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Limbic Circuitry Activation in Ethanol Withdrawal is Regulated by a Chromosome 1 Locus

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Abstract

Physiological dependence and associated withdrawal episodes are thought to constitute a motivational force sustaining alcohol use/abuse and contributing to relapse in alcoholics. Although no animal model exactly duplicates alcoholism, models for specific factors, including the withdrawal syndrome, are useful for identifying potential genetic and neural determinants of liability in humans. We previously identified highly significant quantitative trait loci (QTLs) with large effects on predisposition to withdrawal after chronic and acute alcohol exposure in mice and mapped these loci to the same region of chromosome 1 (*Alcdp1* and *Alcw1*, respectively). The present studies utilize a novel *Alcdp1/Alcw1* congenic model (in which an interval spanning *Alcdp1* and *Alcw1* from the C57BL/6J donor strain [build GRCm38 150.3–174.6 Mb] has been introgressed onto a uniform inbred DBA/2J genetic background) known to demonstrate significantly less severe chronic and acute withdrawal compared to appropriate background strain animals. Here, using c-Fos induction as a high-resolution marker of neuronal activation, we report that male *Alcdp1/Alcw1* congenic animals demonstrate significantly less alcohol withdrawal-associated neural activation compared to appropriate background strain animals in the prelimbic and cingulate cortices of the prefrontal cortex as well as discrete regions of the extended amygdala (i.e., basolateral) and extended basal ganglia (i.e., dorsolateral striatum, and caudal substantia nigra pars reticulata). These studies are the first to begin to elucidate circuitry by which this confirmed addiction-relevant QTL could influence behavior. This circuitry overlaps limbic circuitry involved in stress, providing additional mechanistic information. *Alcdp1/Alcw1* maps to a region syntenic with human chromosome 1q, where multiple studies find significant associations with risk for alcoholism.

Keywords

ethanol; c-Fos; limbic; amygdala; withdrawal

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Introduction

Alcohol (ethanol) abuse and alcoholism are leading causes of global disease burden (Whiteford et al., 2013). Alcohol-use disorders are also one of the most highly heritable addictive disorders (Goldman, Oroszi, O'Malley, & Anton, 2005), with risk estimated at 40%–60% in family and twin studies. Unfortunately, the neural and genetic determinants of alcohol abuse and alcoholism are largely unknown, hindering effective prevention and treatment. Although no animal model duplicates clinically defined alcoholism, models for specific factors are useful for identifying potential genetic and neural determinants of liability in humans. These factors include withdrawal, a hallmark of alcohol physiological dependence that can constitute a motivational force that can perpetuate alcohol use and abuse (Little et al., 2005).

Using robust preclinical models, we have identified significant quantitative trait loci (QTLs) affecting alcohol physiological dependence and associated withdrawal following chronic and acute alcohol exposure in mice. These include proven QTLs affecting predisposition to alcohol withdrawal following chronic alcohol exposure (*Alcdp1*; Buck, Rademacher, Metten, & Crabbe, 2002; Kozell, Belknap, Hofstetter, Mayeda, & Buck, 2008) and after acute alcohol exposure (*Alcw1*; Buck, Metten, Belknap, & Crabbe, 1997; Kozell et al., 2008). Although *Alcdp1* and *Alcw1* map to the same discrete region of chromosome 1 (Kozell et al., 2008), it remains to be elucidated to what degree the underlying gene(s) and mechanism(s) may be shared, in part because more than one plausible candidate gene is located within the QTL interval (Denmark & Buck, 2008), one or more of which may significantly affect one or both phenotypes.

The aim of the present studies was to elucidate neural circuitry associated with ethanol withdrawal and affected in a QTL-(*Alcdp1*/*Alcw1*) dependent manner in mice. c-Fos is a high-resolution histological marker of neuronal stimulation (Herdegen & Leah, 1998; Morgan, Cohen, Hempstead, & Curran, 1987), which identifies a distinct activation pattern associated with alcohol withdrawal (Borlikova, Le Merrer, & Stephens, 2006; Chen & Buck, 2010; Chen, Kozell, Hitzemann, & Buck, 2008; Dave, Tabakoff, & Hoffman, 1990; Knapp, Duncan, Crews, & Breese, 1998; Kozell, Hitzemann, & Buck, 2005; Morgan, Nadi, Karanian, & Linnoila, 1992; Wilce, Beckmann, Shanley, & Matsumoto, 1994). Previous analyses have identified brain regions that differ between standard inbred strains in c-Fos expression associated with withdrawal after chronic and acute ethanol exposure (Chen, Reilly, Kozell, Hitzemann, & Buck, 2009; Kozell et al., 2005). Significant strain differences in withdrawal-associated activation of limbic basal ganglia brain regions were found using acute alcohol exposure models, with an extended limbic circuit (i.e., hippocampus, amygdala, and prefrontal cortex) apparently recruited following chronic alcohol exposure. Because of the near elimination of genetic “noise” from loci elsewhere in the genome, comparisons between congenic and background strain animals are invaluable to address a QTL’s influence on neural activity and identify brain regions potentially involved in mediating its impact on behavior (Chen et al., 2008, 2009).

