littermates (Easton et al., 2013a) and display altered conditioned place preference for alcohol (Easton et al.,

2013b). We have shown that voluntary alcohol drinking increases expression of the active (phosphorylated) form of CaMKIIα$_{T286}$ (pCaMKIIα$_{T286}$) in the mouse amygdala, and that intra-amygdala inhibition of CaMKII activity reduces the positive reinforcing effects of alcohol (Salling et al., 2014). CaMKII is activated upon phosphorylation, allowing it to phosphorylate several downstream targets including the GluA1$_{S831}$ subunit of the AMPA subtype of glutamate receptors (Derkach et al., 1999). Phosphorylation of GluA1$_{S831}$ (pGluA1$_{S831}$) is associated with increased stability of AMPARs in the synapse, which promotes synaptic plasticity and learning (Lee et al., 2010). Our work also shows that AMPAR activity in the amygdala is required for the reinforcing effects of alcohol (Salling et al., 2014). Together, these data suggest a critical role for CaMKIIα-GluA1 signaling in alcohol self-administration and reinforcement. However, age-dependent differences in sensitivity to alcohol-induced modifications of this system have not been explored.

To address this question, the present study was designed to test the hypothesis that adolescents are more sensitive to alcohol-induced changes in CaMKIIα and AMPAR GluA1protein phosphorylation and expression than adults. We chose to investigate protein changes in the amygdala and striatum based on our previous results as well as a wealth of literature indicating that these regions are functionally involved in alcohol self-administration and reinforcement (Hodge et al., 1992, Salling et al., 2014). To evaluate the functional involvement of this system in adolescent and adult alcohol drinking, we used the drug tianeptine, a systemic upregulator of CaMKIIa-dependent GluA1 activation (e.g., phosphorylation). A binge-like model of alcohol access was utilized in these experiments in order to most closely mimic the drinking patterns exhibited by adolescents in the United States.

Materials and Methods

Animals

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were individually housed in standard Plexiglass cages with a small PVC pipe for environmental enrichment. Adolescent mice were postnatal day 21 (PND 21) and adults were (PND 63±2) upon arrival in our facility. Food and water were available ad libitum in home cages except where noted. The colony room was maintained on a 12-h light/dark cycle (lights on at 20:00 in the initial binge exposure experiment and lights on at 19:00 in the pharmacology experiments) at 21°C. All experimental manipulations and testing occurred during the dark cycle. All procedures were carried out in accordance with the NIH *Guide to Care and Use of Laboratory Animals* (NRC, 1996) and approved by the Internal Review Board of the University of North Carolina, Chapel Hill.

Experiment 1: Daily Binge Alcohol Exposure and Protein Changes in the Adolescent and Adult Mouse Brain

Binge Alcohol Exposure Procedure—Adolescent and adult mice (N=40) were allowed one week (PND 21–27) or (PND 63–69) to habituate to our colony and acclimate to the light/dark cycle (Fig 1A). During habituation, mice were handled and weighed daily. Mice were then brought to consume alcohol in a binge-like access procedure adapted from

Rhodes et al. (2005). Beginning on PND 28 or PND 70, mice were weighed and home cage water bottles were removed at 11:00AM and replaced with a single drinking tube containing either 20% (v/v) alcohol (alcohol treated animals) or water (water control animals) for four hours, n=10/treatment/age. The drinking tubes consisted of a 10mL serological pipette fitted with a ball-bearing sipper tube and fastened to the wire cage lid with a medium binder clip. This limited access procedure was repeated daily for two weeks, ending on PND 42 for adolescents or PND 84 for adults.

Tissue Collection—Immediately after the cessation of drinking, on the last day of testing, mice were rapidly decapitated and blood alcohol concentration (BAC) was assessed. Approximately 20μL of trunk blood was centrifuged to obtain 5μL of plasma for use in an AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA). The brains were extracted from the skulls and flash-frozen in −30°C isopentane.

Western Blot Analysis—Brain regions of interest were dissected from coronal brain sections (1mm slices) using a 1mm sterile tissue punch (Stoelting, Wood Dale, IL) and homogenized in buffer (10ml: 1.1g sucrose, 50μL 1M HEPES buffer, 1:100 protease/phosphatase inhibitor cocktail, 1mL 10% SDS, 26.55mL ultra-pure water.) Protein concentration was measured using a calorimetric assay kit (Pierce Biotechnology, Rockford, IL). Protein (5μg) was diluted 4:1 with lithium dodecyl sulfate sample buffer (40–70% glycerol), 10:1 with sample reducing agent, vortexed, loaded onto a Nu-Page 4–15% Tris-glycine polyacrylamide gel (Invitrogen, Carlsbad, CA) for gel electrophoresis separation, and transferred to nitrocellulose membrane using an iBlot dry blotting system (Life Technologies, Grand Island, NY). Membranes were blocked with 3% albumin bovine serum (Sigma-Aldrich, St. Louis, MO) before being incubated with primary antibodies [rabbit anti-pCaMKII$_{T286}$ (1:2 500; Abcam, Cambridge, MA), -pGluR1$_{S831}$ (1:1 000, EMD Millipore, Billerica, MA) -tGluR1 (1:1 000; Abcam) and mouse anti-tCaMKIIα (1:10 000; Millipore), in blocking solution at 4°C overnight and β-actin (mouse monoclonal, 1:5 000; Sigma) in blocking solution 1 h at room temperature] and washed before incubation with secondary antibodies (HRP-conjugated goat anti-rabbit and goat anti-mouse, 1:10 000; Jackson ImmunoResearch, West Grove, PA). Membranes were then visualized using enhanced chemiluminescence substrate (Pierce), and bands were quantified using optical density measurements (NIH/Scion Image).

