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Differential Effects of Chronic Ethanol Consumption and Withdrawal on Homer/Glutamate Receptor Expression in Subregions of the Accumbens and Amygdala of P Rats

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Abstract

Background—Homer proteins are constituents of scaffolding complexes that regulate the trafficking and function of central Group1 metabotropic glutamate receptors (mGluRs) and *N*-methyl-D-aspartate (NMDA) receptors. Research supports the involvement of these proteins in ethanol-induced neuroplasticity in mouse. In this study, we examined the effects of short versus long-term withdrawal from chronic ethanol consumption on Homer and glutamate receptor protein expression within striatal and amygdala subregions of selectively bred, alcohol-preferring P rats.

Methods—For 6 months, male P rats had concurrent access to 15% and 30% ethanol solutions under intermittent (IA: 4 d/wk) or continuous (CA: 7 d/wk) access conditions in their home cage. Rats were killed 24 hours (short withdrawal: SW) or 4 weeks (long withdrawal: LW) after termination of ethanol access, subregions of interest were micropunched and tissue processed for detection of Group1 mGluRs, NR2 subunits of the NMDA receptor and Homer protein expression.

Results—Within the nucleus accumbens (NAC), limited changes in NR2a and NR2b expression were detected in the shell (NACsh), whereas substantial changes were observed for Homer2a/b, mGluRs as well as NR2a and NR2b subunits in the core (NACc). Within the amygdala, no changes were detected in the basolateral subregion, whereas substantial changes, many paralleling those observed in the NACc, were detected in the central nucleus (CeA) subregion. In addition, most of the changes observed in the CeA, but not NACc, were present in both SW and LW rats.

Conclusions—Overall, these subregion specific, ethanol-induced increases in mGluR/Homer2/ NR2 expression within the NAC and amygdala suggest changes in glutamatergic plasticity had taken place. This may be a result of learning and subsequent memory formation of ethanol's rewarding effects in these brain structures, which may, in part, mediate the chronic relapsing nature of alcohol abuse.

Keywords

Homer Proteins; Group1 Metabotropic Glutamate Receptors; NMDA Receptors; Nucleus Accumbens; Amygdala; Ethanol Consumption

Repeated ethanol administration, including ethanol drinking, elevates both pre- and post-synaptic aspects of excitatory neurotransmission within the extended amygdala, including the nucleus accumbens (NAC) and central nucleus of the amygdala (CeA) (Kapasova and Szumlinski, 2008; Lominac et al., 2006; Melendez et al., 2005; Moghaddam and Bolinao, 1994; Piepponen et al., 2002; Roberto et al., 2004, 2006; Szumlinski et al., 2005b, 2007b, 2008b; Zhu et al., 2007; also for detailed reviews see Chandler, 2003; Gass and Olive, 2008; Koob, 2003; Koob and Le Moal, 2008; Krystal et al., 2003; McBride, 2002; Siggins et al., 2003; Szumlinski et al., 2008a; Woodward, 1999). Acutely, ethanol inhibits the function of both *N*-methyl-D-aspartate receptors (NMDA) and non-NMDA ionotropic glutamate receptors, as well as the mGluR5 subtype of Group1 metabotropic glutamate receptors (mGluRs) (e.g., Läck et al., 2007; Minami et al., 2005; Roberto et al., 2004; Zhu et al., 2007). However, repeated ethanol exposure (via inhalation, injection, or ingestion) can sensitize both pre- and postsynaptic aspects of NAC and CeA glutamatergic neurotransmission, as measured by neurochemical and electrophysiological methods (e.g., Kapasova and Szumlinski, 2008; Melendez et al., 2005; Roberto et al., 2004, 2006; Szumlinski et al., 2005b, 2007b, 2008a,b; Zhu et al., 2007). At least within the NAC, extracellular glutamate levels actively regulate ethanol intake in the alcohol-preferring C57BL/6J mouse (Kapasova and Szumlinski, 2008) with intra-NAC blockade of NMDA or mGluR5 receptors attenuating ethanol intake in rats and mice under both operant and nonoperant conditions (Cozzoli et al., 2009; Rassnick et al., 1992). In addition, manipulation of intra-accumbal glutamatergic activity modifies ethanol-induced place-preference in low ethanol-consuming DBA/2J mice (Gremel and Cunningham, 2008). Less is known about the role that amygdalar glutamate receptors play in ethanol-directed behaviors. A recent report indicated that intra-CeA blockade of either NMDA or AMPA receptors reduced the expression of an ethanol-induced place-preference in rats (Zhu et al., 2007). Such observations for ethanol are consistent with considerable data derived from other drugs of abuse, which have implicated a critical role for glutamatergic neurotransmission within NAC and amygdala subregions in regulating various aspects of addiction-related behaviors (for detailed reviews see Belin et al., 2008; Crombag et al., 2008; Everitt et al., 2008; Kalivas and Volkow, 2005). Thus, the existing literature suggests functional constituents mediating neuroplasticity at glutamatergic synapses within the NAC and/or amygdala may mediate high ethanol-consuming behavior.