In order to dissociate the influence of *Alcw1*/*Alcdp1* from that of other ethanol-withdrawal QTLs elsewhere in the genome (Buck et al., 1997, 2002), we compared the pattern of

neuronal activation associated with withdrawal in *Alcdp1/Alcw1* congenic animals to wild-type background strain animals. This genetic model is known to demonstrate significantly less severe withdrawal convulsions following chronic and acute ethanol exposure compared to wild-type background strain animals and captures *Alcdp1/Alcw1* within its introgressed interval (defined by *D1Mit200* and *D1Mit150*, located at 150.3 and 174.6 Mb in GRCm38) from the C57BL/6J (B6) donor strain superimposed on an genetic background that is >98% DBA/2J (D2). Details of the creation of this genetic model are given in Kozell et al., 2008.

Methods

Animals

All of the animals tested were bred in our colony in the Department of Comparative Medicine at Oregon Health & Science University. Adult (60–90 days) male congenic (D2.B6-*Alcdp1/Alcw1*) and wild-type background strain (D2) animals were used. The creation of the D2.B6-*Alcdp1/Alcw1* congenic model (originally referred to as D2.B6-*D1Mit206*) has been described previously (Kozell et al., 2008). D2 breeder stock was originally purchased from the Jackson Laboratory (Bar Harbor, ME). Mice used in the study were group-housed 2–4 per cage by genotype. Mouse chow (Purina #5001) and water were available *ad libitum*. Procedure and colony rooms were kept at a temperature of 21 ± 1 °C. Lights were on in the colony from 6:00–18:00 h. Behavioral testing was initiated between 7:00 and 9:00 h. All procedures were approved by the Oregon Health & Science University and VA Medical Center Care and Use Committees in accordance with USDA and USPHS guidelines.

Immunohistochemistry

c-Fos immunostaining was performed as described in our previous work (Chen et al., 2008; Kozell et al., 2005). Notably, the mice used were not tested for convulsions in order to avoid potential confounds of evoked convulsions on c-Fos immunoreactivity. The study was performed in two experimental passes. Briefly, mice were administered a hypnotic dose of ethanol (4 g/kg, 20% v/v intraperitoneally [i.p.]; n = 7 congenic and n = 8 wild-type) or an equivalent volume of vehicle (sterile 0.9% saline; n = 6 congenic and n = 6 wild-type) and returned to their home cage and left undisturbed for 7 h. This time point was used to assess immediate early gene expression associated with alcohol withdrawal for several reasons. First because previous results using congenic and background strain mice demonstrate that withdrawal-associated handling-induced convulsions begin approximately 4–5 h post-ethanol exposure and peak in severity approximately 6–7 h post-ethanol exposure (Kozell et al., 2008). Second because c-Fos protein induction typically occurs within 1 h of such stimuli (Chang, Kenigs, Moldow, & Zadina, 1995; Morgan et al., 1987), and lastly, to facilitate comparison of analyses using standard inbred strains (Chen et al., 2009; Kozell et al., 2005) and other congenic models for different withdrawal QTLs (Chen & Buck, 2010; Chen et al., 2008).