Experiment 2: Pharmacological Manipulation of Intermittent Binge Drinking

Binge Alcohol or Sucrose Drinking Procedure—Mice (N=24/experiment) were allowed a one-week (PND 21–27) or (PND 63–69) habituation period (Fig 4A). During habituation, mice were handled and weighed daily and given saline injections to habituate to the injection procedure. Beginning on PND 28 (adolescent) or PND 70 (adult), mice were weighed and the home cage water bottles were removed at 10:00AM and replaced with a drinking tube containing 20% (v/v) alcohol (alcohol experiment) or 0.5% sucrose (sucrose experiment). Mice had access to alcohol or sucrose for four hours every other day. Beginning on PND 36 or PND 80, mice were treated with 0, 3, 10 or 17 mg/kg tianeptine (i.p.) 30 minutes prior to alcohol or sucrose tube access according to a Latin square dosing regimen. Drug pretreatment and drinking continued on PND 38, 40, and 42 (adolescent) or

PND 82, 84, and 86 (adult). Tianeptine dose range and pretreatment time were determined from a study showing behavioral effects in alcohol-exposed rodents (Uzbay et al., 2006).

Locomotor Testing—On PND 46 or PND 90, mice were pretreated with either saline or the effective dose of tianeptine (10 mg/kg for adolescents, 17 mg/kg for adults) for 2 hours prior to a locomotor activity test (n=6/treatment/age). Open field activity was measured in Plexiglas activity monitor chambers (27.9 cm^2; ENV-510, Med Associates, Georgia, VT). Two sets of 16 pulse-modulated infrared photobeams were located on opposite walls and recorded X–Y ambulatory movements. Distance traveled (in meters) throughout the session was quantified by assessing the mouse's position in the open field every 100 miliseconds. Data from each chamber were collected by a computer.

Blood Alcohol Clearance—On PND 47–48 or PND 91–92, adolescent (n=6) and adult (n=6) mice were pretreated with the effective dose of tianeptine (10 mg/kg for adolescents, 17 mg/kg for adults) or saline. Thirty minutes later, mice were injected with 4.0 g/kg alcohol (20% w/v) i.p. Beginning at 10 minutes post-alcohol injection, mice were confined in clear Plexiglas restraint tubes (Braintree Scientific, Braintree, MA), tail blood was collected using a heparinized microcapillary tube. Additional samples were collected at 30 minutes, 1 hour, 2 hours and 4 hours post-alcohol injection. Blood samples were analyzed as in Experiment 1.

Drugs—Alcohol solutions (v/v) were prepared by diluting 95% ethanol (Pharmco Products Inc., Brookfield, CT) with tap water (for drinking) or 0.9% saline (for injection). The GluA1 modulator tianeptine (Tocris Bioscience; Ellisville, MO) was freshly dissolved in saline before each day of testing.

Data Analysis—All analyses were performed using Prism v. 6.0 (GraphPad, La Jolla, CA). For drinking experiments, alcohol intake data were reported as grams of intake per kilogram of body weight and water intake data were reported as milliliters of fluid consumed per kilogram of body weight. Intake of both solutions was analyzed via two-way repeated measures ANOVA (Age × Day). BAC was analyzed via t-test to compare adolescent and adult values.

Western blot data were expressed as percent change from age-matched water drinking controls, and all protein levels were expressed as a ratio to β-Actin. Data were analyzed via independent t-tests comparing alcohol and water treated animals separately within each age group.

In the two tianeptine pretreatment experiments, alcohol and sucrose intake were analyzed via two-way repeated measures ANOVA (Age × Dose) respectively. Tukey's LSD test was used for all post-hoc analyses. BEC was analyzed via two-way repeated measures ANOVA (Dose × Time) separately within each age. Open field locomotor data were collapsed into 20 minute time bins and activity was assessed via two-way repeated measures ANOVA (Dose × Time) separately within each age. Locomotor data were further analyzed for potential age or drug-induced differences in anxiety-like behavior in an open-field test. Thigmotaxis was evaluated by comparing distance (cm) traveled in the center zone (inner 25% of the area) to

distance traveled in the periphery (outer 75% of the area) as previously described (Hodge et al., 2002). α was set at 0.05 for all comparisons.

Results

Experiment 1

Binge-like Alcohol Consumption—Adolescent and adult mice consumed equivalent amounts of alcohol and water over the two-week daily access period (adolescents averaged 5.01 ±.22 g/kg and adults averaged 5.09 ±.13 g/kg, Figures 1B and C). Alcohol drinking did not alter body weight in adolescents or adults (Figure 1D). Blood alcohol levels immediately after drinking on the last experimental day exceeded the NIAAA criteria for a binge drinking session (80 mg/dL), with no significant differences between the two age groups (Figure 1E) (NIAAA, 2004).

CaMKIIα Changes in the Amygdala—Western blot analysis of adolescent amygdala revealed that a two-week history of alcohol drinking significantly decreased the phosphorylation of CaMKIIα_{T286} by approximately 30%, $t(17)= 1.916$, $p < 0.05$ (Figure 2A). No differences in total CaMKIIα expression emerged in adolescents (Figure 2B). In contrast, neither pCaMKIIα_{T286} nor tCaMKIIα were affected by alcohol drinking in the adult amygdala (Figure 2C and D).

CaMKIIα Changes in the Striatum—Analysis of the adolescent and adult nucleus accumbens via Western blot showed no effect of alcohol exposure on pCaMKIIα_{T286} in either age (Table 1). Similarly, pCaMKIIα_{T286} levels were unaltered by alcohol drinking in both ages in the dorsal striatum. Total expression of CaMKIIα was also unaffected by alcohol exposure in adolescents and adults in both regions.

GluA1 Changes in the Amygdala—To examine a downstream target of phosphorylated CaMKII activity, the GluA1 subunit of the AMPAR receptor was analyzed in the adolescent and adult amygdala. Western blot analysis showed that in the adolescent amygdala, a two-week history of alcohol drinking had a non-significant trend to decrease pGluA1$_{Ser831}$, $p= 0.11$ (Figure 3A). In the adult amygdala, alcohol drinking significantly increased pGluA1$_{Ser831}$ by approximately 65%, $t (17)= 1.262$, $p =0.05$ (Figure 3C). No effect of alcohol drinking on total GluA1 expression emerged in either age (Figure 3B and D).