One of these constituents is Homer2, a member of the Homer family of postsynaptic scaffolding proteins (for detailed, recent reviews, see Shiraishi-Yamaguchi and Furuichi, 2007; Szumlinski et al., 2008b; Worley et al., 2007). In vitro, Homer proteins regulate various aspects of pre- and postsynaptic glutamatergic transmission, in particular the trafficking and function of NMDA and Group1 mGluRs (Kammermeier, 2008; Sala et al., 2005; Tu et al., 1999; Xiao et al., 2000). In vivo, both Homer1 and Homer2 proteins stimulate and regulate basal glutamate release within limbic regions, as well as the expression, localization and function of Group1 mGluRs and NMDA receptors at the synaptic membrane (Lominac et al., 2005; Szumlinski et al., 2004, 2005a,b, 2008b). Pertaining to drugs of abuse, both *Homer1* and *Homer2* gene products regulate behavioral and neurochemical sensitivity to cocaine (Ghasemzadeh et al., 2003; Lominac et al., 2005; Szumlinski et al., 2004, 2006). Regarding ethanol, behavioral genetic studies indicate an active and necessary role for *Homer2* gene products in regulating various aspects of ethanol reward, as well as ethanol-induced neurochemical sensitization within the NAC (see Cozzoli et al., 2009; Szumlinski et al., 2005b, 2008b). Homer2a/b, but not Homer1b/c, expression levels are sensitive to ethanol experience; and, consistent with earlier in vitro data (e.g., Tu et al., 1999; Xiao et al., 1998), ethanol-induced increases in Homer2a/b levels are accompanied by elevations in Group1 mGluR and NR2 subunit expression (Cozzoli et al., 2009; Szumlinski et al., 2008b). Unlike the co-regulation of forebrain and temporal lobe Homer2a/b and glutamate receptor levels reported following cocaine (Ary and Szumlinski, 2007; Swanson et al., 2001), this protein co-regulation induced by ethanol treatment has only been reported in the NAC thus far (Cozzoli et al., 2009; Szumlinski et al.,

2008b). Importantly, observed increases in NAC mGluR/Homer2/NR2 protein expression produced by chronic, continuous ethanol consumption persists for at least 2 weeks after cessation of ethanol access (Szumlinski et al., 2008b). These findings suggest that a history of ethanol drinking can produce relatively long-lasting increases in Homer2/glutamate receptor-mediated signaling within this, and perhaps other extended amygdala, brain regions.

The first aim of this study was to extend our previous immunoblotting findings derived from studies conducted in mice (Cozzoli et al., 2009; Szumlinski et al., 2008b). Here, we examined the effects of short-term (24 hours; SW) versus long-term (4 weeks; LW) periods of withdrawal from chronic (6 months) ethanol consumption upon changes in Homer and glutamate receptor protein expression within striatal subregions of selectively bred, alcohol-preferring P rats and compared these effects between rats with intermittent (IA: 4 d/wk) versus continuous (CA: 7 d/wk) ethanol access. The second aim of this study was to extend our knowledge regarding ethanol-induced changes in limbic Homer/glutamate receptor expression to include subregions of the amygdala, as converging evidence indicates a critical role for ethanol-induced neuroplastic changes within the CeA subregion in the development of ethanol dependence (for detailed reviews, see Koob and Le Moal, 2008; McBride, 2002; Siggins et al., 2003).

MATERIALS AND METHODS

Animals

Male, alcohol-preferring P rats ($n = 68$) from the 42nd to 43rd generations weighing approximately 120 g (6 weeks of age) at the start of the experiment were used. Rats were singly housed in plastic, HEPA-filtered, micro-isolators (40×40×20 cm) in ventilated banks within a temperature- (21°C) and humidity- (50%) controlled vivarium at the Indiana University School of Medicine (Indianapolis, IN). The vivarium room was maintained on a 12 h/12 h light/dark cycle (lights off at 19:00 hours) in a facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Food and water were available in the home cage ad libitum throughout the experiment. All research protocols were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine (Indianapolis, IN) and were in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse and the NIH *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 80-23, 1996).

Ethanol Drinking Regimens

For 6 months, P rats had 24-hour free-choice access to ethanol (15% and 30% v/v available concurrently) under continuous access (CA: 7 d/wk) or intermittent access (IA: 4 d/wk) conditions in their home cages (see Fig. 1 for drinking behavior). A 3 bottle-choice procedure was employed to induce higher daily ethanol intakes than that attained with more conventional 2 bottle-choice procedures (Bell et al., 2003, 2004, 2006a). To be consistent with previous immunoblotting studies conducted in mice (Cozzoli et al., 2009; Szumlinski et al., 2008b), half of the rats in each group (water: $n = 22$; CA: $n = 16$; IA: $n = 14$) were killed 24 hours after termination of ethanol access (short withdrawal or SW). To examine for persistent changes in protein expression during a time when physical withdrawal is negligible, the remaining rats in each group were killed at 4 weeks after termination of ethanol access (long withdrawal or LW)—a time intermediate between those employed in our previous mouse study (Szumlinski et al., 2008b). Brain regions of interest were micropunched from frozen sections (300 μ m) prepared in a chilled cryostat, according to the rat brain atlas of Paxinos and Watson (1998). The following brain regions were excised: the shell (NACsh) and core (NACc) subregions of the NAC, the basolateral (BLA) and central (CeA) subregions of the amygdala, as well as the central dorsal striatum (see Fig. 2 for locations of micropunches). All micropunch samples

were stored frozen at -80°C and then shipped to the University of California at Santa Barbara for immunoblotting.