The mice were killed by cervical dislocation and the brains were removed and placed in ice-cold 4% paraformaldehyde in 0.1-M phosphate buffer (PB) overnight. The paraformaldehyde solution was then replaced with 0.1-M PB containing 30% sucrose until the brain was completely submerged (typically within 48 h). Brains were coronally

sectioned (30 μ m) using a cryostat, and the tissue was stored in 10-mM PB containing 0.02% sodium azide until it was processed for immunohistochemical analysis. Within an experimental pass, all of the experimental groups were processed simultaneously, and under the same conditions. The sections were first rinsed three times in 10-mM PB before being incubated in 1.5% hydrogen peroxide in 10-mM PB in 0.9% saline solution (PBS) for 15 min to block endogenous peroxidase activity. All immunohistochemical processing steps took place on a rotating table for rinses, and antibody and peroxidase incubations took place while rotating on a rotisserie shaker. Tissue was washed six times in 10-mM PBS. Next, the sections were blocked for 2 h in immunoreaction buffer (10-mM PBS containing 0.25% Triton-X 100 and 5% dry milk). Rabbit anti-c-Fos antibody (1:10,000; Oncogene Science Inc., Cambridge, MA) was then added and the incubation continued for 72 h at 4 C. The sections were rinsed three times in 10-mM PBS and incubated for 1 h at room temperature with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) in 10-mM PBS. The sections were then incubated with horseradish peroxidase avidin-biotin complex in 10-mM PBS for 1.5 h at room temperature (ABC Elite peroxidase kit, Vector Laboratories). The sections were rinsed three times in 10-mM PBS and placed in 0.05-M Tris buffer (pH 7.4) for 5 min. The chromatic reaction was completed with fresh diaminobenzidine (50 mg/100 mL of 0.05-M Tris, Sigma, St. Louis, MO) in the presence of 0.01% nickel ammonium sulfate solution and 0.035% hydrogen peroxide. Omission of the primary antibody to the sections was used as a staining control. The sections were mounted onto slides, dehydrated, and cover-slipped in Permount (Fisher Scientific, Pittsburgh, PA).

For quantitative morphometric analysis of c-Fos immunoreactive cells, an Olympus BX60 light microscope and LEICA DFC 480 imaging system were used to obtain a permanent record of cell distribution. Results using mean densities across a brain region and representative sections are comparable (Chen et al., 2008), so representative sections were analyzed for each brain region as follows (from Paxinos & Franklin, 2001): cingulate and prelimbic cortices (Cg1 and PrL, plate 18), ectorhinal-perirhinal cortex (EcP, plate 49), the central and basolateral nuclei of the amygdala (CeA and BLA, plate 42), the bed nucleus of the stria terminalis (BNST, plate 30), rostral substantia nigra pars reticulata (SNr, plate 55), caudal SNr (plate 61), subthalamic nucleus (STN, plate 48), lateral globus pallidus (LGP, plate 35) and rostromedial LGP (plate 33), medial globus pallidus (MGP, plate 42), ventral pallidum (VP, plate 30), discrete regions (dorsomedial dorsolateral and rostroventral) of the striatum (STR) (plate 23), ventral tegmental area (VTA, plate 56), nucleus accumbens (NAc) shell and core (plate 23), intermediate layers of superior colliculus (SCi, plate 61), lateral and medial septum (plate 27), and hippocampal formation CA1, CA2, CA3, stratum radiatum, dentate gyrus (DG), and DG molecular cell layer (plate 47). All images were taken at 10X and signals were quantified using Image Pro Plus (Media Cybernetics). Standardized brain region templates based on established anatomical markers were used to assess c-Fos-positive cell labeling within each region (i.e., counting the number of c-Fos immunoreactive cells within the template). Standardized threshold parameters (160, light intensity range from 0 to 255) were employed to identify and quantify individual c-Fos immunoreactive neurons. Sample size estimates were based on previous studies (Chen et al., 2008; Hitzemann & Hitzemann, 1997; Kozell et al., 2005) and are sufficient to overcome potential

artifacts associated with assessing representative sections (Hitzemann & Hitzemann, 1997). The technician was blind to the experimental condition and genotype for each subject.

Data analysis

The c-Fos expression in *Alcdp1/Alcw1* congenic and background strain mice was not normally distributed based on a significant Shapiro-Wilkes test, so a square-root transformation was performed. The resulting data were normally distributed as appropriate for analyses using two-way analysis of variance (ANOVA), with genotype and treatment as between-subject factors. Experimental passes were combined for statistical analysis, with outliers removed for final analysis, and the results reported. When appropriate, the Tukey HSD test was used for *post hoc* analyses. All data were analyzed using Systat 13 statistical software (Systat Statistical Inc.). The significance level was set at $p < 0.05$ (two-tailed) throughout for the ANOVA and *post hoc* analyses.