Experiment 2

Effect of Tianeptine Pretreatment on Binge-like Alcohol Consumption—Prior to drug treatment, baseline 4-hour every-other-day alcohol intake was significantly greater among adolescents (5.6 g/kg ±0.69) than adults (3.7 g/kg ±0.69) [Main effect of Age, $F(1, 22) = 8.896$, $p < 0.01$]. Over the course of the entire experiment, adolescent body weight increased steadily from an average of 14g on PND 28 to an average of 21g on PND42. The rate of body weight gain did not change following tianeptine pretreatment and is consistent with the published literature for mouse body weight at these ages (Huang et al., 2012).

Following 30 minute pretreatment with tianeptine, a Dose × Age interaction emerged, $F(3, 66) = 5.062$, $p < 0.001$. Tukey's multiple comparisons test revealed that among adolescent

mice, the 10mg/kg dose of tianeptine significantly increased alcohol consumption relative to vehicle (p <0.05, Figure 4B). Adolescent alcohol intake increased from 5.4 g/kg to 6.6 g/kg (22%). In contrast, the 17mg/kg dose of tianeptine significantly decreased alcohol consumption relative to vehicle in adults (p <0.05). Adult alcohol intake decreased from 4.2 g/kg to 3.2 g/kg (25%). Dose effects were only apparent during the last two hours and total four-hour intake period (data not shown.) A main effect of Age also emerged [$F(1, 22) = 45.37$, p <0.0001] with adolescents consuming significantly more alcohol than adults.

Selectivity of Tianeptine Effects—To assess behavioral (alcohol) specificity, we evaluated effects of tianeptine pretreatment on binge-like consumption of sucrose, a non-drug solution that has rewarding properties. Analysis of 4-hour sucrose consumption following 30-minute pretreatment with tianeptine revealed a main effect of Dose, $F(3,63) = 3.579$, p <0.05, indicating that tianeptine decreased sucrose consumption similarly in adolescent and adult mice. No Age × Dose interaction emerged (p >0.05, Figure 4C).

To determine if tianeptine effects were associated with nonspecific locomotor effects, adolescent and adult mice were pretreated with either vehicle or the effective dose of tianeptine (10mg/kg for adolescents and 17mg/kg for adults) 2 hours prior to a 2 hour open-field locomotor activity test in order to correspond with the time interval during which tianeptine effects on alcohol drinking were observed. Analysis of cumulative locomotor activity in both adolescents and adults failed to detect any significant effect of tianeptine pretreatment (p >0.05, Figure 4D and E).

To evaluate anxiety-like behavior, locomotor data were analyzed for time spent in the center versus perimeter of the chamber. A main effect of zone emerged, such that mice in both age groups spent more time in the perimeter of the chamber than in the center zone, $F(1, 10) = 504.0$, $p < 0.0001$. Under vehicle treatment conditions, both adolescents and adults spent approximately 80% of their time in the perimeter zone. No significant effect of tianeptine pretreatment or age emerged in either zone ($p > 0.05$, data not shown.)

To determine if tianeptine affected alcohol clearance, adolescent and adult mice were pretreated with tianeptine 30 minutes prior to a 4g/kg intraperitoneal injection of alcohol. Analysis of blood alcohol samples collected 10, 30, 60, 120, and 240 minutes after the alcohol injection failed to reveal any significant effects of tianeptine pretreatment on BACs in either age (p >0.05, Figure 4F and G).

Discussion

Although human (Hingson et al., 2006, Grant and Dawson, 1998) and rodent studies (Pascual et al., 2009, Rodd-Henricks et al., 2002, Alaux-Cantin et al., 2013) indicate that adolescent alcohol exposure increases the risk for lifetime AUDs, the neurobiological mechanisms of this age-specific vulnerability remain to be determined. To address this gap in knowledge, we first evaluated effects of voluntary binge-like alcohol drinking on protein expression and activation (e.g., phosphorylation) of CaMKIIα_{T286} and the GluA1$_{S831}$ subunit of AMPARs in adolescent and adult mice. To determine if CaMKIIα-GluA1 signaling mechanistically regulates age-dependent binge drinking, we next assessed the

effects of systemic administration of the antidepressant Tianeptine, which potentiates AMPAR GluA1 activity via activation of CaMKIIα (Szegedi et al 2011), in adolescent and adult mice. Results indicate that voluntary binge-like alcohol drinking produces age-dependent differential effects on pCaMKIIα$_{T286}$ and pGluA1$_{S831}$ in the amygdala. Accordingly, positive modulation of CaMKII-dependent AMPA signaling via systemic Tianeptine administration increased binge-like alcohol drinking specifically in adolescent mice and reduced drinking in adults. Together, these data indicate that binge drinking produces age-dependent effects on CaMKIIα and GluA1 signaling that regulate the amount of alcohol consumed during binge drinking episodes.

Effects of Binge-Drinking on CaMKIIα and GluA1 Expression and Phosphorylation

Adolescent and adult male C57BL/6J mice voluntarily consumed similar doses of alcohol during the two-week exposure period, each achieving blood alcohol levels consistent with binge exposure (e.g., > 80 mg/dL). Binge drinking produced no effects on overall health as indexed by body weight comparisons to parallel water-only controls. The lack of age differences in alcohol intake in Experiment 1 was advantageous because it ruled out alcohol dose-dependent differences in protein expression, allowing us to more clearly interpret age-dependent differences (i.e., without the confound of differential alcohol dose).

Adolescent—Binge-like alcohol drinking produced an age-dependent decrease in pCaMKIIα$_{T286}$ in adolescent amygdala with no change in total protein expression. Since pCaMKIIα$_{T286}$ is a primary molecular mechanism of synaptic plasticity that is required for associative learning and memory processes mediated by the amygdala (Lisman et al., 2002, Rodrigues et al., 2004), binge alcohol-induced downregulation of CaMKIIα activity in the adolescent amygdala may underlie age-dependent inhibition of memory processes as previously observed following alcohol exposure (Broadwater and Spear, 2013, Spanos et al., 2012, Markwiese et al., 1998). This observation is highly significant from a translational perspective since estimates indicate that over 50% of college-age frequent binge drinkers have experienced significant associative memory loss, including complete blackouts (White and Hingson, 2013). Moreover, emerging adults (age18–24) who have experienced binge-induced blackouts have lower levels of glutamate in the anterior cingulate cortex as compared to light drinkers (Silveri et al., 2014). Thus, binge drinking may produce adolescent-specific cognitive deficits by disruption of basic molecular mechanisms of memory and plasticity in the amygdala.