Immunoblotting

The immunoblotting procedure employed for the detection of glutamate receptors and Homers was identical to that previously described by our group (Ary and Szumlinski, 2007; Ary et al., 2007; Cozzoli et al., 2009; Szumlinski et al., 2008b). Frozen micropunches were homogenized in a medium consisting of 0.32 M sucrose, 2 mM EDTA, 1% w/v sodium dodecyl sulfate, 50 μM phenyl methyl sulfonyl fluoride and 1 $\mu\text{g}/\text{ml}$ leupeptin ($\text{pH} = 7.2$) and 50 mM sodium fluoride, 50 mM sodium pyrophosphate, 20 mM 2-glycerol phosphate, 1 nM *p*-nitrophenyl phosphate, and 2 μM microcystin LR were included to inhibit phosphatase activity. Samples were then subjected to low-speed configuration at 10,000 *g* for 20 min. For immunoblotting, tissue homogenates (10 μl of each sample) were subjected to SDS–polyacrylamide gel electrophoresis, following incubation in a reducing agent (Invitrogen, Carlsbad, CA). Tris–acetate gradient gels (3% to 8%) (Invitrogen) were used for the separation of Homers, mGluR1a, mGluR5, NR2a and NR2b. Proteins were transferred to PVDF membranes, preblocked with phosphate-buffered saline containing 0.1% (v/v) Tween-20 and 5% (w/v) nonfat dried milk powder for at least 2 hours before overnight incubation with primary antibodies. The following rabbit polyclonal antibodies were used: anti-Homer1b/c and anti-Homer2a/b (Dr. Paul F. Worley, Johns Hopkins University School of Medicine; 1:1,000 dilution), anti-mGluR5 (Millipore, Billerica, MA; 1:1,000 dilution), anti-NR2a and anti-NR2b (EMD Chemicals, Gibbstown, NJ; 1:1,000 dilution). An anti-mGluR1a mouse polyclonal antibody (Millipore; 1:1,000 dilution) was also used. A rabbit anti-calnexin polyclonal primary anti-body (Stressgen, Ann Arbor, MI; 1:1,000 dilution) was also used as a control to indicate protein loading, migration and transfer (e.g., Cozzoli et al., 2009; Szumlinski et al., 2008b; Swanson et al., 2001). Membranes were washed, incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary anti-body (Millipore, Billerica, MA; 1:5,000 to 1:10,000 dilution) or anti-mouse secondary anti-body (Jackson Immuno Research Laboratories, West Grove, PA; 1:10,000) for 90 min, washed again, and immunoreactive bands were detected by enhanced chemiluminescence using either ECL Plus (Amersham Biosciences, Inc., Piscataway, NJ) or Pierce SuperSignal West Femto (Thermo Fisher Scientific, Rockford, IL). Primary antibodies were stripped off PVDF membranes with Millipore's ReBlot Plus Strong Antibody Stripping Solution (Millipore). The level of immunoreactivity for all proteins were quantified by integrating area/band density using computer-assisted densitometry (NIH Image J; NIH, Bethesda, MD) and the immunoreactivity for each protein of interest for each animal was first normalized to that of its appropriate calnexin signal to provide a protein/calnexin ratio. An analysis of the immunoreactivity exhibited by the water-drinking controls killed at the SW and LW time-points failed to reveal significant group differences (data not shown). Therefore, the data for these groups were combined into 1 water-drinking control group for final analyses of ethanol's effects. The protein/calnexin ratios for the animals within each of the 4 alcohol-drinking experimental groups ($n = 8$ to 14/group) were then normalized to the mean ratios for each protein of the water-drinking control ($n = 19$) for each individual gel.

Statistical Analyses

Body weight, water intake, ethanol intake, and preference were analyzed using a 2-way pretreatment (water, CA, IA) by block (data are collapsed across 4 day periods) mixed analysis of variance (ANOVA), with pretreatment being the between-groups factor and block being the within-groups factor.

Due to technical issues during homogenization and protein transfer, not all brain regions or proteins were examined for every animal. Thus, the immunoblotting data were analyzed using one-way analyses of variance (ANOVAs), with the between-subjects factor of treatment (5

levels: [1] water; [2] ethanol 7 d/wk-24 hours withdrawal [CA-SW]; [3] ethanol 7 d/wk-4 weeks withdrawal [CA-LW]; [4] ethanol 4 d/wk-24 hours withdrawal [IA-SW]; and [5] ethanol 4 d/wk-4 weeks withdrawal [IA-LW]). Significant main effects were followed by protected Fisher's least significant difference post-hoc tests ($\alpha < 0.05$).

RESULTS

Body Weight, Water Consumption, Ethanol Consumption, and Ethanol Preference

As seen in Fig. 1, the 3 Nat Rev Neurosci groups displayed similar body weights [pretreatment by block interaction: $F(52,1274) = 0.641$, $p = 0.978$; pretreatment main effect: $F(2,49) = 0.522$, $p = 0.597$] across the entire study period (panel A); the ethanol-consuming groups drank significantly less water [pretreatment by block interaction: $F(52,1274) = 3.19$, $p < 0.001$; pretreatment main effect: $F(2,49) = 50.9$, $p < 0.001$] than the water control group after the 5th week (panel B); the IA group drank significantly [pretreatment by block interaction: $F(52,1274) = 24.58$, $p < 0.001$; protected Fisher's LSD tests for first 8 blocks, $p < 0.05$] more ethanol than the CA group, during each 4-day block when both groups had ethanol access, through the 8th week (panel C); both ethanol groups had similar preference scores [pretreatment (CA vs. IA) main effect: $F(1,27) = 0.071$, $p = 0.791$], with 50% or higher preference values after the 11th week (panel D). These data are similar to previously published data with the alcohol-preferring P rat (e.g., Bell et al., 2006a,b,2008).

