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Voluntary Binge-like Ethanol Consumption Site-specifically Increases c-Fos Immunoexpression in Male C57BL6/J Mice

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Abstract—The assessment of binge ethanol-induced neuronal activation, using c-Fos immunoreactivity (IR) as a marker of neuronal activity, is typically accomplished via forced ethanol exposure, such as intraperitoneal injection or gavage. Neuronal activity using a voluntary binge-like drinking model, such as "drinking-in-the-dark" (DID), has not been thoroughly explored. Additionally, studies assessing ethanol-elicited neuronal activation may or may not involve stereotaxic surgery, which could impact c-Fos IR. The experiments detailed herein aimed to assess the effects of voluntary binge-like ethanol consumption on c-Fos IR in brain regions implicated in ethanol intake in animals with and without surgery experience. Age-matched male C57BL/6J mice underwent either stereotaxic surgery (Study 1) or no surgery (Study 2). Then, mice experienced one 4-day DID cycle, tail blood samples were collected immediately after test conclusion on day 4, and mice were subsequently sacrificed. In each study, mice that drink ethanol were sorted into those that achieved binge-equivalent blood ethanol concentrations (BECs $\geq$ 80 mg/dl) versus those that did not. Relative to water-consuming controls, mice with BECs $\geq$ 80 mg/dl showed significantly elevated c-Fos IR in several brain regions implicated in neurobiological responses to ethanol. In general, the brain regions exhibiting binge-induced c-Fos IR were the same between studies, though differences were noted, highlighting the need for caution when interpreting ethanol-induced c-Fos IR when subjects have a prior history of surgery. Altogether, these results provide insight into the brain regions that modulate binge-like ethanol intake stemming from DID procedures among animals with and without surgery experience.

Key words: c-Fos, drinking-in-the-dark (DID), voluntary consumption, binge, ethanol, surgery.

INTRODUCTION

The National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines a binge episode as a 2-h period in which men and women consume $\geq$5 or 4 alcoholic beverages, respectively, eliciting blood ethanol concentrations (BECs) exceeding 0.08% (80 mg/dL) (NIAAA, 2004). Several existing rodent models of binge drinking involve forced exposure techniques, such as intragastric gavage and intraperitoneal administration (i.p.), though these methods inherently fail to model voluntary consumption observed in humans. A well-developed preclinical model of voluntary binge-like ethanol consumption has been developed, called "drinking-in-the-dark" (DID), which promotes high levels of consumption and reliably generates BECs exceeding 80 mg/dL over a 4-day paradigm (Rhodes et al., 2005; Rhodes et al., 2007; Thiele and Navarro, 2014). Though most commonly utilized in mice, researchers have adapted the DID paradigm for use in rat studies (Bell et al., 2011; Holgate et al., 2017; Larraga et al., 2017). With DID procedures researchers have shown that several brain regions are implicated in modulation of binge-like ethanol intake, recruiting a variety of neurochemical systems (Sprow and Thiele, 2012). For example, in response to binge-like ethanol drinking, corticotropin releasing factor (CRF) and neuropeptide Y (NPY) levels are increased or decreased, respectively, in the bed nucleus of the stria terminalis (BNST; Pleil et al., 2015),

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Abbreviations: A2, A2 region of the NTS; AcbC, nucleus accumbens core; BEC, blood ethanol concentration; BLA, basolateral amygdala; CeA, central amygdala; CeMPV, medial posterioventral portion of the central nucleus of the amygdala; CRF, corticotropin releasing factor; dBNST, dorsal bed nucleus of the stria terminalis; DID, "drinking-in-the-dark" paradigm; EW, Edinger–Westphal nucleus; hr, hour; i.p., intraperitoneal injection; IR, immunoreactivity; LC, locus coeruleus; LH, lateral hypothalamus; lPBn, lateral parabrachial nucleus; NPY, neuropeptide Y; NTS, nucleus tractus solitarius; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PVA, paraventricular nucleus of the thalamus, anterior; PVN, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; TH, tyrosine hydroxylase; vBNST, ventral bed nucleus of the stria terminalis; VTA, ventral tegmental area.

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CRF levels are increased within the central amygdala (CeA) and ventral tegmental area (VTA; Lowery-Gionta et al., 2012; Albrechet-Souza et al., 2015), NPY levels are reduced in the CeA (Sparrow et al., 2012), and orexin levels are reduced in the lateral hypothalamus (LH; Olney et al., 2015).