At the molecular and cellular level, alcohol-induced inhibition of pCaMKIIα$_{T286}$ expression in adolescents may disrupt numerous functions subserved by the kinase including regulation of membrane current, neurotransmitter synthesis and release, cytoskeletal organization, dendrite maturation, and gene expression (Colbran and Brown, 2004, Hanson and Schulman, 1992, Lisman et al., 2002, Metzger, 2010). Further, a crucial function of CaMKIIα is to phosphorylate AMPARs at the GluA1$_{S831}$ site, which leads to potentiation of AMPA-mediated synaptic activity (Mammen et al., 1997, Barria et al., 1997), promotes AMPAR membrane insertion, and enhances their function (Colbran and Brown, 2004, Hanson and Schulman, 1992, Lisman et al., 2002). Here, we observed a trend for decreased pGluA1$_{S831}$ following adolescent binge-like alcohol intake. Binge drinking may disrupt a variety of

critical neural and behavioral functions in the adolescent brain via inhibition of CaMKIIα activity and downstream mechanisms. This suggests that binge-induced changes in CaMKIIα activity may be associated with subtle changes in AMPAR signaling.

Adult—In contrast to the adolescent, a two-week history of daily binge alcohol drinking specifically increased $pGluA1_{S831}$ in adult amygdala with no change in $pCaMKII\alpha_{T286}$ or total protein expression. These findings are consistent with previous evidence showing that $pGluA1_{S831}$ is increased by voluntary 24-h home-cage drinking or low-dose operant alcohol self-administration in adult mice (Salling et al., 2014), and by chronic intermittent high-dose alcohol vapor in adult rats (Christian et al., 2012). Phosphorylation of the GluA1 subunit of the AMPAR results in increased single-channel conductance (Derkach, 2003, Derkach et al., 1999) and has been shown to be critical for the induction of LTP (Lee et al., 2010). Recent work in our lab has demonstrated that CaMKII and GluA1 activity in the amygdala functionally regulate alcohol self-administration (Salling et al., 2014). Thus, increased $pGluA1_{S831}$ may be indicative of increased activity of AMPARs in the adult amygdala after alcohol exposure, and may contribute to long-term plastic adaptations to alcohol. Since CaMKIIα phosphorylation was not increased in adults, it is plausible binge-like alcohol drinking does not specifically alter CaMKII signaling in this age group. Thus, $GluA1_{S831}$ phosphorylation may have been mediated by another alcohol-sensitive kinase, such as PKC (Besheer et al., 2006, Hodge et al., 1999, Ren et al., 2013, Wilkie et al., 2007). These results add weight to a growing body of evidence suggesting that glutamate signaling in the amygdala is a target of alcohol self-administration.

Overall, these data show differential age-dependent effects of binge drinking on $CaMKII\alpha_{T286}$ and $GluA1_{S831}$ phosphorylation between adolescent and adult mice. Although the specific mechanism(s) for these effects are unknown, it is plausible that well-documented age-dependent maturation of the target systems is partly accountable. For instance, CaMKIIα expression increases linearly in rat forebrain by 10-fold from PND 5 through 25 (Kelly and Vernon, 1985). Similarly, CaMKIIα mRNA increases 10-fold between PND 1 and 21 with an additional 5-fold increase by PND 90, which spans the adolescent and early adult periods examined in the present study. Additionally, the subcellular distribution of CaMKII shifts from primarily cytosolic to membrane localization during development (Sahyoun et al., 1985, Weinberger and Rostas, 1986). Developmentally regulated protein concentration and localization in neural tissue may influence the effects of alcohol on CaMKIIα phosphorylation.

Tissue for these experiments was collected immediately following the last drinking session, when mice had achieved a binge-level of alcohol consumption. Therefore, the results should be considered in the context of alcohol-induced brain protein changes in the presence of alcohol. The present findings were also selective for the amygdala, with no changes in $pCaMKIIa_{T286}$ or $pGluA1_{S831}$ observed in the nucleus accumbens or the dorsal striatum. Therefore, glutamatergic signaling in the striatum may be less relevant than in the amygdala in terms of the regulation of binge-like alcohol self-administration. The amygdala is a complex region comprised of several subnuclei, including the central, basolateral and lateral subregions, and these nuclei may play different roles in the regulation of alcohol drinking behavior (McCool et al., 2010, McBride, 2002). The current experiment is limited by the use

of whole amygdala tissue, making it difficult to establish the relevance of different sub-nuclei in these findings. Future studies utilizing immunohistochemistry would clarify the contributions of different subregions of the amygdala to the effects observed in the present study.

Effects of the pGluA1$_{S831}$ Upregulator Tianeptine on Binge-Drinking

In Experiment 2, adolescent animals consumed more alcohol than adult animals both at baseline and during drug treatment under vehicle conditions. This result is in contrast to Experiment 1, where adolescent and adult alcohol intake was equivalent. Although adolescents have been reported to consume more alcohol than adults in some studies (Holstein et al., 2011, Hargreaves et al., 2009), other reports have failed to find differences in adolescent and adult intake (Siegmund et al., 2005, Hefner and Holmes, 2007). Procedural differences between experiments are likely to account for discrepancies in age differences across studies. Interestingly, the studies above that failed to find age differences in alcohol self-administration utilized daily or continuous access procedures (as in Experiment 1) whereas the studies that demonstrated increased alcohol consumption during adolescence made use of intermittent drinking protocols (as in Experiment 2). These findings suggest that intermittent drinking procedures may be advantageous for experiments in which age differences in alcohol consumption are under investigation, whereas daily drinking procedures may be useful when dose differences between ages represent a confounding variable.