Results

c-Fos expression in *Alcdp1/Alcw1* congenic and wild-type mice

In order to isolate the influence of *Alcdp1/Alcw1* from that of other withdrawal QTLs elsewhere in the genome (Buck et al., 1997, 2002), alcohol withdrawal-associated c-Fos induction was compared in *Alcdp1/Alcw1* congenic and wild-type mice. c-Fos was selected because alcohol withdrawal is associated with a distinct pattern of neuronal activation as assessed by its induction, and to facilitate comparison to studies using other genetic models, including other congenic models (Chen et al., 2008, 2009; Kozell et al., 2005 and references therein). In contrast, expression of other immediate early gene products, i.e., zif268, is largely insensitive to alcohol withdrawal although its expression is induced in the central amygdala (Borlikova et al., 2006). In order to avoid potential confounds of evoked convulsions on c-Fos expression, none of the mice used in the immunohistochemical analyses were tested for withdrawal convulsions, and none of the mice used exhibited spontaneous convulsions in their home cage pre- or post-ethanol administration.

Table 1 summarizes the number of c-Fos positive neurons in ethanol-withdrawn and control (saline) congenic and wild-type mice across 28 brain regions, emphasizing cortical, limbic, and basal ganglia circuitry. As expected, little or no c-Fos expression was detected in control animals for most of the brain regions evaluated. The data were examined for main effects of strain (congenic or wild-type) and treatment (ethanol or saline control), and for strain $\times$ treatment (SXT) interactions. Statistically significant main effects of treatment and/or strain and SXT interactions are also indicated in Table 1. Seven of the 28 brain regions assessed demonstrated significant SXT interactions (i.e., Cg1, PrL, BLA, BNST, caudal SNr, dorsolateral STR, and NAc shell) (Table 1). *Post hoc* analyses confirmed significantly lower withdrawal-associated c-Fos induction in congenic mice compared to wild-type mice in four of these regions (i.e., PrL, BLA, dorsolateral STR, and caudal SNr). These regions are part of an extended limbic circuit illustrated in Fig. 1, which highlights the connectivity within this circuit based upon anatomical and electrophysiological studies. Representative immunohistochemical results for selected brain regions are shown as follows: the PrL and Cg1 cortices (Fig. 2), the CeA and BLA (Fig. 3), and the SNr (Fig. 4).

Cerebral cortex—A main effect of treatment was apparent in all three cortical regions assessed, i.e., the PrL, Cg1, and EcP (for details, see Table 1). Significant SXT interactions were apparent in the prefrontal cortical regions assessed, the PrL and Cg1. *Post hoc* analyses confirmed significantly less c-Fos induction in withdrawn congenic mice compared to wild-type mice in the PrL ($p = 0.004$), but did not achieve statistical significance for the Cg1 ($p = 0.074$, NS). In the EcP, where the effect of treatment was particularly dramatic, only a main effect of treatment was apparent.

Extended amygdala—A main effect of treatment was apparent in all three regions evaluated, the CeA, BLA, and BNST (Table 1). The BLA also showed a main effect of strain and a significant SXT interaction, with significantly less c-Fos induction in withdrawn congenic mice compared to wild-type mice ($p = 0.005$). In the BNST, although no main effect of strain was detected, a significant SXT interaction was apparent. Neither a main effect of strain nor a SXT interaction was detected in the CeA.

Extended basal ganglia—Significant SXT interactions were found in three of the 13 regions evaluated, i.e., the caudal SNr, dorsolateral STR, and the NAc shell (Table 1). *Post hoc* analyses confirmed significantly less c-Fos induction in withdrawn congenic mice compared to wild-type mice in the caudal SNr ($p = 0.004$) and dorsolateral STR ($p = 0.01$). Main effects of strain were also evident in the caudal SNr and dorsolateral STR. Significant treatment effects were apparent in all but one (i.e., NAc core) of the extended basal ganglia regions evaluated. A significant main effect of treatment was apparent in the rostral SNr, caudal SNr, STN, LGP, rostromedial LGP, MGP, VP, dorsomedial STR, dorsolateral STR, rostroventral STR, VTA, NAc shell, and superior colliculus.

Septum—No treatment or strain effects were detected in the lateral or medial septum.

Hippocampal formation—There was almost no detectable c-Fos immunoreactivity in any of the six regions assessed, and no treatment or strain effects were detected (Table 1).

Discussion

The human relevance of preclinical data depends on using robust animal models and the high degree of homology to the human genome at the gene, linkage, and network levels. Using a preclinical model, the present studies are the first to elucidate neural circuitry by which a confirmed QTL for both chronic and acute alcohol withdrawal influences behavior, and point to *Alcdp1/Alcw1* effects on behavior involving its actions in an extended limbic circuit.