The data summarizing the changes in CC-Homer and glutamate receptor expression within the NACsh and NACc, as well as CeA and BLA, observed in SW and LW rats after either IA or CA ethanol consumption are presented respectively in Figs. 3–6. To facilitate subregional comparisons, all data are summarized in Table 1.

Effects of CA and IA Ethanol Consumption on Protein Expression Changes in the NAC

As seen in Fig. 3 (NACsh) and Fig. 4 (NACc), the effects of CA and IA ethanol consumption upon protein expression, within the NAC, were selective for the core subregion. The analysis of NACsh protein expression (Fig. 3) revealed a moderate, but insignificant, reduction in Homer1b/c expression, which was greatest following SW (24 hours) from CA ethanol consumption (24 h/d, 7 d/wk) [main effect: $F(4,61) = 2.30$, $p = 0.07$]. However, no effects of ethanol consumption on NACsh levels of Homer2a/b, mGluR1, or mGluR5 were observed ($p > 0.05$). IA ethanol drinking (4 d/wk) reduced NACsh NR2b levels in general [main effect: $F(4,60) = 3.61$, $p = 0.01$, post-hoc tests $p < 0.05$], but post-hoc tests indicated that this reduction was transient and primarily present in the SW group. Finally, IA ethanol drinking elicited a rise in NACsh NR2a levels that was significant in both the SW and LW groups [main effect: $F(4,58) = 3.18$, $p = 0.02$, post-hoc tests $p < 0.05$].

Inspection of NACc protein expression (Fig. 4) suggested that both CA and IA ethanol consumption elevated Homer1b/c levels, but this did not reach statistical significance ($p = 0.11$). However, the analysis did reveal that both CA and IA ethanol consumption significantly elevated NACc Homer2a/b mRNA levels in the SW group, whereas this effect was only seen in the LW group of CA animals [main effect: $F(4,59) = 5.00$, $p = 0.002$; post-hoc tests $p < 0.05$]. As illustrated in Fig. 4, the NACc levels of mGluR1, NR2a, and NR2b expression were elevated in the SW groups of both CA and IA animals [main effects for mGluR1: $F(4,58) = 6.18$, $p < 0.0001$; for NR2a: $F(4,58) = 4.71$, $p = 0.002$; for NR2b: $F(4,58) = 4.07$, $p = 0.006$, post-hoc tests $p < 0.05$], but these effects were not significant in the LW groups. In contrast, the level of mGluR5 expression was elevated in SW groups of both CA and IA drinking rats, but the effect persisted in the LW group of IA rats only [main effect: $F(4,59) = 3.44$, $p = 0.01$, post-hoc tests $p < 0.05$].

In contrast to either NAC subregion, an analysis of ethanol-induced changes in protein expression within the more dorsal aspect of the striatum failed to reveal any effect of ethanol consumption for the proteins examined (all p -values >0.05 ; data not shown).

Effects of CA and IA Ethanol Consumption on Protein Expression Changes in the Amygdala

As observed for the NAC (Figs. 3 and 4), an analysis of the effects of ethanol drinking upon amygdala Homer and glutamate receptor expression also revealed sub-regional differences, with changes observed in the CeA (Fig. 5), but not in the BLA (Fig. 6), subregion. As depicted in Fig. 5, both CA and IA ethanol consumption elevated CeA levels of Homer2a/b, mGluR1, and NR2b in both the SW and LW groups [main effects: for Homer2a/b: $F(4,63) = 4.69$, $p = 0.002$; for mGluR1: $F(4,63) = 7.84$, $p < 0.0001$; for NR2b: $F(4,63) = 3.13$, $p = 0.02$, post-hoc tests $p < 0.05$]. Both CA and IA ethanol consumption also elevated CeA levels of NR2a, although this effect was only seen in the SW group [main effect: $F(4,63) = 6.96$, $p < 0.0001$; post-hoc tests $p < 0.05$]. In addition, a transient elevation in CeA mGluR5 levels was observed only in the IA group [main effect: $F(4,63) = 5.37$, $p = 0.001$; post-hoc tests $p < 0.05$]. Homer1b/c levels did not significantly change under either of the ethanol conditions ($p > 0.05$).

In contrast to the robust and often enduring changes in CeA protein expression (Fig. 5), analyses for ethanol-induced changes in the BLA (Fig. 6) failed to reveal any significant effects of ethanol consumption for any of the proteins examined (all p -values >0.05 ; Fig. 6). In addition, our anti-mGluR1 antibody failed to detect significant levels of this protein in our BLA samples and, thus, the effects of ethanol consumption and length of withdrawal could not be assessed for this protein.