Binge alcohol consumption increased pGluA1$_{S831}$ in the adult amygdala. Mimicking this effect via systemic pretreatment with the AMPAR positive modulator Tianeptine dose-dependently decreased binge-like alcohol consumption in adult mice. Since Tianeptine increases pGluA1$_{S831}$ in a CaMKII-dependent manner (Szegedi et al., 2011), it may have substituted for a pharmacological effect of alcohol in adult mice, leading to decreased alcohol consumption. This finding is consistent with previous studies which have shown that tianeptine pretreatment decreases alcohol intake (Daoust et al., 1992) and attenuates alcohol withdrawal symptoms in adult rats (Uzbay et al., 2006). Moreover, alcohol self-administration increases pGluA1$_{S831}$ expression in adult mouse amygdala where AMPAR activity is required for the positive reinforcing effects of alcohol (Salling et al., 2014) and increased non-NMDA glutamatergic signaling in the amygdala is associated with alcohol conditioned reward (Zhu et al., 2007). Importantly, tianeptine pretreatment also decreased sucrose consumption in adult mice, suggesting that tianeptine may globally reduce the consumption of palatable solutions. These findings add growing evidence that AMPAR activity is critical for the reinforcing effects of alcohol and other palatable solutions in adults.

In contrast to adults, binge-like alcohol drinking resulted in a trend for decreased pGluA1$_{S831}$ in the adolescent amygdala. Opposing this alcohol-induced effect via systemic pretreatment with tianeptine increased binge-like alcohol consumption in this age group. Tianeptine may therefore have blocked a pharmacological effect of alcohol at AMPARs in adolescent mice, leading to a compensatory increase in alcohol drinking. The effects of tianeptine on adolescent binge drinking formed an inverted U-shaped dose-response curve,

with an intermediate dose leading to increased alcohol consumption and a high dose returning to baseline. It is possible that testing a higher dose would reveal decreased alcohol consumption in adolescents, indicating a rightward shift of the dose-response curve in adolescents corresponding to the effect in adults. Significantly, tianeptine pretreatment produced comparable decreases in sucrose consumption in adolescent and adult mice, indicating that the increase in alcohol drinking seen after tianeptine pretreatment in adolescents is selective for alcohol and not generalized to other reinforcing solutions. The alterations in glutamate signaling reported here in the adolescent mouse may therefore be both selective for the adolescent brain and specific for alcohol. Together, these data provide evidence that CaMKIIα-GluA1 signaling differentially regulates binge-like drinking by adolescent and adult mice. Upregulation of AMPAR activity in the amygdala may promote increased drinking during adolescence but inhibit this behavioral pathology during adulthood. Future studies that target AMPAR activity specifically in the amygdala would clarify the anatomical basis of this age-dependent differential response.

Tianeptine pretreatment failed to significantly alter open-field activity in both adolescents and adults, and blood alcohol clearance was similarly unaffected by tianeptine pretreatment in both ages, indicating a lack of effect of tianeptine on gross locomotor activity and alcohol metabolism. It is possible that tianeptine and alcohol may interact to alter locomotor behavior in adolescent and adult mice. While no obvious locomotor effects were observed during drinking, this possibility limits the interpretation of the locomotor results. Tianeptine may alter the metabolism of lower doses of alcohol but not the higher doses tested here, limiting the interpretation of the blood alcohol clearance experiments. Additionally, the injections administered in these experiments may have represented a significant stressor in the experimental design, potentially contributing to the age differences observed in alcohol drinking. However, zone analysis of adolescent and adult locomotor behavior under vehicle conditions revealed no evidence for anxiety-like behavior in either age, and tianeptine pretreatment did not alter time spent in the center or perimeter of the open field. Therefore, stress-related explanations for these findings are unlikely.

A potential limitation of these findings is the possibility for tianeptine activity at targets other than CaMKII/GluA1 signaling. Unlike most antidepressants, tianeptine has very low affinity for monoamine receptors or transporters (Pineyro et al., 1995) and the drug has not been found to interact directly with NMDA receptors (Svenningsson et al., 2007). However, activation of CaMKII also prompts the protein to bind with the NR2B subunit of the NMDA receptor (Lisman et al., 2012). Therefore, activation of CaMKII via tianeptine pretreatment could alter NMDA receptor activity as well as $GluA1_{Ser831}$ phosphorylation. Additionally, a recent report suggests that tianeptine may have some affinity for the μ-opioid receptor (Gassaway et al., 2014), which could represent another mechanism for tianeptine's effects on alcohol drinking. However, activation of μ-opioid receptors has been shown to be an upstream trigger of the signaling cascade that stimulates CaMKII activation (Garzon et al., 2008), and therefore may be part of the $pGluA_{Ser831}$ potentiation seen after tianeptine treatment.

In conclusion, the findings from this study indicate that binge-like alcohol drinking impacts amygdala glutamate signaling systems of adolescent and adult animals in very different

ways. Glutamate signaling in the amygdala, as indexed by CaMKIIα and GluA1 phosphorylation, appears to be downregulated by alcohol in adolescence and upregulated in adulthood. Results from this study suggest that CaMKIIα-GluA1 signaling in the adolescent amygdala is especially vulnerable to binge-induced insult. Understanding the age-dependent differences in the impact of binge alcohol drinking on glutamate signaling and how these systems, in turn, regulate intake is critical to understanding adolescent vulnerability to alcohol addiction.