Four of the 28 brain regions assessed in the present studies (i.e., the PrL, dorsolateral STR, BLA, and caudal SNr) demonstrate significant withdrawal-associated neuronal activation in an *Alcdp1/Alcw1*-dependent manner. It is informative to compare these results to those for a different proven alcohol withdrawal QTL (*Alcw2* on chromosome 4), in which a distinct pattern of neural activation is observed. *Alcw2* congenic mice exhibited significantly less withdrawal-associated neuronal activity than wild-type mice in the SNr, STN, VP, rostromedial LGP, and rostroventral STR (Chen et al., 2008, 2011), with more intense neural

activation in subregions of the basal ganglia associated with limbic function than in those subregions associated with sensorimotor function (Chen et al., 2008). Clearly, the *Alcdp1/Alcw1* and *Alcw2* congenic results show marked specificity (presumably owing to different mechanisms of action for these QTLs), making it unlikely that their distinct patterns of neuronal activation are due to a nonspecific effect, such as generalized hyperexcitability. Nor are the patterns apparent in *Alcdp1/Alcw1* or *Alcw2* congenic animals consistent with response due to handling-induced stress or injections (Ryabinin, Wang, & Finn, 1999), providing further evidence that genetically determined differences in c-Fos induction are not due to a nonspecific or stress response.

The present studies provide the first evidence for withdrawal QTL-dependent neural activation in the prefrontal cortex. Our results using the *Alcdp1/Alcw1* congenic model expand upon our previous work comparing the two progenitor strains, in which the D2 strain showed significantly greater withdrawal-associated c-Fos induction than the B6 strain in the PrL following chronic alcohol exposure (Chen et al., 2009), and in the Cg1 using an acute withdrawal model (Kozell et al., 2005). Notably, the medial prefrontal cortex is also implicated in withdrawal-enhanced alcohol self-administration in humans and animal models (Carlson & Drew Stevens, 2006; George et al., 2001; Meinhardt et al., 2013). Additionally, in dependent rats, withdrawal-associated increases in c-Fos immunoreactive neurons in the medial prefrontal cortex were associated with alcohol intake as well as impairment of working memory (George et al., 2012). Given that our results demonstrate that withdrawal-associated neural activation in prefrontal cortical areas is *Alcdp1/Alcw1*-dependent, it is intriguing to speculate that *Alcdp1/Alcw1* may have a broader role in behavioral responses to alcohol beyond its confirmed role in withdrawal convulsions. The PrL also has a well-documented role in the inhibition of hypothalamo-pituitary-adrenal responses to emotional stress *via* influences on neuroendocrine effector mechanisms (Diorio, Viau, & Meaney, 1993; Figueiredo, Bruestle, Bodie, Dolgas, & Herman, 2003; Radley, Arias, & Sawchenko, 2006). It is therefore important for future work to rigorously test the potential role of *Alcdp1/Alcw1* in behavioral measures of working memory and emotional stress as well as alcohol behaviors beyond withdrawal convulsions (including depression-like and anxiety-like behaviors).

Our results also provide the first evidence for *Alcdp1/Alcw1*-dependent neural activation in the BLA and dorsolateral STR. This work expands upon our previous work comparing the two progenitor strains, with D2 strain mice showing significantly greater withdrawal-associated c-Fos induction than the B6 strain in the PrL and BLA following chronic alcohol exposure (Chen et al., 2009), and the Cg1 and dorsolateral STR using an acute withdrawal model (Kozell et al., 2005). The PrL has direct projections to the amygdala including the BLA (McDonald, Mascagni, & Guo, 1996) and other limbic structures (Deniau, Menetrey, & Charpier, 1996; Gabbott, Warner, Jays, Salway, & Busby, 2005), whereas the Cg1 projects to the dorsal STR and other structures (Gabbott et al., 2005; Mitrofanis & Mikuletic, 1999; Sesack, Deutch, Roth, & Bunney, 1989), and receives inputs from the BLA and SNr (Zeng & Stuesse, 1991). Taken together, our results point to the involvement of an extended limbic circuit in mediating *Alcdp1/Alcw1* actions, providing mechanistic (brain regional) information.