DISCUSSION

In this study, immunoblotting was conducted to assess the interaction between chronic (6 months) CA (7 d/wk) versus IA (4 d/wk) ethanol self-administration and withdrawal upon Homer1/2, Group1 mGluR, and NR2a/b subunit expression within subregions of the striatum and amygdala from selectively bred, alcohol-preferring P rats. The data presented here extend the results of our earlier immunoblotting studies conducted in mice. Our laboratory's previous studies found that chronic, continuous ethanol intake over a 3-month period (Szumlinski et al., 2008b), as well as intermittent bouts of binge (i.e., BACs >0.8 mg/ml in 30 minutes) ethanol consumption (Cozzoli et al., 2009), elevated protein expression of members of the mGluR/Homer2/NMDA signaling complex within the ventral, but not dorsal, aspects of the striatum. To the best of our knowledge, this is the first published report of subregion-specific changes in amygdala Homer/glutamate receptor expression following a 6-month history of ethanol drinking, under both CA and IA conditions. In particular, increased protein expression was observed in the CeA, but not the BLA, subregion of this structure. Finally, the ethanol-induced increases in CeA Homer2a/b, mGluR1 and NR2b expression were detected in both the SW and LW groups, suggesting that, as observed in the NAC, chronic ethanol consumption produces enduring increases in the efficiency of postsynaptic glutamate receptor signaling that persist well beyond the time when signs of physical withdrawal have dissipated. Therefore, it is reasonable to hypothesize that these persistent changes may be a factor, at least in part, underlying the chronic relapsing nature of alcoholism. While our use of one-way ANOVAs to analyze the immunoblotting data may have introduced some α error, the trend of the data across the regions suggests that these findings are consistent with our statistical results. Thus, if any error occurred, it is most likely due to moderate power, thus β error, rather than due to false positive findings.

Our earlier immunoblotting studies conducted in mice revealed robust ethanol-induced changes in NAC Homer2/glutamate receptor expression, which, based on the results of pharmacological, genetical, and neurochemical studies, are necessary for glutamatergic

sensitization. Research from our laboratory and that of others indicates this glutamatergic sensitization mediates, in part, high ethanol-drinking and seeking phenotypes (e.g., Bäckström et al., 2004; Cozzoli et al., 2009; Hodge et al., 2006; Olive et al., 2005; Schroeder et al., 2005; Szumlinski et al., 2005b, 2008b). However, our earlier ethanol-drinking studies examined for protein expression changes within the entire NAC of the mouse (Cozzoli et al., 2009; Szumlinski et al., 2008b), thereby preventing conclusions regarding possible subregional differences in the effects of chronic ethanol consumption. Detection of a subregional dissociation between the effects of different ethanol-drinking paradigms and length of withdrawal would advance our understanding of the role for Homers and their associated glutamate receptor signaling molecules in the neurobiology of addiction. This stems from the fact that the NACsh and NACc subregions are components of functionally and anatomically distinct neural subcircuits (e.g., Alexander et al., 1990; Heimer et al., 1997). In particular, the NACsh mediates the primary reinforcing properties of drugs of abuse and the ability of addictive drugs to modulate conditioned reinforcement (factors affecting “drug-taking”), whereas the NACc subserves various aspects of associative learning involved in the acquisition and expression of instrumental responses involved in “drug-seeking” behavior (e.g., Cardinal et al., 2002; Corbit et al., 2001; Di Chiara et al., 2004; Di Ciano and Everitt, 2001; Di Ciano et al., 2001; Hall et al., 2001; Ikemoto et al., 2005; Ito et al., 2004; Parkinson et al., 1999, 2000; Wise, 2004).

As is clear from the present data (Fig. 3 vs. Fig. 4), 6 months of CA ethanol intake in P rats increased the co-expression of mGluR1/5, NR2a/b and Homer2a/b within the NACc, but not the NACsh, subregion. The present findings indicate that the effects of chronic ethanol intake, under either CA or IA conditions, on NACc mGluR1/5 and NR2a/b expression in P rats did not persist into protracted withdrawal (i.e., present in the SW group only), whereas Homer2a/b levels remained elevated at 4 weeks following cessation of CA ethanol drinking (Fig. 4). While it remains to be determined whether or not such subregion-specific changes in Homer2/glutamate receptor expression occur in mice after chronic ethanol consumption as well, it is interesting to note that a number of similarities exist between the findings for the NACc in this study (Fig. 4) and those observed in the whole NAC of C57BL/6J mice (Szumlinski et al., 2008b). These similarities include not only the specific proteins affected by chronic ethanol ingestion (e.g., Homer2a/b vs. Homer1b/c), but also the relative magnitude and deprivation time-courses of ethanol's effects upon NAC protein expression (e.g., shorter term increases in glutamate receptor expression vs. longer term changes in Homer2a/b expression). The similar effects of chronic ethanol drinking upon NAC Homer2/glutamate receptor expression observed between rats and mice are consistent with an earlier report by our group demonstrating across-species generalization of the effects of cocaine withdrawal upon Homer/glutamate receptor expression within NACsh, NACc, hippocampus, and prefrontal cortex (Ary and Szumlinski, 2007). Such data argue that drug-induced changes in Homer/glutamate receptor expression, at least within glutamate-rich frontal cortical and basal forebrain structures are not likely species specific.