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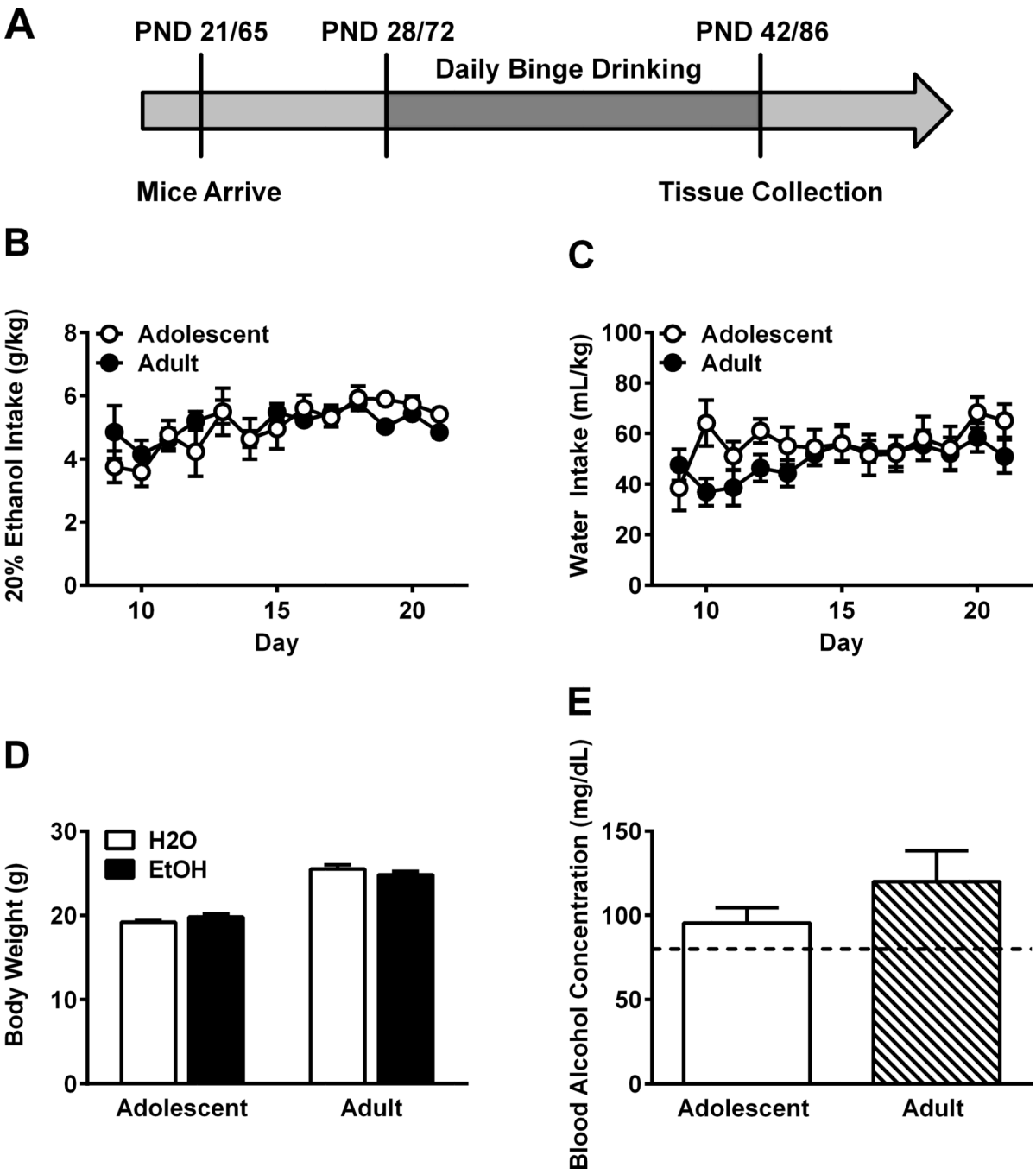

Figure 1. Adolescent and adult mice achieve binge levels of alcohol consumption in a daily limited access procedure

(**A**) Timeline of binge-like drinking procedure. Adolescent and adult alcohol (**B**) and water (**C**) intake did not differ over two weeks of daily drinking sessions. (**D**) Alcohol drinking did not alter body weight in either adolescents or adults. (**E**) Blood ethanol concentration did not differ between alcohol-drinking adolescent and adult mice immediately following the drinking session on the last drinking day. Dashed line indicates the NIAAA criteria for a binge drinking session (≥ 80 mg/dL).