Thus far, only one brain region (i.e., caudal SNr) has been identified that exhibits alcohol withdrawal-associated activation that is both *Alcdp1/Alcw1*- and *Alcw2*-dependent, and it is tempting to speculate that this region may be crucially involved in a final common pathway by which *Alcdp1/Alcw1* and *Alcw2* affect withdrawal. The caudal SNr receives projections from brain areas that exhibit evidence of *Alcdp1/Alcw1*-dependent neural activation (i.e., the BLA, dorsolateral STR) or *Alcw2* dependence (i.e., rostromedial STR, STN, and VP). Notably, electrolytic lesions of caudolateral SNr significantly attenuate withdrawal after acute and repeated alcohol exposures, but not pentylenetetrazol-(PTZ, a chemiconvulsant that blocks GABA_A receptor-operated channels) enhanced convulsions, demonstrating a selective role for the caudolateral SNr in risk for alcohol-withdrawal convulsions but not generalized seizure susceptibility (Chen et al., 2008). Chemical lesions restricted to this region also significantly attenuated alcohol withdrawal (and pentobarbital withdrawal) without affecting PTZ-enhanced convulsions, thus demonstrating that neurons intrinsic to the caudolateral SNr are crucially involved in withdrawal (Chen et al., 2011). In contrast, lesions restricted to rostromedial SNr did not affect alcohol withdrawal convulsion severity (Chen et al., 2008). Numerous analyses support the premise that there are functional differences between caudal and rostral SNr in seizure control (Fan, Zhang, Yu, Li, & Juorio, 1997; Velísková, Garant, Xu, & Moshé, 1994; Velísková, Löscher, & Moshé, 1998; Velísková & Moshé, 2001). The caudal SNr displays greater expression of NMDA and metabotropic glutamate receptor densities (Hedberg, Velísková, Sperber, Nunes, & Moshé, 2003) and 5-HT_{2C} receptor mRNA (Eberle-Wang, Mikeladze, Uryu, & Chesselet, 1997) than the rostral SNr. The rostral and caudal SNr mediate distinct facilitatory and inhibitory effects on seizures (Fan et al., 1997; Velísková & Moshé, 2001), with the caudal SNr active during the pre-clonic period and thought to facilitate seizure initiation/propagation, while the rostral SNr becomes involved after a motor seizure occurs (Velísková, Miller, Nunes, & Brown, 2005). Thus, regionally specific receptor expression and/or intrinsic properties of SNr neurons may influence site-specific effects on withdrawal.

Our studies contribute significantly to understanding the genetic and neural determination of alcohol withdrawal, providing mechanistic information and a foundation upon which to rigorously test the mechanism(s) by which *Alcdp1/Alcw1* affects alcohol-withdrawal behavior and beyond, but there are some limitations. Not all neuronal populations express c-Fos protein during stimulation (Strassman & Vos, 1993; Willoughby, Mackenzie, Medvedev, & Hiscock, 1995), and future studies using other immediate early genes and assessing more time points may identify additional brain regions of interest. An unbiased stereological counting procedure may also provide more accurate estimation of the activated cells (Mura, Murphy, Feldon, & Jongen-Relo, 2004). Additionally, the *Alcdp1/Alcw1* congenic tested harbors two discrete QTLs affecting alcohol withdrawal within its introgressed region (Kozell et al., 2008; Kozell, Walter, Milner, Wickman, & Buck, 2009), so we are currently developing smaller donor segment D2.B6 congenic models to disentangle the actions of these two QTLs on the pattern of neural activation. This will provide additional mechanistic (brain regional) information to facilitate rigorous studies to increase understanding of the mechanistic pathways involved. Finally, *Alcw1/Alcdp1* homology to humans remains to be established. The *Alcdp1/Alcw1* region of mouse chromosome 1 is syntenic with human 1q, and multiple clinical studies have found significant associations with risk for alcohol

dependence on human 1q (Edenberg et al., 2010; Ehlers, Walter, Dick, Buck, & Crabbe, 2010; Heath et al., 2011), including a recent genome-wide association study (GWAS) (Zuo et al., 2012). It is worth noting that clinical studies have generally sought markers associated with the diagnosis and endophenotypes (maximum drinks, metabolism, brain oscillations) rather than withdrawal *per se*, are often confounded (e.g., by co-dependence to additional drugs and/or psychiatric disorders), and that a known limitation of GWAS thus far is that they are often underpowered. Thus, although the relevance of *Alcw1/Alcdp1* to alcohol dependence in humans is not certain, currently available data are quite encouraging.