The deprivation time course (SW vs. LW) of the observed ethanol-induced increases in NACc Homer2a/b and mGluR5 expression varied as a function of ethanol access. That is, IA induced changes at SW for Homer2a/b and at both withdrawal times for mGluR5, whereas CA induced changes at both withdrawal times for Homer 2a/b and only SW for mGluR5. Contrarily, ethanol-induced changes in mGluR1/NR2a/b expression were similar under the 2 access conditions (see Fig. 4), with changes observed in the SW, but not LW group, much like those observed upon 24 hour withdrawal from ethanol drinking in a limited-access binge mouse model of ethanol intake (30 min/d, every 3rd day; Cozzoli et al., 2009). While it cannot be discerned from the present experiment or those conducted previously in mice whether or not the changes in NAC glutamate receptor expression observed at 24 hour withdrawal from alcohol consumption reflect responses to ethanol withdrawal or to chronic ethanol consumption

per se (or both), increases in NAC mGluR and NR2 expression persist for at least 2 weeks following chronic, CA ethanol in mice—a time when signs of physical withdrawal are negligible (Szumlinski et al., 2008b). Together, these data support the notion that ethanol-induced inhibition of NMDA and Group1 mGluR function (Lovinger et al., 1989; Minami et al., 2003) during ethanol ingestion (regardless of the ethanol access paradigm) produces a compensatory elevation in NAC (core) glutamate receptor/Homer signaling.

An alcohol-induced increase in glutamate receptor/Homer signaling is predicted to facilitate the control of associative processes mediating approach and ingestive behavior and render an animal more likely to seek and self-administer ethanol upon subsequent availability. In support of this line of reasoning, virus-mediated NAC over-expression of Homer2b facilitates both appetitive and consummatory aspects of behaviors within operant paradigms and facilitates the development of an ethanol-conditioned place-preference, whereas *Homer2* deletion or NAC knockdown reduces ethanol intake, as well as conditioned-approach behavior (Cozzoli et al., 2009; Szumlinski et al., 2005b, 2008b). Similarly, mGluR5 blockade or *mGluR5* deletion attenuates (i) free-access ethanol intake, (ii) the expression of an ethanol-conditioned place-preference, (iii) the discriminative stimulus properties of ethanol, (iv) instrumental responding for ethanol, and (v) cue-induced reinstatement of ethanol seeking in both rats and mice (Bäckström et al., 2004; Besheer and Hodge, 2005; Besheer et al., 2006, 2008; Blednov and Harris, 2008; Hodge et al., 2006; Lominac et al., 2006; McMillen et al., 2005; Olive et al., 2005; Schroeder et al., 2005, 2008). Additionally, regarding other drugs of abuse, systemic or intra-NAC core pretreatment with AMPA, NMDA/glycine or mGluR5 antagonists attenuates cue-induced cocaine-seeking in rats (Bäckström and Hyttiä, 2006, 2007).

Whereas the short-term effects of ingested ethanol upon NAC Homer2/glutamate receptor expression do not appear to be highly dependent upon the ethanol-access regimen (Fig. 4; Cozzoli et al., 2009; Szumlinski et al., 2008b), the P rats in the CA group exhibited an increase in NACc Homer2a/b expression following protracted withdrawal (1 month), but Homer2a/b levels returned to that of water-drinking controls in rats drinking under the IA-LW condition. This occurred despite the overall higher average daily intakes of the IA group relative to their continuous drinking counterparts (~7 g/kg/d for the CA group vs. ~9 g/kg/d for the IA group). Although the protracted effects of different ethanol-access regimens upon Homer/glutamate receptor expression have yet to be examined systematically in mice, it is noteworthy that the ethanol-induced increases in Homer2a/b levels observed in the entire NAC of C57BL/6J mice drinking under CA conditions (4 bottle-choice; 7 d/wk; daily intakes ~11 g/kg/d) persisted for at least 2 months into withdrawal (Szumlinski et al., 2008b). Moreover, in both cases of CA, whether by mice or rats, the enduring rise in Homer2a/b expression was observed at a time when Group1 mGluR and NR2 subunit expression had returned to that of water controls (see Fig. 4; also, Szumlinski et al., 2008b). While requiring further investigation, these 2 data sets support the hypothesis that the pattern of ethanol intake or ethanol availability plays a potentially important role in mediating the protracted effects of ethanol drinking upon NAC (core) expression of glutamatergic scaffolding and signaling molecules. Therefore, given the role of glutamatergic neurotransmission, and its plasticity, in learning and memory, the hypothesized mechanism outlined above may mediate, at least in part, the chronic, relapsing nature of alcoholism.

Regarding the amygdala, chronic ethanol exposure, at least via ethanol inhalation, sensitizes both pre- and postsynaptic aspects of glutamate release within the CeA subregion (Roberto et al., 2004, 2006; Zhu et al., 2007). Extending the results of these earlier electrophysiological and neurochemical studies, we observed an increase in the CeA expression of Homer2a/b, which co-occurred with elevations in both Group1 mGluRs and NR2 subunits (Fig. 6). As was observed for the NAC, the effects of CA upon amygdala protein expression were subregion-specific occurring within the CeA, but not within the BLA (Fig. 5 vs. Fig. 6). However, in

contrast to our observations for the NACc where ethanol induced a persistent increase in Homer2a/b only, CeA levels of mGluR1 and NR2b expression remained elevated in the LW group in concert with the rise in Homer2a/b expression. Moreover, the enduring increase in Homer2/mGluR1/NR2b expression was observed in both the CA and IA ethanol-drinking groups, indicating that the schedule of ethanol access was not a significant factor affecting CeA expression for these proteins, at least within the confines of this study.