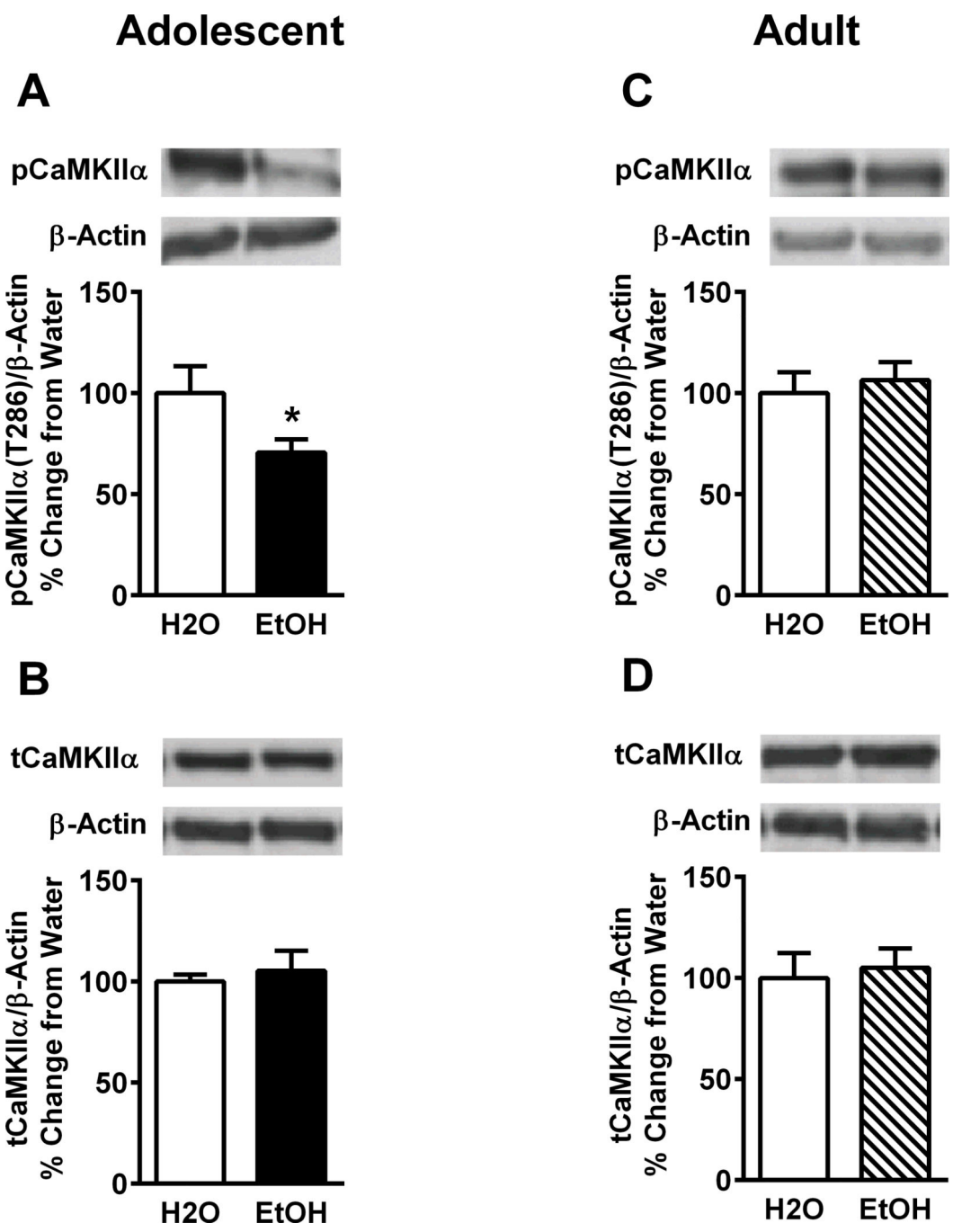

Figure 2. CaMKII phosphorylation is altered by two weeks of daily binge-like alcohol drinking in the adolescent but not adult amygdala

(**A**) Phospho-CaMKIIα_{T286} (pCaMKIIα_{T286}) was decreased in the amygdala of adolescent mice exposed to alcohol in the daily binge drinking procedure (*p <0.05). (**B**) Total CaMKIIα expression was not different between alcohol and water drinking adolescents. (**C**) pCaMKIIα_{T286} did not differ in the amygdala of adult mice exposed to alcohol or water in the daily binge drinking procedure. (**D**) tCaMKIIα expression was also not altered by alcohol treatment in adults.

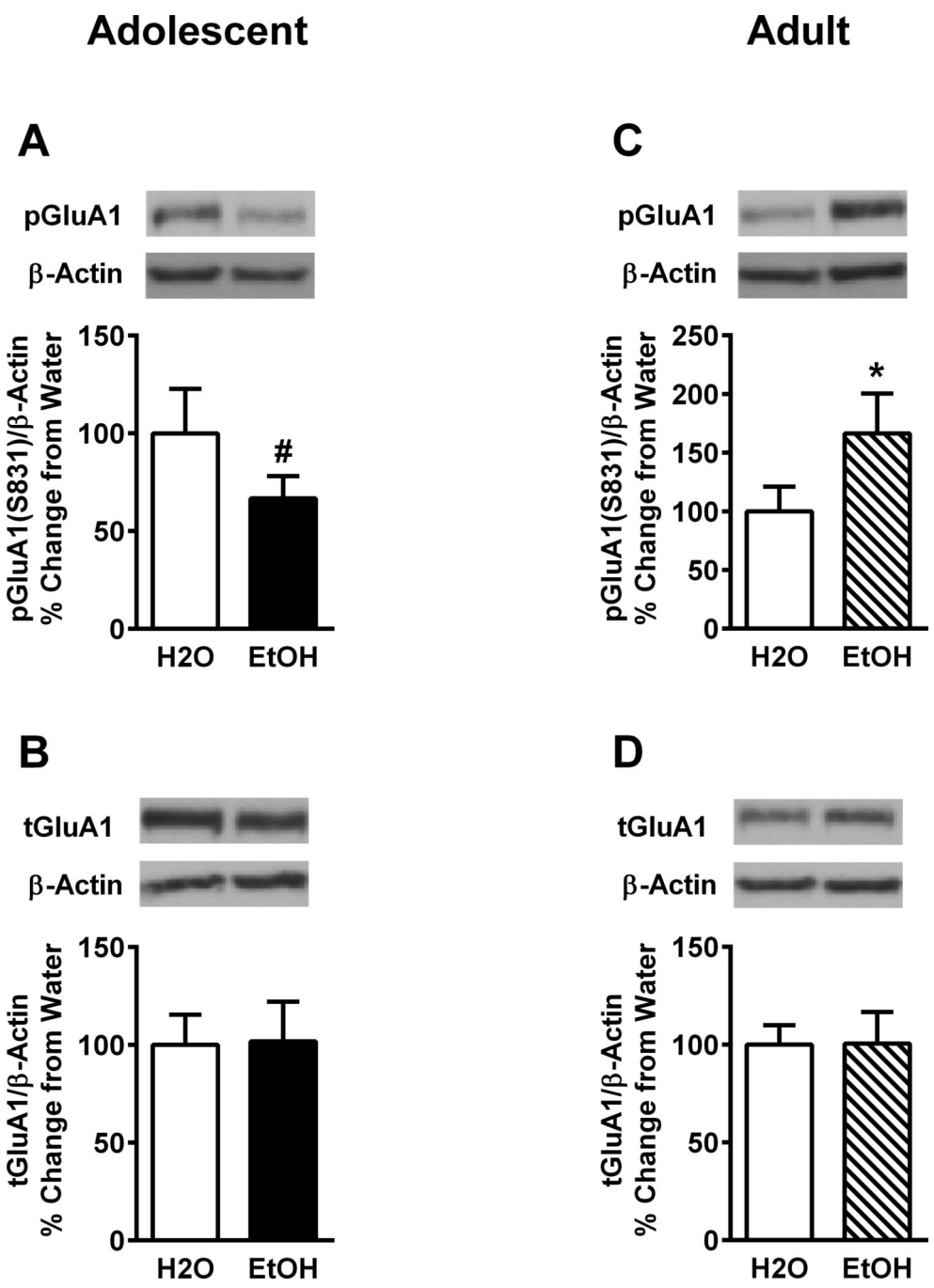

Figure 3. Differential effects of a two-week history of daily binge alcohol exposure on GluA1 phosphorylation in the adolescent and adult amygdala

(**A**) Phospho-GluA1$_{S831}$ (pGluA1$_{S831}$) had a tendency to decrease in the amygdala of adolescent mice exposed to alcohol in the daily binge drinking procedure (#p <0.1). (**B**) Total GluA1 expression was not different between alcohol and water drinking adolescents. (**C**) pGluA1$_{S831}$ was increased in the amygdala of adult mice exposed to alcohol in the daily binge drinking procedure (*p =0.05). (**D**) Total GluA1 expression was not different between alcohol and water drinking adults.

