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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.

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1. 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

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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.

2. Methods

2.1. 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.

2.2. 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 μm) 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 coverslipped 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),