That being said, a 2-week history of continuous ethanol inhalation (BACs ~1.8 mg/ml) also elevates CeA mRNA and protein expression of NR2a, NR2b, as well as that of the obligatory NR1 subunit of the NMDA receptor, but these effects dissipate within 2 weeks of ethanol cessation (Roberto et al., 2006). While further studies are necessary to better understand the influence of chronic ethanol experience and withdrawal upon amygdala glutamate receptor expression and its relation to glutamate transmission and drinking behavior, it is clear that (i) NR2a- and NR2b-containing NMDA receptors are more sensitive to inhibition by ethanol (e.g., Allgaier, 2002; Lovinger et al., 1989; Masood et al., 1994; Mirshahi and Woodward, 1995); (ii) Homer2 regulates the plasma membrane trafficking of NR2a/b subunits in vivo (Szumlanski et al., 2005b); and (iii) the heightened ethanol sensitivity of CeA NMDA receptors produced by chronic ethanol inhalation can be blocked by the NR2b-selective antagonist ifenprodil (Roberto et al., 2004). Thus, it is reasonable to speculate that ethanol-mediated inhibition of CeA glutamate receptors during chronic ethanol consumption elicits an enduring rise in CeA Homer2a/b expression, which facilitates the plasma membrane trafficking of NR2b-containing NMDA receptors and mGluR1. Such a neuroadaptation would be predicted to augment CeA sensitivity to ethanol, heighten the severity of withdrawal symptoms in the shorter term and contribute to rebound ethanol self-administration during protracted abstinence (e.g., Roberts et al., 2000).

The subregional specificity of the observed effects of chronic ethanol ingestion upon Homer/glutamate receptor within the NAC and amygdala is intriguing and raises questions as to the precise neurocircuitry affected during early and protracted withdrawal from free-access ethanol consumption. As it shares a variety of histochemical features and connections with the CeA and the medial nucleus of the amygdala, the NACsh (but not NACc) is considered a component of the “extended amygdala” (Alheid and Heimer, 1988; Heimer et al., 1997). The observation that enduring glutamate-associated neuroadaptations co-occur within the CeA and NACc, but not NACsh, during withdrawal from free-access ethanol consumption suggests that chronic ethanol consumption does not produce some general increase in glutamate signaling throughout the entire extended amygdala. While there is no direct anatomical connection between the CeA and NACc, both structures receive glutamatergic projections from the anterior cingulate cortex. Anterior cingulate innervation of the NACc is involved in associative processing that is hypothesized to direct behavior towards appetitive stimuli (in the case of drugs of abuse), while simultaneous activation of projections to the CeA are predicted to not only evoke autonomic/neuroendocrine responses but also engage behavioral arousal via activation of dopamine release within the NACc (Everitt et al., 1999). While disconnection studies have provided some support for this cortico-striatal loop in mediating auto-shaping/Pavlovian-approach behavior within the confines of noncontingent food reward delivery (see Belin et al., 2008), its relevance to the establishment and maintenance of chronic ethanol intake requires further experimentation.

In summary, data presented here demonstrate that both CA and IA ethanol consumption by P rats produce selective increases in Group1 mGluR/Homer2/NR2 expression within the NACc and CeA—2 brain regions highly implicated in mediating various aspects of the addiction process. The effects of chronic ethanol intake upon NACc Homer2a/b and CeA mGluR1/Homer2/NR2b expression persist into protracted withdrawal, implicating these molecular adaptations in the persistent behavioral and neurochemical effects of chronic ethanol exposure.

Moreover, the present data show for the first time that IA can produce short-term increases in Homer/glutamate receptor expression within both the NACc and the CeA, which may increase the aversiveness of early ethanol withdrawal and consequently augment the negative reinforcing properties of ethanol. Together, these findings support a hypothesized important role for ethanol-induced increases in Homer/glutamate receptor expression in mediating both the short- and longterm behavioral consequences of chronic ethanol intake and implicate for the first time a potential role for Homer-associated glutamate signaling within the CeA in the neuropathology of alcoholism.

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Fig. 1.

Depiction in 4-day blocks (when both intermittent [IA] and continuous [CA] access groups had access to ethanol [15% and 30% concurrently with water]) of mean $\pm$ SEM for body weight (g) across the ethanol treatment period (**A**); for water consumption (ml/kg/d) across the ethanol treatment period (**B**); for ethanol consumption (g/kg/d) across the ethanol treatment period (**C**); and for overall ethanol preference (% of total fluids) across the ethanol treatment period (**D**). In general, body weight did not differ between the groups, ethanol consuming rats drank significantly ($p < 0.05$) less water than the water control rats after the 5th week, the IA rats drank significantly ($p < 0.05$) more ethanol (during the 4 days that both groups had ethanol access) than the CA rats for the first 8 weeks, and CA and IA rats did not differ in overall ethanol preference and both groups had 50% ethanol preference values or greater after the 11th week of ethanol access. To facilitate ease of presentation, significance is not indicated on the graph.

Fig. 2.

Loci of micropunches. Represented here are schematics of coronal sections of the rat brain and the representative site of micropunches for the accumbens core (AcbC), the accumbens shell (AcbSh), the central amygdala (CeA), and the basolateral amygdala (BLA). Below each coronal section is the approximate bregma level from which the punches were acquired. For all nuclei, bilateral punches and punches from each level were combined. aca = anterior commissure, anterior part, mfb = medial forebrain bundle, LV = lateral ventricle, 3V = third ventricle (D, dorsal), opt = optic tract. Modified from Paxinos and Watson, 2005. Due to the absence of significant findings for the dorsal striatum samples, their micropunch sites are not presented.