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Highlights

- Alcohol withdrawal-associated neuronal activation was assessed in a congenic model.
- An extended limbic circuit was identified as uniquely involved in the congenic strain.
- The congenic interval maps to a region syntenic with human chromosome 1q.
- Multiple human studies find significant associations with risk for alcoholism on 1q.

Fig. 1. Schematic representation of alcohol withdrawal-associated neural activation that is *Alcdp1/Alcw1* dependent

Regions showing differential alcohol withdrawal-associated c-Fos induction between *Alcdp1/Alcw1* congenic and wild-type mice are shown in dark red ($p < 0.05$). The dashed and solid black arrows represent glutamatergic and GABAergic projections, respectively, within an extended limbic circuit. Of course, additional projections exist that are not shown. Abbreviations: BLA, basolateral amygdala; BNST, bed nucleus stria terminalis; Cg1, cingulate cortex; CeA, central nucleus of the amygdala; LGP, lateral globus pallidus; NAc, nucleus accumbens; PFC, prefrontal cortex; PrL, prelimbic cortex; SNr, substantia nigra pars reticulata; VP, ventral pallidum.

Fig. 2. Photomicrographs of c-Fos immunoreactive neurons in the medial prefrontal cortex Representative photomicrographs for (A) control (saline) *Alcdp1/Alcw1* congenic, (B) alcohol-withdrawn congenic, (C) control wild-type, and (D) alcohol-withdrawn wild-type mice are shown. The PrL showed withdrawal-associated neuronal activation in both genetic models (with a trend in Cg1), but to a lesser degree in *Alcdp1/Alcw1* congenic mice compared with wild-type mice. Scale bar = 100 μ m. Abbreviations: Cg1, cingulate cortex; PrL, prelimbic cortex.

Fig. 3. c-Fos immunoreactive neurons in the amygdala

Shown are representative photomicrographs for (A) control (saline) *Alcdp1/Alcw1* congenic, (B) alcohol-withdrawn congenic, (C) control wild-type, and (D) alcohol-withdrawn wild-type mice. The BLA showed withdrawal-associated neuronal activation in both genetic models, but to a lesser degree in *Alcdp1/Alcw1* congenic mice compared to wild-type mice. Scale bar = 100 μ m. Abbreviations: BLA, basolateral amygdala; CeA, central nucleus of the amygdala.

Fig. 4. Photomicrographs of c-Fos immunoreactive neurons in the caudal SNr
Representative photomicrographs for (A) control (saline) *Alcdp1/Alcw1* congenic, (B) alcohol-withdrawn congenic, (C) control wild-type, and (D) alcohol-withdrawn wild-type mice are shown. The caudal SNr showed withdrawal-associated neuronal activation in both genetic models, but to a lesser degree in *Alcdp1/Alcw1* congenic mice compared with wild-type mice. Scale bar = 100 μ m. Abbreviation: SNr, substantia nigra pars reticulata.

Table 1Brain regional numbers of c-Fos immunoreactive neurons in *Alc1p1/Alcw1* congenic and wild-type mice.