Numerous studies have measured ethanol administration-elicited neuronal activation via quantification of inducible transcription factors (ITFs), such as c-Fos (Curran and Morgan, 1995). The use of c-Fos expression has been useful for not only assessing neuronal activity in response to ethanol consumption and administration, but also to study phenotypes associated with increased risk of excessive consumption. For example, adolescent mice with a history of prenatal ethanol exposure exhibit reduced c-Fos activity in the infralimbic cortex (Fabio et al., 2013) while prenatal ethanol exposure reduces and elevates ethanol injection-primed c-Fos IR in the prelimbic cortex and VTA, respectively (Fabio et al., 2015). Brain mapping of nuclei involved in ethanol's effects via quantification of ethanol-induced c-Fos expression has been studied using a variety of ethanol-exposure paradigms, though ethanol exposure at binge-like levels has most frequently been modeled via intragastric and i.p. administration techniques. For instance, researchers have shown that intragastrically administered binge-like episodes increase c-Fos immunoreactivity (IR) in various brain regions including the CeA (Leriche et al., 2008; Lee et al., 2011), the locus coeruleus (LC), the A1-A2 cell groups, and adrenergic C1-C3 cell groups (Lee et al., 2011). Likewise, ethanol administered i.p. increases c-Fos IR in the LC and A2 subregion of the nucleus tractus solitarius (NTS; Thiele et al., 2000), Edinger–Westphal nucleus (EW; Chang et al., 1995; Turek and Ryabinin, 2005), and the paraventricular nucleus of the hypothalamus (PVN), CeA, dBNST, and EW (Knapp et al., 2001).

Assessment of c-Fos induction following voluntary ethanol consumption in limited-access consumption or operant paradigms (Ryabinin et al., 2003) or chronic consumption in two-bottle choice paradigms (Li et al., 2010; Sajja and Rahman, 2013) indicates that voluntary consumption can region-specifically alter c-Fos expression. However, c-Fos IR resulting from DID-elicited binge-like ethanol drinking has not been examined in mice. This is a critical gap in the literature given the popular use of DID procedures in pre-clinical studies (Sprow and Thiele, 2012). Accordingly, the goal of the present study was to assess the effects of binge-like ethanol intake, using DID procedures, on neuronal activation in various brain regions implicated in alcohol use and abuse. Candidate regions for quantification included noradrenergic brainstem structures, extended amygdaloid structures (BNST, CeA, & basolateral amygdala (BLA)), the LH, and the EW, regions that have previously been shown to exhibit ethanol-induced c-Fos expression using other consumption or exposure paradigms. To this end, c-Fos IR in mice with BECs exceeding 80 mg/dl was compared to c-Fos IR from mice with BECs below 80 mg/dl and water-consuming control mice. BECs and tissue collection occurred following test conclusion (day 4), providing insight into patterns of neuronal activity present during experimental manipulations typically performed on day 4 of the DID procedure. Since preclinical studies assessing ethanol consumption may or may not utilize stereotaxic surgery procedures prior to testing, and given recent evidence that exposure to the anesthetic drug isoflurane impacts ethanol-induced c-Fos expression (Smith et al. 2016) we assessed binge-induced c-Fos expression in both surgery-naïve and surgery-exposed mice in two separate studies. Finally, we assessed tyrosine hydroxylase (TH)/c-Fos co-expression in the LC and A2 nucleus of the NTS among surgery-exposed mice to determine the percentage of noradrenergic cells activated within each region and to compare with one of our previous studies (Thiele et al., 2000) that examined similar labeling in rats receiving an ethanol injection.

EXPERIMENTAL PROCEDURES

Animals

Male C57BL/6J mice (n = 50, stock # 000664, Jackson Laboratory), 6 – 8 weeks old were housed in individual home cages with a room temperature maintained at 22 °C and a 12:12-h (hr) reverse light/dark cycle with lights off at 0830 h. Prolab® RMH 3000 (Purina labDiet®; St. Louis, MO) and water were available *ad libitum* except where noted. All protocols were conducted under National Institute of Health guidelines and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Voluntary consumption: "drinking-in-the-dark" procedure

A four-day DID paradigm was used. Briefly, animal weights were collected 30 min prior to homecage water bottle removal. Beginning 3 h into the dark cycle, homecage water bottles were removed and replaced with 10 mL plastic pipettes (calibrated to 0.1 mL) containing either unsweetened ethanol [20%, v/v; diluted from 95% (Decon Labs, King of Prussia, PA)] or tap water. Following the two hour free-access period, pipettes were removed and homecage water bottles were returned. Pipette volume was measured to the nearest 0.1 mL at homecage water bottle removal and replacement. Ethanol consumption was assessed as the difference in volume measured at the beginning of the session versus the end. On the fourth day tail blood samples were collected from each animal 2–4 min after ethanol or water access, and BECs from ethanol-consuming mice were assessed via an alcohol analyzer (Analox Instruments, Lunenburg, MA).

We elected to provide mice with 2 h of access over all 4 days as opposed to 4-access on day 4 of the DID procedure as our lab has found that chemogenetic and pharmacological procedures often produce effects that subside within 2 h (Navarro et al., 2016; Olney et al., 2017; Rinker et al., 2017), and despite the more limited access period, mice undergoing 2 h of access achieve binge-equivalent BECs similar in magnitude to BECs achieved with 4 h of access on day 4 (Olney et al., 2017).

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