Fig. 3.

Representative immunoblots for, and summary of, the total protein levels of Homer1b/c, Homer2a/b, mGluR1/5, and NR2a/b in the nucleus accumbens shell (NACsh) of P rats killed 24 hours (SW) or 4 weeks (LW) after continuous (CA: 7 d/wk) or intermittent (IA: 4 d/wk) ethanol drinking. IA and/or CA ethanol consumption elevated NACsh protein expression of NR2a and decreased the protein expression of NR2b. The data are expressed as a percent of the average optical band density of water-drinking P rats controls. All bars represent the mean $\pm$ SEM of 9 to 19 animals/time point. The asterisk (*) denotes significance versus controls $*p < 0.05$ (ANOVA followed by Protected Fisher's LSD post-hoc *t*-tests).

Fig. 4.

Representative immunoblots for, and summary of, the total protein levels of Homer1b/c, Homer2a/b, mGluR1/5, and NR2a/b in the nucleus accumbens core (NACc) of P rats killed 24 hours (SW) or 4 weeks (LW) after continuous (CA: 7 d/wk) or intermittent (IA: 4 d/wk) ethanol drinking. IA and/or CA ethanol consumption elevated NACc protein expression of Homer1b/c, Homer2a/b, mGluR1/5, and NR2a/b subunits. The data are expressed as a percent of the average optical band density of water-drinking P rats controls. All bars represent the mean $\pm$ SEM of 9 to 19 animals/time point. The asterisk (*) denotes significance versus controls $*p < 0.05$ (ANOVA followed by Protected Fisher's LSD post-hoc *t*-tests).

Fig. 5.

Representative immunoblots for, and summary of, the total protein levels of Homer1b/c, Homer2a/b, mGluR1/5, and NR2a/b in the central amygdala (CeA) of P rats killed 24 hours (SW) or 4 weeks (LW) after continuous (CA: 7 d/wk) or intermittent (IA: 4 d/wk) ethanol drinking. IA and/or CA ethanol consumption elevated CeA protein expression of Homer2a/b, NR2a/b subunits, and mGluR1/5. The data are expressed as a percent of the average optical band density of water-drinking P rats controls. All bars represent the mean $\pm$ SEM of 9 to 19 animals/time point. The asterisk (*) denotes significance versus controls $*p < 0.05$ (ANOVA followed by Protected Fisher's LSD post-hoc *t*-tests).

Fig. 6. Representative immunoblots for, and summary of, the total protein levels of Homer1b/c, Homer2a/b, mGluR1/5, and NR2a/b in the basolateral amygdala (BLA) of P rats killed 24 hours (SW) or 4 weeks (LW) after continuous (CA: 7 d/wk) or intermittent (IA: 4 d/wk) ethanol drinking. IA and/or CA ethanol consumption did not alter BLA protein expression of Homer2a/b, NR2 subunits, or mGluR1/5. The data are expressed as a percent of the average optical band density of water-drinking P rats controls. All bars represent the mean $\pm$ SEM of 9 to 19 animals/time point.

Table 1

Summary of the findings from alcohol-preferring (P) rats given either intermittent (IA: 4 d/wk) or continuous (CA: 7 d/wk) ethanol (15% and 30% concurrently with water) access regarding Homer and glutamate receptor protein expression levels assayed in the nucleus accumbens shell (NACsh) and core (NACc), in the dorsal striatum (dSTR), as well as in the central nucleus (CeA) and basolateral nucleus (BLA) of the amygdala following short-term (24 hour) withdrawal (SW) or longer-term (4 weeks) withdrawal (LW)

	Homer1b/c			Homer2a/b			mGluR1			mGluR5			NR2a			NR2b		
	IA	CA	nc	IA	CA	nc	IA	CA	nc	IA	CA	nc	IA	CA	nc	IA	CA	nc
NACsh	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	+ ^{SW} , lw	nc	nc	— ^{SW} , lw	— ^{SW}	nc
NACc	nc	nc	+ ^{SW}	+ ^{SW}	+ ^{SW} , lw	+ ^{SW}	+ ^{SW}	+ ^{SW} , lw	+ ^{SW}	+ ^{SW}	+ ^{SW}	+ ^{SW}	+ ^{SW}	+ ^{SW}	+ ^{SW}	+ ^{SW}	+ ^{SW}	+ ^{SW}
dSTR	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc
CeA	nc	nc	+ ^{SW} , lw	+ ^{SW} , lw	+ ^{SW} , lw	+ ^{SW} , lw	+ ^{SW} , lw	+ ^{SW} , lw	+ ^{SW}	nc	+ ^{SW}	+ ^{SW}	+ ^{SW}	+ ^{SW}	+ ^{SW}	+ ^{SW} , lw	+ ^{SW} , lw	+ ^{SW} , lw
BLA	nc	nc	nc	nc	nc	nd	nd	nd	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc

+ indicates significant ($p < 0.05$) increase in the level of protein relative to control group; — indicates a significant ($p < 0.05$) decrease in the level of protein relative to control group; “nc” indicates no change relative to the control group; “nd” indicates the protein was not detected; sw indicates significant ($p < 0.05$) changes detected after the short-term withdrawal (24 hours); lw indicates significant ($p < 0.05$) changes detected after the long-term withdrawal (4 weeks).