	Wild-type		Congenic		Significant	
	Saline	Ethanol	Saline	Ethanol	T, S and SxT results	
Prefrontal Cortex						
Cgl	10 ± 3	47 ± 4	24 ± 3	33 ± 3	T	$F_{(1,22)}=48.4, p=5.5 \times 10^{-7}$
					SxT	$F_{(1,22)}=19.9, p=2.0 \times 10^{-4}$
PrL	24 ± 6	100 ± 11	35 ± 6	54 ± 6	T	$F_{(1,22)}=35.2, p=5.7 \times 10^{-6}$
					SxT	$F_{(1,22)}=13.8, p=0.001$
EcP	60 ± 11	255 ± 14	64 ± 3	221 ± 27	T	$F_{(1,20)}=94.9, p=4.9 \times 10^{-9}$
Extended Amygdala						
CeA	16 ± 6	79 ± 12	15 ± 2	58 ± 6	T	$F_{(1,23)}=610, p=6.5 \times 10^{-8}$
BLA	29 ± 5	83 ± 5	25 ± 4	52 ± 8	T	$F_{(1,22)}=43.8, p=1.2 \times 10^{-6}$
					S	$F_{(1,22)}=9.2, p=0.006$
					SxT	$F_{(1,22)}=4.5, p=0.046$
BNST	3 ± 2	71 ± 11	15 ± 4	64 ± 14	T	$F_{(1,23)}=67.6, p=2.7 \times 10^{-8}$
					SxT	$F_{(1,22)}=4.3, p=0.05$
Extended basal ganglia						
SNr						
Rostral	0 ± 1	263 ± 24	1 ± 1	250 ± 19	T	$F_{(1,22)}=955, p=1.5 \times 10^{-11}$
Caudal	0 ± 1	363 ± 12	1 ± 1	291 ± 20	T	$F_{(1,21)}=2280, p=1.3 \times 10^{-11}$
					S	$F_{(1,21)}=7.3, p=0.013$
					SxT	$F_{(1,21)}=7.3, p=0.013$
STN	0 ± 1	308 ± 19	0 ± 1	284 ± 28	T	$F_{(1,22)}=1378, p=1.4 \times 10^{-11}$
LGP	0 ± 1	196 ± 16	0 ± 1	167 ± 10	T	$F_{(1,22)}=1234, p=1.4 \times 10^{-11}$
Rostromedial LGP	0 ± 1	272 ± 20	0 ± 1	261 ± 17	T	$F_{(1,23)}=1378, p=1.4 \times 10^{-11}$
MGP	0 ± 1	113 ± 17	0 ± 1	144 ± 22	T	$F_{(1,23)}=243, p=1.8 \times 10^{-11}$
VP	0 ± 1	203 ± 13	0 ± 1	163 ± 13	T	$F_{(1,23)}=1240, p=1.4 \times 10^{-11}$
STR						
Dorsomedial	0 ± 1	12 ± 2	0 ± 1	14 ± 1	T	$F_{(1,23)}=244, p=1.8 \times 10^{-11}$
Dorsolateral	0 ± 1	14 ± 1	0 ± 1	21 ± 2	T	$F_{(1,22)}=639, p=1.6 \times 10^{-11}$
					S	$F_{(1,22)}=5.6, p=0.027$
					SxT	$F_{(1,22)}=5.6, p=0.027$
Rostroventral	0 ± 1	36 ± 3	0 ± 1	41 ± 3	T	$F_{(1,23)}=898, p=1.5 \times 10^{-11}$
VTA	0 ± 1	2 ± 1	0 ± 1	2 ± 1	T	$F_{(1,23)}=25.8, p=1.5 \times 10^{-5}$
NAc						
shell	41 ± 4	99 ± 7	60 ± 10	72 ± 9	T	$F_{(1,23)}=21.4, p=1.2 \times 10^{-4}$

	Wild-type		Congenic		Significant	
	Saline	Ethanol	Saline	Ethanol	T, S and SxT results	
					SxT	$F_{(1,23)}=8.6, p=0.007$
core	36 ± 7	54 ± 7	55 ± 12	39 ± 6		
Superior Colliculus						
Sci	20 ± 9	126 ± 13	18 ± 6	136 ± 7	T	$F_{(1,23)}=19, p=2.3 \times 10^{-4}$
Septum						
Lateral	31 ± 9	26 ± 5	41 ± 12	29 ± 4		
Medial	4 ± 2	4 ± 1	4 ± 2	5 ± 2		
Hippocampus						
CA1	1 ± 1	1 ± 1	1 ± 1	1 ± 1		
CA2	1 ± 1	1 ± 1	1 ± 1	1 ± 1		
CA3	1 ± 1	1 ± 1	1 ± 1	1 ± 1		
Stratum radiatum	1 ± 1	1 ± 1	1 ± 1	1 ± 1		
DG	3 ± 1	5 ± 1	3 ± 1	3 ± 1		
DG, molecular layer	1 ± 1	1 ± 1	1 ± 1	2 ± 1		

Congenic and wild-type (D2) mice were administered 4 g/kg ethanol or saline ($n = 6-8$ per strain per treatment). Seven hours later, the brains were removed and processed for immunohistochemical analysis. Values represent the number of c-Fos immunoreactive cells (mean ± SEM) in representative sections. Significant ($p < 0.05$) main effects of treatment (T), strain (S), and strain × treatment interactions (SXT) are shown; with trends ($p < 0.10$) also indicated in italics. Abbreviations: BLA, basolateral amygdala; BNST, bed nucleus stria terminalis; CeA, central nucleus of the amygdala; Cg1, cingulate cortex area 1; DG, dentate gyrus; EcP, ectorhinal-perirhinal cortex; LGP, lateral globus pallidus; MGP, medial globus pallidus; NAc, nucleus accumbens; PrL, prelimbic cortex; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; SCi, superior colliculus intermediate layers; VP, ventral pallidum; VTA, ventral tegmental area.