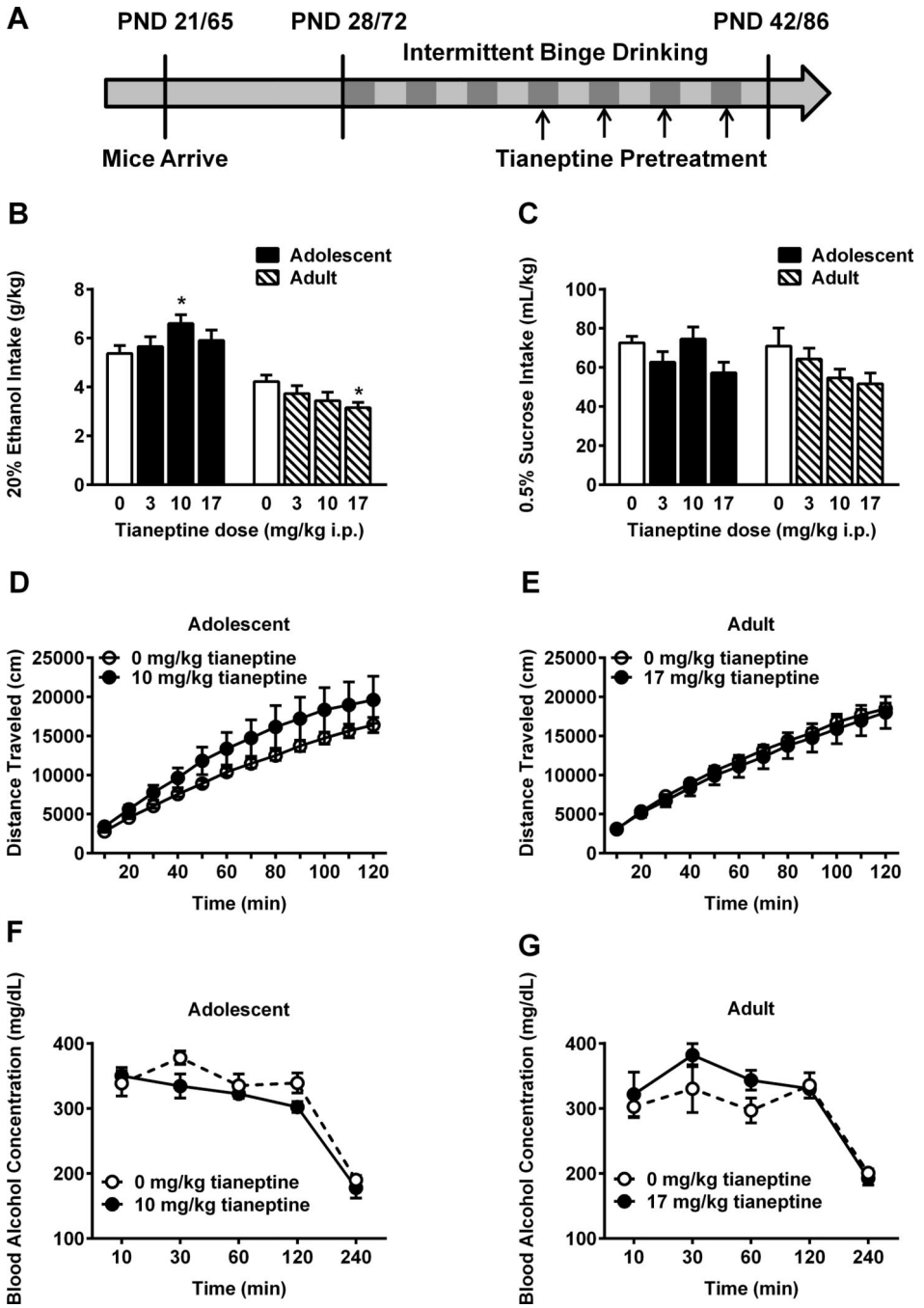

Figure 4. Tianeptine pretreatment alters alcohol, but not sucrose, intake in opposite directions in adolescent and adult male mice

(**A**) Timeline of tianeptine pretreatment binge drinking procedure. (**B**) Tianeptine dose-dependently increased alcohol intake in adolescent mice but decreased alcohol intake in adult mice (*p <0.05). (**C**) Tianeptine comparably decreased sucrose intake in adolescent and adult mice. The open-field locomotor behavior of adolescent (**D**) and adult (**E**) mice injected with the effective dose of tianeptine in each age did not differ from age-matched

controls injected with vehicle. Adolescent (**F**) and adult (**G**) mice pretreated with tianeptine did not differ in blood alcohol concentration following an acute alcohol injection.

Table 1

Phospho-and total-CaMKIIα is unaffected by alcohol drinking in the adolescent and adult nucleus accumbens and dorsal striatum. Data are represented as the mean optical density/β-Actin for each age and treatment condition ±SEM.

	pCaMKIIαT286		tCaMKIIα	
Brain region	**Water**	**Alcohol**	**Water**	**Alcohol**
ADOLESCENT				
Nucleus Accumbens	100±5	101±17	100±8	99±8
Dorsal Striatum	100±9	98±20	100±3	105±3
ADULT				
Nucleus Accumbens	100±15	82±14	100±6	99±4
Dorsal Striatum	100±18	102±13	100±3	107±6